Regulation of fluoride stress response in indica rice cultivars: damage, defence and amelioration

Thesis submitted for the degree of
Doctor of Philosophy (Sc.)
in
BIOTECHNOLOGY

Submitted by
ANKUR SINGH
Registration No. Ph.D/18/BMBT/02
Date of Registration: 14.01.2020

Supervisor:
Dr. Aryadeep Roy Choudhury
Assistant Professor, St. Xavier's College

Post Graduate & Research Department of Biotechnology
St. Xavier's College (Autonomous) Kolkata
Affiliated to
University of Calcutta

Dedicated to my

Beloved Mother

Acknowledgement

First and foremost, praises and thanks to the God, the Almighty, for His showers of blessings throughout my research work to complete the research successfully. I have experienced your guidance day by day.

I am forever grateful to Rev. Father Dr. Dominic Savio S.J., Principal, St. Xavier's College (Autonomous), Kolkata, and Prof. Bertram Da Silva, Vice-principal, Arts and Science for being a constant source of support and motivation to me at this institution. I also acknowledge the administrative staff, the IQAC staff and all my worthy acquaintances in the college for making my research tenure hassle-free.

I would like to express my deep and sincere gratitude to my research supervisor, Prof. Aryadeep Roy Choudhury, Discipline of Life Sciences, School of Sciences, Indira Gandhi National Open University, Maidan Garhi, New Delhi for giving me the opportunity to do research and providing invaluable guidance throughout my research. His dynamism, vision, sincerity and motivation have deeply inspired me. He has taught me the methodology to carry out the research and to present the research works as clearly as possible. It was a great privilege and honor to work and study under his guidance. I am extremely grateful for what he has offered me. I would also like to thank him for his friendship, empathy, and great sense of humor. I am also very thankful for his patience during the discussion I had with him on research work and thesis preparation.

I would also like to show gratitude to my research advisory committee, including Dr. Samrat Roy [Ph.D. co-ordinator, St. Xaviers's College (Autonomous), Kolkata], Prof. (Dr.) Maitree Bhattacharyya [Director, Jagadis Bose National Science Talent Search, Kolkata], Prof. (Dr.) Sanghamitra Sengupta [Department of Biochemistry, Ballygunge Science College, University of Calcutta, Kolkata] and Dr. Dipankar Chakraborti [Department of Genetics, Ballygunge Science College, University of Calcutta, Kolkata] for suggesting crucial points that enhances the quality of my doctoral thesis.

Acknowledgement

I would take the opportunity to thank all my other teachers and professors in the undergraduate and postgraduate course for whom I have reached this destination.

I would like to express my sincere gratitude to all the enthusiastic professors of the Postgraduate Department of Biotechnology, Dr. Uma Siddhanta, Dr. Sudipa Saha, Dr. Chandana Barat, Dr. Aniruddha Banerji, Dr. Jhimli Dasgupta (Head), Dr. Ronita Nag Chaudhuri, Dr. Priyanka De, Dr. Souvik Roy and Dr. Sayak Ganguli for their co-operation and kind nature, which conferred a conductive environment for working.

I am grateful to Dr. Rina Ghosh [Director, Central Research Facility, St. Xaviers's College (Autonomous), Kolkata] and staffs like Mr. Arup Mondal and Mr. Pallab Palta for their support and co-operation.

I would like to sincerely express my gratitude to the laboratory assistants, Mr. Abhijit Ghosal, Mr. Rajkumar Dolui, Mr. Bidesh Makhal, Mr. Bittu Baidya and Mr. Debasish Das for their ever helping nature and kind co-operation.

I am fortunate to gratefully acknowledge the support of my other laboratory mates, Dr. Puja Ghosh, Dr. Aditya Banerjee and Dr. Santanu Samanta for their help and constant co-operation throughout my research tenure. I am also grateful to the different members of the research laboratory of our Department, Dr. Arnab Purohit, Dr. Shreeparna Ganguly, Dr. Anagh Ray, Dr. Saurabh Sengupta, Dr. Aheli Majumder, Dr. Sejuti Banerjee,, Dr. Shehnaz Ferdosh, Ms. Shrestha Chakraborty, Ms. Indrila Saha, Ms. Peeali Mukherjee, Mr. Sanatan Ghosh, Ms. Preeti Khan, Ms. Drishti Mandal and Mr. Swarnavo Chakraborty for their co-operation and ever negotiating behavior which created a positive environment of harmony, while working in the laboratory together.

Special thanks must go to Dr. Mathummal Sudarshan of UGC-DAE Consortium for Scientific Research, Kolkata Centre, India, for providing the ED-XRF facility for calcium and sodium ions analyses.

Acknowledgement

I express my distinguished gratitude to the Science and Engineering Research Board Government of India for providing me with financial assistance through fellowship grants during the tenure of my research.

Getting through my doctoral research required more than academic support, and I must thank my friends for listening to and, at times, having to tolerate me over the past five years. I cannot begin to express my gratitude and appreciation for their friendship. They have been unwavering in their personal support during the time I needed them. For many memorable moments, I must thank everyone.

Finally, I must express my very profound gratitude to my family for providing me with immense support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

Ankur Singh

Septermber 2023

AAO	ABA aldehyde oxidase
ABA	Abscisic acid
ABA8ox	ABA-8-oxidase
ABRE	ABA-responsive element
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ADC	Arginine decarboxylase
ANOVA	Analysis of variance
APS	Adenosine 5'-phosphosulfate
APX	Ascorbate peroxidase
AsAO	Ascorbic acid oxidase
ATP	Adenosine triphosphate
ATP-S	ATP-sulfurylase
BADH	Betaine aldehyde dehydrogenase
BSA	Bovine serum albumin
Ca	Calcium
$Ca(NO_3)_2$	Calcium nitrate
$Ca(OH)_2$	Calcium hydroxide
$CaCl_2$	Calcium chloride
CaM	Calmodulin
CAT	Catalase
CBL	Calcineurin-B-like
CDNB	1-chloro-2,4-dinitrobenzene
CLC	Chloride channel
CS	Citrate synthase
$CuSO_4$	Copper sulfate
DAO	Diamine oxidase
DES	Cysteine desufhydrase
DMSO	Dimethyl sulfoxide
DNPH	2,4-dintrophenylhydrazine
DREB	Dehydration-responsive element binding
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ED-XRF	Energy dispersive X-ray fluorescence
ETBR	Ethidium bromide
$FADH_2$	Flavin adenine dinucleotide (reduced)
$FeCl_3$	Ferric chloride
FEX	Fluoride exporter
FRAP	Ferric-reducing antioxidant potential
GA	Gibberellin
GDH	Glutamate dehydrogenase
Gly I	Glyoxalase I
Gly II	Glyoxalase II

GOGAT	Glutamate synthase
GPoX	Guaiacol peroxidase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutamine synthase
GSH	Reduced glutathione
GST	Glutathione S-transferase
h	Hour
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
HMA	Heavy Metal ATPase
HNO_3	Nitric acid
IDH	Isocitrate dehydrogenase
INT	Iodonitrotetrazolium chloride
INV	Acid invertase
JV	Jarava
KCl	Potassium chloride
KH	Khitish
KI	Potassium iodide
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
LEA	Late embryogenesis abundant
LOX	Lipoxygenase
Lsi	Low silicon
MDA	Malondialdehyde
MDH	Malate dehydrogenase
MG	Methylglyoxal
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MT	Matla
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTU	MTU1010
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Sodium phosphate dibasic
Na_2SiO_3	Sodium metasilicate
Na_2WO_4	Sodium tungstate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide hydrogen
NaF	Sodium fluoride
$NaHCO_3$	Sodium bicarbonate

NaOH	Sodium hydroxide
NB	Nonabokra
NBT	Nitro blue tetrazolium chloride
NCED	9-cis-epoxy carotenoid di-oxygenase
NEDA	N-(1-Naphthyl)Ethylene diamine Dihydrochloride
NH_4^+	Ammonium ions
NH_4OH	Ammonium hydroxide
NiR	Nitrite reductase
NO_2^-	Nitrite
NO_3^-	Nitrate
NOX	NADPH oxidase
NR	Nitrate reductase
$O_2^{\cdot-}$	Superoxide radical
OAA	Oxaloacetic acid
OAS-TL	O-acetylserine(thiol) lyase
ODC	Ornithine decarboxylase
Osem	*Oryza sativa* embryonic abundant
P5CS	Δ^1-pyrroline-5-carboxylate synthetase
PAL	Phenylalanine ammonia lyase
PAO	Polyamine oxidase
PAR	Photosynthetically active radiation
PHO	Starch phosphorylase
PMSF	Phenylmethylsulfonyl fluoride
POD	Peroxidases
ppm	parts per million
PVP	Polyvinylpyrrolidone
PyDH	Pyruvate dehydrogenase
Rab16A	Responsive to ABA 16A
RL	Root length
ROS	Reactive oxygen species
RT-PCR	Reverse-transcriptase polymerase chain reaction
RuBisCo	Ribulose 1,5-biphosphate carboxylase
S_2^-	Sulfide
SAMDC	S-adenosyl-methionine decarboxylase
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
Si	Silicon
SL	Shoot length
SLG	S-lactoyl-D-glutathione
SO_4^{2-}	Sulfate ions
SOD	Superoxide dismutase
SOS	Salt overly sensitive
SPDS	Spermidine synthase

SPMS	Spermine synthase
SPS	Sucrose phosphate synthase
SS	Sucrose synthase
Taq	*Thermus aquaticus*
TBA	2-thiobarbituric acid
TDC	L-tryptophan decarboxylase
TEMED	Tetramethylethylenediamine
TISAB	Total ionic strength adjustment buffer
TKM	Tris-KCl-$MgCl_2$
TPTZ	2,4,6-tris(2-pyridyl)-s-triazine
WHO	World Health Organization
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

Contents

Chapter 3

Chapter 3

Chapter 3

Abstract of the thesis

Population explosion in recent decades has led to severe environmental issues. One such significant drawback of unregulated population growth is unwanted release of xenobiotics such as fluoride in the environment. Excess deposition of fluoride in the ground and surface water bodies affects plant health and development. The aim of this Doctoral Thesis was to highlight the effects of fluoride toxicity on rice varieties. Exposure to sodium fluoride led to higher accumulation of fluoride ions due to higher expression of chloride channels in 10 and 20 day-old Khitish and MTU1010, respectively that significantly hampered the growth of seedlings. Additionally, fluoride toxicity also triggered the formation of superoxide ions and H_2O_2 that caused membrane peroxidation leading to higher electrolyte leakage, formation of cytotoxic metabolites like ammonium ions, methylglyoxal and malondialdehyde along with higher LOX and NOX activity. Fluoride accumulation also led to degradation of the chlorophyll. Contrastingly, higher adaptability against fluoride stress was noted in 20 and 10 day-old Khitish and MTU1010 seedlings, respectively which was attributed to higher expression of FEX and H^+-ATPase along with efficient uptake and utilization of macroelements such as nitrogen and sulfur. Exogenous application of protective chemicals such as calcium and silicon lowered the uptake of fluoride ions by chelating them and by forming a protective barrier in the roots, respectively, that inhibited the absorption of fluoride ions. Moreover, higher endogenous level of calcium and silicon also induced the formation of osmolytes and non-enzymatic antioxidants and activity of enzymatic antioxidants that detoxified the reactive oxygen species accumulated in the tissues which in turn reduced the effects of fluoride-induced oxidative stress. Priming of seeds with calcium compounds also improved carbon metabolism in seedlings by restoring the activity of enzymes involved in tricarboxylic acid cycle along with sucrose and starch metabolism that provided sufficient energy to overcome the stressed environment. Furthermore, the role of abscisic acid (ABA) in fluoride stress was also studied since its protective role in case of other abiotic stress is widely known. Reduced ABA accumulation led to higher accumulation of gibberellic acid and melatonin in fluoride-stressed Nonabokra and Matla seedlings that promoted their tolerance level. However, higher ABA content in Jarava seedlings upregulated the expression of chloride channels that stimulated the uptake of fluoride ions, leading to severe fluoride-mediated damage in spite of higher accumulation of polyamines and osmolytes like proline and glycine betaine via concerted action of genes like *ODC*, *ADC*, *DAO*, *PAO*, *SAMDC*, *SPDS*, *SPMS* and *PDH*. Treatment of seedlings with ABA inhibitor (Na_2WO_4) promoted

fluoride tolerance in seedlings, whereas supplementation with exogenous ABA further aggravated the symptoms of fluoride toxicity. Overall, the studies undertaken in this thesis highlight the toxic effects of fluoride in rice plants and showcase the promising role of calcium and silicon in reverting back the normal growth of seedlings. Furthermore, data obtained also highlighted that fluoride tolerance is negatively regulated or is independent of ABA and ABA signaling pathway, but is rather efficiently regulated by the ABA antagonists like melatonin and gibberellic acid.

Chapter 1

Introduction

Fluorine (F^-) is the ninth element of the periodic table and was identified by Professor Henry Moissan in 1886. Due to its electronic configuration ($1s^2$, $2s^2$, $2p^5$), fluorine is the most electronegative and highly reactive element. It is pale yellow irritable gas with pungent smell and do not exist freely in nature. Due to high electronegativity, it has strong affinity to combine with other elements to form compounds called fluoride. Fluoride occurs naturally in rocks particularly that are associated with phosphate. According to Radosites et al. (1994), soil that is derived from rocks may contain toxic amount of fluoride. In addition, rapid industrialization and population growth have also significantly contributed towards the deposition of fluoride in the environment. In India, fluoride poisoning is one of the major problems faced by several states due to the presence of excess amount of fluoride in drinking water (Choubisa 1997; Choubisa et al. 2001). According to Choubisa (2012), 50-100% districts of Gujarat, Rajasthan, Uttar Pradesh and Andhra Pradesh are suffering from fluoride contamination, whereas 30-50% districts of Bihar, Karnataka, Haryana, Maharashtra, Tamil Nadu, West Bengal, Madhya Pradesh and Punjab are challenged with fluoride pollution. Level of fluoride in drinking water in some parts of India is as high as 5.1−15 mg L^{-1} (Dausa, Rajasthan) and 3.2−3.8 mg L^{-1} (Birbhum, West Bengal) which is significantly higher than that of recommended level of fluoride (1.5 mg L^{-1}) by World Health Organization (Bhattacharya and Samal 2018). On being cultivated with fluoride-contaminated water, plants easily absorbs fluoride from water and soil, leading to eventual accumulation in their tissues and grains. Prolonged consumption of these intoxicated crops along with contaminated water led to serious health-related problems in humans.

Fluoride is an emerging pollutant that originates from various sources and pollutes the surrounding water bodies and soil, affecting plant growth and nutrient bioavailability of the soil. Plants uptake fluoride from the contaminated soil and water bodies when exposed for a prolonged period. The endogenous fluoride level in plants is estimated to increase by 3 ppm for each 100 ppm increase in soil fluoride up to the 2200-ppm level (Bharti et al. 2017). The baseline of fluoride deposition in plants is usually <10 mg kg^{-1}, however, it varies depending on the species (Zuo et al. 2018). Prolonged exposure of plants to fluoride leads to its absorption via fluoride channels such as chloride channel (CLC) 1 and CLC 2. Higher deposition of fluoride in the tissues significantly impacts the productivity of plants and also causes extensive yield loss. In addition, excessive bioavailability of fluoride incites various toxicity effects at biochemical and metabolic level, such as nutrient absorption, mineral

homeostasis and alteration in water status within the plant tissues (Farouk and Al-Amri 2019). Absorption of fluoride beyond the threshold level in plants led to the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot-}$) that causes peroxidation of lipid membrane and also hampers its integrity (Yadu et al. 2016). Higher accumulation of ROS also provokes the activity of lipoxygenase (LOX) which eventually causes membrane perturbation, leading to higher leakage of electrolytes along with higher formation of other cytotoxic species like malondialdehyde (MDA) and methylgloxal (MG). The elevated level of ROS and MG generated due to excess accumulation of fluoride leads to the modifications of structural property of proteins (protein carbonylation) and also enhances their proteolytic breakdown via enhanced protease activity (Farouk and Al-Amri 2019). Higher fluoride deposition within the tissues also enhances chlorophyll breakdown and inhibits its synthesis (Banerjee and Roychoudhury 2019a).

In order to survive under harsh conditions, plants are equipped with protective metabolites such as osmolytes and antioxidants. Osmolytes such as proline, glycine-betaine and amino acids are small electrically neutral, non-toxic metabolites that help in scavenging of free radicals in the cells. Additionally, they also enhance the tolerance capability of the plants by maintaining the osmotic balance of the cells and integrity of the lipid membranes (Paul et al. 2017). The activity of Δ^1-pyrroline-5-carboxylate synthetase (P5CS) that controls the rate limiting step of proline biosynthesis was also found to be enhanced during abiotic stresses (Zhang and Becker 2015).

In addition to osmolytes, plants are equipped with enzymatic and non-enzymatic antioxidants that detoxify the ROS generated within the cells, thus protecting its overall structure and function. The major enzymatic antioxidants present in cells are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), guaiacol peroxidase (GPoX) and glutathione reductase (GR). During stressed condition, superoxide radicals generated in the cells are converted into less toxic H_2O_2 due to the catalytic activity of SOD; H_2O_2 again decomposes into non-toxic metabolites by the enzymatic action of CAT, APX, GPX and GPoX (Chakraborty and Patra 2015). The synthesis of non-enzymatic antioxidants such as phenolics, anthocyanins, carotenoids, flavonoids, ascorbate, reduced glutathione and xanthophylls is also elevated in cells as a protective measure to abrogate the negative effects of oxidative stress, occurring due to the formation of ROS in response to abiotic stresses (Ahmad et al. 2010). The role of phenylalanine ammonia lyase (PAL) that

enhances the formation of flavonoids by controlling the committed step of flavonoid biosynthesis is also well known during fluoride stress in rice seedlings (Banerjee and Roychoudhury 2019a). The formation of MG is nullified in the cells by the action of glyoxalase I (Gly I) and glyoxalase II (Gly II) that together forms the glyoxalase system (Hong et al. 2016). During stressed conditions, the ascorbic acid pool is also used in various reactions, as well as by ascorbic acid oxidase (AsAO) to produce dehydroascorbate.

Tricarboxylic acid (TCA) cycle is regarded as the major path of carbon metabolism. It is considered as a vital pathway that connects carbon and nitrogen metabolism along with the release of energy in plants (Schnarrenberger and Martin 2002). Various metabolic acids (pyruvate, citrate and malate) formed during this cycle collectively helps to mitigate the effects of abiotic stresses by taking part in energy production and acting as the precursor of various amino acids that quench excess cations and also maintain the homeostasis of the cells (Lopez-Bucio et al. 2000). According to Petrovič et al. (2002), abiotic stress hampers the activity of enzymes involved in TCA cycle that eventually influences the carbon content of the cells. Previously, Banerjee et al. (2019a) reported that fluoride stress hampered the activity of three major enzymes of TCA cycle, i.e., pyruvate dehydrogenase (PyDH), succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) which in turn lowered the tolerance capability of the rice seedlings. Along with organic acids, sugar and starch produced during the growing phase of plants also act as osmoregulatory molecules to protect various biomolecules from the toxic effects of ROS. Sucrose helps in the translocation of carbon molecules, whereas starch acts as a carbon reservoir in leaves and gets finally deposited in fruits and grains (Zhou et al. 2002). In photosynthetic tissues, the level of sucrose is highly regulated due to the action of sucrose phosphate synthase (SPS). Sugar formed due to the action of SPS on d-fructose 6-phosphate and uridine diphosphate glucose is broken down by acid invertase (INV) and sucrose synthase (SS) (Geigenberger and Stitt 1993). Breaking-down of starch due to the catalytic action of starch phosphorylase and α-amylase provides energy which is utilized by the plants to overcome stressed condition and maintain their proper growth (Krasensky and Jonak 2012).

Mineral elements play a critical role in ameliorating the negative effects of abiotic stress by regulating the formation of protective metabolites (Bhardwaj et al. 2023). Nitrogen and sulfur are two such important nutrients that act as a major stress reducer in plants. Nitrogen is considered as a macronutrient in plants due to its wide involvement in the formation of

photosynthetic pigments, polyamines and secondary metabolites. Additionally, it also forms an integral part of various enzymes, amino acids and proteins (Zhang et al. 2013). Nitrogen is mostly absorbed as nitrate (NO_3^-) from the soil and is readily converted in nitrite (NO_2^-) by the action of nitrate reductase (NR). In the cytoplasm, NO_2^- is transformed into ammonium ions (NH_4^+) by the action of nitrite reductase (NiR) (Gangwar and Singh 2011). Ammonium ions being highly toxic, plants rapidly convert it into glutamate and glutamine by the action of glutamate synthase (GOGAT) and glutamine synthase (GS), respectively. Glutamate is further transformed to 2-oxoglutarate by the catalytic action of glutamate dehydrogenase (GDH) that acts as a substrate of TCA cycle. Various earlier reports have shown that abiotic stress hinders the accumulation of nitrogen in plants that significantly reduces their tolerance level. Banerjee et al. (2019a) reported that fluoride stress inhibited the activity of NR in susceptible rice variety that drastically hampered its survival capability, as compared to that of tolerant cultivar where the activity of NR was marginally affected. Similar results were also shown by Sil et al. (2020) where they demonstrated that arsenic stress negatively influenced nitrogen assimilation in wheat seedlings showing hindered growth.

Sulfur is recognized as the fourth most important plant macronutrient after nitrogen, phosphorus and potassium (Kumaran et al. 2008). Thorough observation of various plant process and biomolecules has recognized sulfur as one of the most abundant molecules in organic structures within plants. Sulfur is mostly involved in the formation of proteins, chlorophyll, sulfur-containing amino acids (cysteine), vitamins and various protective metabolites such as glutathione and phytochelatins that are involved in stress tolerance in plants (Spadaro et al. 2010). Similar to that of nitrogen, sulfur is also taken up by the roots form the soil as sulfate ions (SO_4^{2-}). For its incorporation into metabolic pathway, sulfur needs to be activated by the enzymatic action of ATP-sulfurylase (ATP-S) (first enzyme of sulfur assimilatory pathway) to yield adenosine 5'-phosphosulfate (APS). APS is converted to sulfide (S_2^-) that finally reacts with O-acetyl serine to form cysteine in presence of O-acetylserine(thiol) lyase (OAS-TL). According to Gill and Tuteja (2011), cysteine is the first organic compound of sulfur assimilatory pathway that contains reduced sulfur in cells. Further, cysteine can be converted to hydrogen sulfide (H_2S) by the catalytic action of cysteine desufhydrase (DES) that in turn regulates the level of cysteine in the cells (Vojtovič et al. 2020). H_2S thus formed also plays a major role in regulating the tolerance capability of plants under harsh conditions. In past, several reports have shown the positive role of

exogenous application of sulfur in plants exposed to stress environment (Hasanuzzaman et al. 2018; Sheng et al. 2016).

Previous reports have shown that treatment of plants with diverse chemicals maintain their growth on being exposed to harsh environmental condition by enhancing the formation of protective metabolites and triggering the uptake of minerals from soil. Calcium (Ca) and silicon (Si) are two such elements whose beneficial role has been widely reported against abiotic stresses. Sulzbach and Pack (1972) reported that application of divalent metals like calcium in soil significantly reduces the uptake of fluoride ions by the plants. Divalent metals like Ca bind with the fluoride ions, thus forming their salt, lowering the absorption of F^- ions by the roots. Dey et al. (2012a) reported that exogenous application of 10 mM calcium chloride ($CaCl_2$) lowered the extent of fluoride accumulation in Bengal gram seedlings, exposed to 20 mM NaF (sodium fluoride) solution, resulting in higher tolerance level of the seedlings. Similarly, Ruan et al. (2004) demonstrated that exogenous application of calcium nitrate [$Ca(NO_3)_2$] checked the uptake and accumulation of fluoride ions in tea plants. Ca also acts as a secondary messenger in plants during unfavorable environmental conditions (Mahajan et al. 2008). During salt stress, calmodulin (CaM) and calcineurin-B-like (CBL) proteins serve as important Ca sensors that help in ROS detoxification by transmitting stress signals (Li et al. 2009a). Another major signaling components that are activated by Ca^{2+} ions constitute the salt overly sensitive (SOS) 1, SOS 2 and SOS 3 (Halfter et al. 2000).

Along with Ca, the protective role of Si against abiotic stresses has also been widely demonstrated by various research groups. Initially, Si was considered to be not so important for plant growth, but recent studies have shown that it plays a significant role in inducing the tolerance capability of the plants and hence considered as a quasi-essential element (Epstein and Bloom 2005). Graminaceous plants such as rice uptake Si from soil and accumulate them in their epidermal tissues. Katz (2018) demonstrated that upon application, Si is absorbed by the plants and is deposited in the internal parts as amorphous silica or in sites like phytoliths that in turn stabilizes the chlorophyll structure and facilities the entry of light during photosynthesis. Recently, Sogarwal et al. (2023) reported that exogenous application of Si (200 and 300 mg per kg of soil) restricted fluoride uptake and its translocation from roots to shoot and also triggered the antioxidant defense mechanism in wheat plants exposed to 400 and 500 mg fluoride per kg of soil. In another study, Banerjee et al. (2021) reported that application of Si nanoparticles lowered the extent of fluoride accumulation in rice plants as

well as triggered the level of protective metabolites and extent of macronutrient absorption from the soil that enabled proper maintenance of the agronomic traits of the plants.

Phytohormones are identified as cellular signaling molecules that, at low concentrations, act as chemical messenger and have a paramount role in regulating various physiological activities of the plants (Fleet and Sun 2005). Phytohormones also play a pivotal role in regulating various biochemical and physiological processes that govern the growth and yield of the plants under optimal and stressed environment. Of the various phytohormones identified in plants, abscisic acid (ABA), melatonin and gibberellins (GAs) are among the few that have a central role in plant response to environmental stresses (Maheshwari et al. 2015). ABA was initially identified in plants in early 1960s and was found to responsible for seed dormancy. Its potential role in stress adaptation was demonstrated later by various scientific communities (Iqbal 2015). Under harsh conditions, stress stimuli triggers ABA formation from β-carotene using 9-cis-epoxy carotenoid di-oxygenase (NCED) enzyme. Of all the NCEDs identified till date, NCED3 is considered to be the rate limiting enzyme of ABA biosynthesis (Tan et al. 2003). ABA-8-oxidase1 (ABA8ox1) is another major enzyme of ABA metabolic pathway that regulates degradation of ABA into phaseic acid (Roychoudhury and Banerjee 2017).

It is worth mentioning that ABA triggers and mobilizes a wide array of biochemical defenses such as biosynthesis of proline, glycine betaine and polyamine such as spermine, spermidine and putrescine that enable plants to ward-off negative effects of abiotic stress (Liu et al. 2018; Singhal et al. 2021). Earlier, Shevyakova et al. (2013) demonstrated that treatment of roots of *Mesembryanthemum crystallinum* with ABA (1 µM) lowered the activity of proline dehydrogenase, leading to higher proline accumulation in plants exposed to 100 and 300 mM salt stress. Moreover, ABA not only regulates physiological responses, but also interacts with other phytohormones in plants exposed to unfavorable conditions (He et al. 2018). Melatonin antagonistically lowers the level of ABA in apple seedlings exposed to drought by up regulating the expression of ABA catabolic genes (Li et al. 2015). Similarly, Zhang et al. (2014) reported that melatonin lowers ABA formation in cucumber seedlings by down regulating the expression of ABA anabolic genes during salt stress. Gomez-Cadenas et al. (2001) reported the GA negatively interacts with ABA to regulate internodal growth and germination.

In past, several reports have shown that fluoride, when present above the threshold level in soil, can be easily absorbed and deposited in the rice. On being deposited in the tissues, fluoride led to the higher formation of ROS that compromised the growth and yield of plants. In order to survive under these inappropriate conditions, plants produce a wide array of protective metabolites that reduces the extent of damage in seedlings by detoxifying the cytotoxic metabolites. However, the exact mechanism regarding how these metabolites operate in various rice cultivars exposed to fluoride stress is still not fully understood. Additionally, the effect of fluoride stress on the metabolic pathways of nitrogen, sulfur and carbon (TCA cycle, sugar and starch) assimilation still remain obscure. Previously, various reports have established ABA as an important phytohormone that plays a major role in mediating stress tolerance capability of the plants; however in case of fluoride stress, no such detailed studies have been conducted till date. Because of such lacuna in knowledge, an extensive elaborative work was conducted in the present Thesis to investigate the detrimental effect of fluoride stress in rice plants. The effect of this emerging pollutant in rice cultivars has been investigated at the biochemical and molecular level and feasible cost-effective approaches for fluoride stress management in rice have been experimentally analyzed.

Objectives:

On the basis of available literature and previous works, this Thesis is aimed to fulfill the following objectives.

1. Biochemical and molecular mechanism of fluoride-mediated oxidative damages and defense mechanism in indica rice cultivars, viz., Khitish and MTU-1010, along with the effect of fluoride toxicity in metabolic pathways like TCA cycle and nitrogen and sulphur metabolism.
2. Potential role of exogenous application of calcium compounds as ameliorating agents to counteract fluoride stress in rice.
3. To decipher the effect of exogenously applied silicon in abrogating the negative effect of fluoride toxicity in rice.
4. Validation of the mechanism of actions of ABA and ABA-mediated pathways in response to fluoride stress in rice.

Chapter 2

Review of Literature

2.1. Overview

The quality of life and health of surrounding environment are directly related to each other. Unprecedented growth of human population along with rise in standard of living and other anthropogenic activities have drastically affected the sustainability of the environment. Unregulated and untreated dumping of industrial wastes in biosphere has further worsened the condition. Toxic pollutant, released in the environment and taken up the plant, eventually accumulates in the food chain. On being consumed, the contaminated food grains led to human and animal health problem (Shahab et al. 2017). Higher incidence of health problem due to environmental change has attracted the attention of various environmentalist and research communities. The major sources of pollution in the biosphere is attributed to non-regulated use of chemical fertilizers (pesticides, herbicides and insecticides), sewage wastes, untreated industrial effluents and household wastes (Chouhan and Flora 2010; SCHER 2011). Most toxic wastes, released from the industries, consist of large amount of inorganic compounds along with hazardous metal and non-metal wastes. Fluorine is one of such severe hazardous elements whose release from the industries has significantly enhanced in the past two decades.

Fluorine ($^{9}F_{18.998}$) is the 13th most available element on the earth crust with an average concentration of about 625 mg kg^{-1} (Camargo and Alonso 2006; Selinus et al. 2016). It is most reactive of all the halogens due to its small size and high electronegativity. Fluorine-rich mineral rocks such as fluorite (CaF_2), amphiboles ($A_{0-1}B_2C_5T_8O_{22}(Cl,F,OH)$), sellaite ($MgF_2$), cryolite ($Na_3AlF_6$), topaz ($Al_2SiO_4(F,OH_2)$), apatite ($Ca_5(Cl,F,OH)(PO_4)_3$) and micas [$(AB_{2-3}[X,Si]_4)_{10}(O,F,OH)_2$] are some of the major geogenic sources of fluoride that are widely found all over the world (Hem 1985; Pickering 1985). Apart from industrial and household wastes, weathering and leaching of fluoride-rich mineral rocks can release fluorides in water bodies. According to the Bureau of Indian Standards and World Health Organization, the permissible limit of fluoride in drinking water is 1.5 mg L^{-1} (WHO 2004; BIS 2012; WHO 1997). According to the recent reports of World Health Organization, more than 260 million individuals are consuming water, contaminated with fluoride, having concentration of about 1 mg L^{-1} (WHO 2004). Fluoride toxicity occurs globally in many countries, such as Sri Lanka, Pakistan, India, Turkey, Mexico, China, Iran, Italy, Algeria, United States, Korea, Kenya, Malawi, Ethiopia, Norway, Ghana and Jordan. In India, the level of fluoride in drinking water ranges between 0.1-61.4 mg L^{-1} (Kumar et al. 2021).

2.2. Sources of fluoride in environment

According to the reports of World Health Organization (1984), fluoride is naturally occurring elements which accounts for about 0.32% of earth's crust. The major sources of fluoride in the environment are geological, natural and anthropogenic by nature.

2.2.1. Geological sources

The major sources of fluoride in the environment constitute weathering and leaching of fluoride containing rocks, atmospheric sources and geothermal vents.

2.2.1.1. Weathering of fluoride-bearing mineral rocks

The major reserve of fluoride is in the rocks containing fluoride-rich minerals. The average value of fluoride in the earth crust is 625 mg kg^{-1}. According to Ozsvanth (2009), the level of fluoride in different rocks varies with their composition such as 1000 mg kg^{-1} in alkaline igneous rocks, 100 mg kg^{-1} in ultramafic rocks and 1300 mg kg^{-1} in marine shales. Metamorphic and igneous rocks are the major storehouse of fluoride-containing minerals; however, very small quantity is found in the sedimentary rocks. Edmunds and Smedley (2005) reported that the concentration of fluoride in sedimentary rocks containing fluorapatite and fluoride-enriched clay may be higher than that found in normal sedimentary rocks. Weathering of granite rocks such as hornblende, amphiboles and muscovite are also one of the major sources of fluoride in groundwater (Vithanage and Bhattacharya 2015). In distant past, Churchill et al. (1948) reported that coal burning also significantly contributes towards deposition of fluoride in the environment since per kg of coal contains 295 mg of fluoride.

2.2.1.2. Geothermal sources

Geothermal and volcanic activities are one of the primary sources of fluoride in some regions of the world. According to Camargo (2003), eruption from volcanoes can contribute to about 60-6000 kilotons of inorganic fluoride annually. India, South Africa, Ethiopia, Ghana, Jordan, Senegal and Kenya are highly affected with fluoride contamination due to the presence of crystalline metamorphic and igneous rocks beneath the earth's surface (Dey et al. 2004). Hydrogen fluoride that is naturally released from volcanic eruption can be present in water and surrounding environment even after the activity of volcano ceased long time back (Araya et al. 1993). Volcanic rocks can lead to a deposition of around 2000 mg kg^{-1} of

fluoride at the subduction zone boundary (Anazawa 2006). Precambrian rocks with composition of schist, amphibolites and granite are more responsible for the high concentration of fluoride in the hot spring water (Marbaniang et al. 2014).

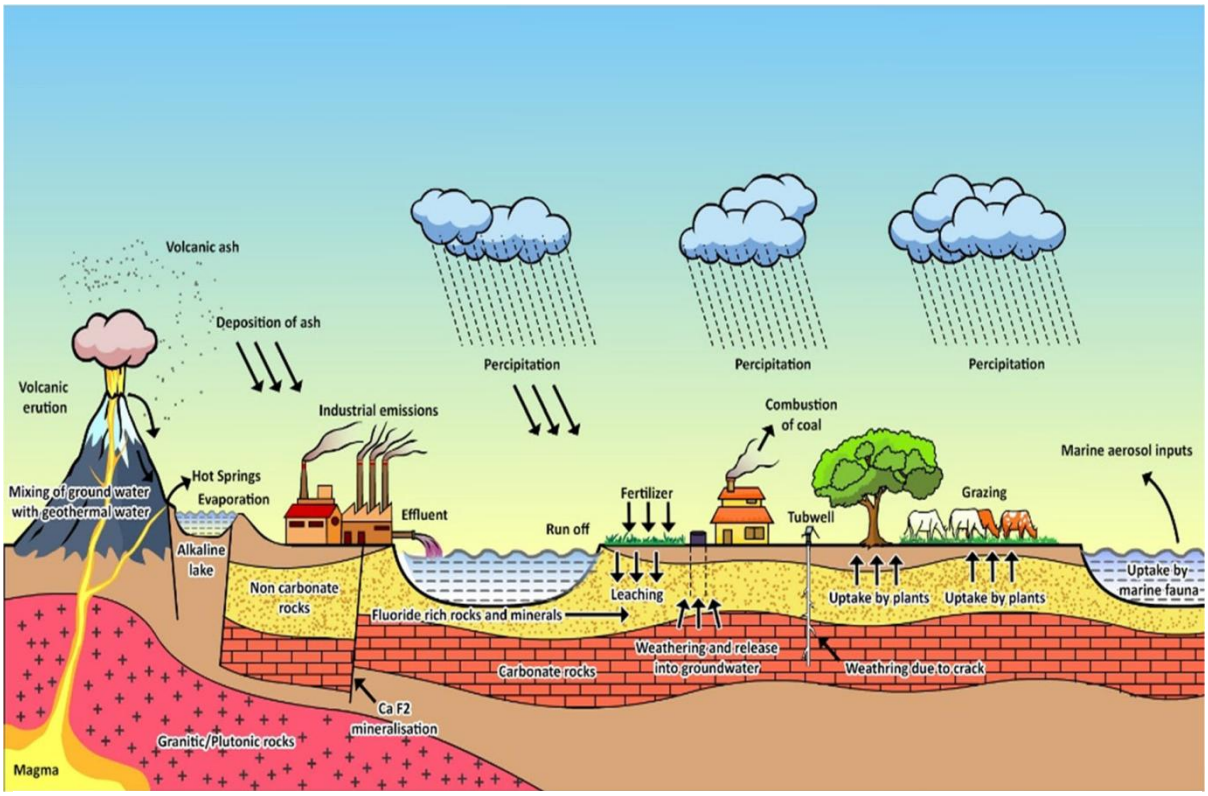
2.2.1.3 Atmospheric sources

Gupta et al. (2005) reported that precipitation and atmospheric air also contributes towards the deposition of fluoride in the environment, ranging from almost below the detection limit to 0.089 mg L^{-1} and 0.01-0.4 µg m^{-3}, respectively. According to the reports of World Health Organization (2002), lead fluoride, aluminum fluoride, calcium fluoride, calcium phosphate fluoride and sodium hexafluorosilicate are the major sources of fluoride-based particulates present in the environment, whereas major gaseous forms are hydrogen fluoride, sulfur hexafluoride, fluorosilicic acid and silicon tetrafluoride. During rainfall or precipitation, atmospheric fluoride reaches the earth surface. Other factors which controls the deposition and distribution of fluoride in the environment are temperature, wind turbulence, wind speed and wind direction (Scheringer 2009). Among all the airborne fluorides, silicon tetrafluoride and hydrogen fluoride are the most important damaging agents causing air pollution, affecting the natural vegetation and agricultural field (Malayeri et al. 2012).

2.2.2. Anthropogenic sources

In recent decades, anthropogenic activities are one of major causes of fluoride deposition in the biosphere. Industries such as aluminum, steel and iron, thermal power plants and ceramic and fertilizer industries chiefly emit fluorides in the form of ash, fluoride-rich dust, fumes and airborne particles in the environment (Kundu et al. 2001; Dey et al. 2012b). On being deposited on the earth's surface, these pollutants significantly affect the quality of water and soil that eventually lead to a loss of vegetation in the areas located in the vicinity of the above mentioned industries (Ranjan and Ranjan 2015). According to the reports of Skjelkvale (1994), deposition from aluminum industries might lead to 10 fold rise in the level of fluoride in the streams, as compared to that of normal background level of 0.05 mg L^{-1}. In addition, fluoride-based pollutants, emitted from ceramic industries, thermal power plants, brick making industries, aluminum smelting factories and glass industries are highly reactive and readily undergo hydrolysis to yield hydrogen fluoride in the environment (Vike 2005). Extensive use of non-renewable fossil fuel in the industries leads to emission of gaseous fluoride in the environment. Clay, used for brick making, contains very high level of fluoride

(Cape et al. 2003). Deposition from phosphate fertilizer industries drastically increases the level of fluoride by more than 100 times in water bodies, as compared to the level of fluoride naturally present in these water bodies (Camargo 2003). Various chemical making factories produce calcium fluoride, hydrogen fluoride and sodium fluoride that also increase the concentration of fluoride in the environment. In addition to industries, one of major factors, contributing toward fluoride pollution in soil and water bodies, includes the unprecedented use of fluoride-based phosphate fertilizers (Kundu and Mandal 2009). Wash-away from the agricultural field leads to the deposition of fluoride in the water bodies which on being used for irrigation further aggravate the conditions (Pettenati et al. 2013). According to the reports of Rao (1997), the use of superphosphate fertilizers can lead to a deposition of about 0.34 mg L^{-1} of fluoride in the water bodies (Fig. 2.1).



"Fig 2.1: Natural and anthropogenic sources of fluoride in the environment, significantly contaminating the biosphere, thus leading to its higher deposition in soil and water bodies. [Reprinted with permission from Springer Nature, Environmental Geochemistry and Health, Groundwater fluoride contamination, probable release, and containment mechanisms: a review on Indian context. Mukherjee I and Singh UK, 2018 vide license number 5587751124472]"

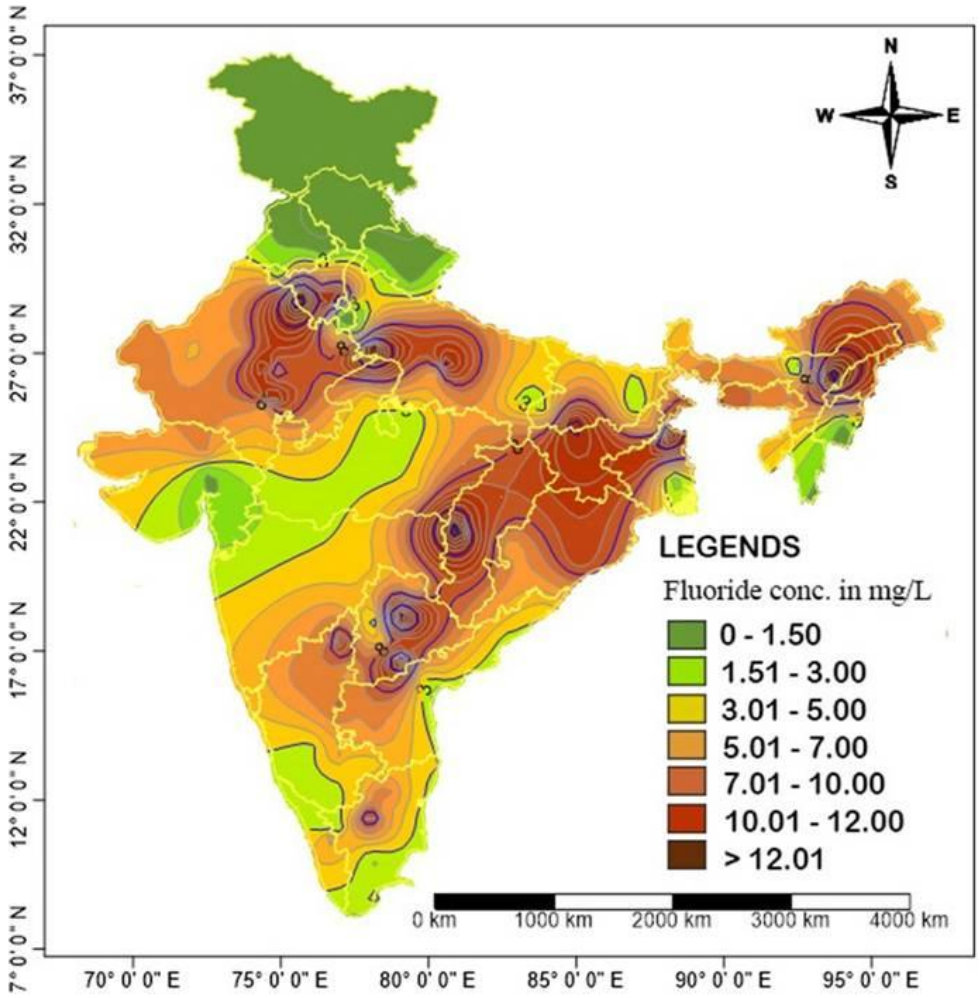
2.2.3. Natural sources

The natural sources of fluoride are marine aerosols, volcanic gases and airborne dust particles (Tavener and Clark 2006). According to Camargo (2003), marine aerosols can lead to an annual deposition of about 20 kilotons of inorganic fluoride globally. Volcanic eruptions release a significant amount of fluoride in the environment along with volcanic ash that readily gets admixed with the water vapour present in environment and finally comes down to earth's surface, thus contaminating a huge topographical area (Araya et al. 1993). In a recent report, Bellomo et al. (2007) stated that volcanic activities in Southern Italy by Mount Etna volcano releases 200 mg of hydrogen fluoride daily which is deposited by dry and wet deposition on the earth's crust (Fig. 2.1).

2.3. Extent of fluoride pollution in context to Indian subcontinent

According to the reports of World Bank (2010), groundwater accounts for about 85% of drinking water supplies in India. Around 50% urban and 80% rural population of India is directly or indirectly dependent on groundwater source for domestic purpose (Pemmaraju and Rao 2011). Thus, contaminations of groundwater with fluoride have significantly affected the Indian population. According to the reports of Chakraborti et al. (2016), more than 66 million of Indian population including 6 million children spreading across 17 different states is suffering from acute dental and skeletal problem due to consumption of fluoride-contaminated groundwater. According to the reports of Shankar et al. (2011), around 90% of the total consumable water in rural areas comes from groundwater sources such as tube wells (56%) and open wells (14%) which further worsen the conditions of people residing in these areas. Almost all the major states of India is reported to have fluoride contamination in groundwater among which Gujarat, West Bengal, Rajasthan, Andhra Pradesh, Telangana, Uttar Pradesh, Assam, Bihar, Jharkhand, Orissa, Chhattisgarh, Punjab, Madhya Pradesh, Haryana, Karnataka, Manipur and Tamil Naidu are most affected (Mukherjee and Singh 2018) (Fig. 2.2). In addition to groundwater, atmospheric deposition of fluoride due to various anthropogenic activities is also noticeable in several parts of India. Earlier report by Saxena et al. (1994) demonstrated the atmospheric deposition of fluoride by dust (30 kg km^{-2} $year^{-1}$) and rainwater (60 kg km^{-2} $year^{-1}$) in Agra due to thermal power plants, burning of fossil fuels and smelting of aluminum ore. Similar report was also published by Chandrawanshi and Patel (1999) where they showed atmospheric deposition of fluoride over

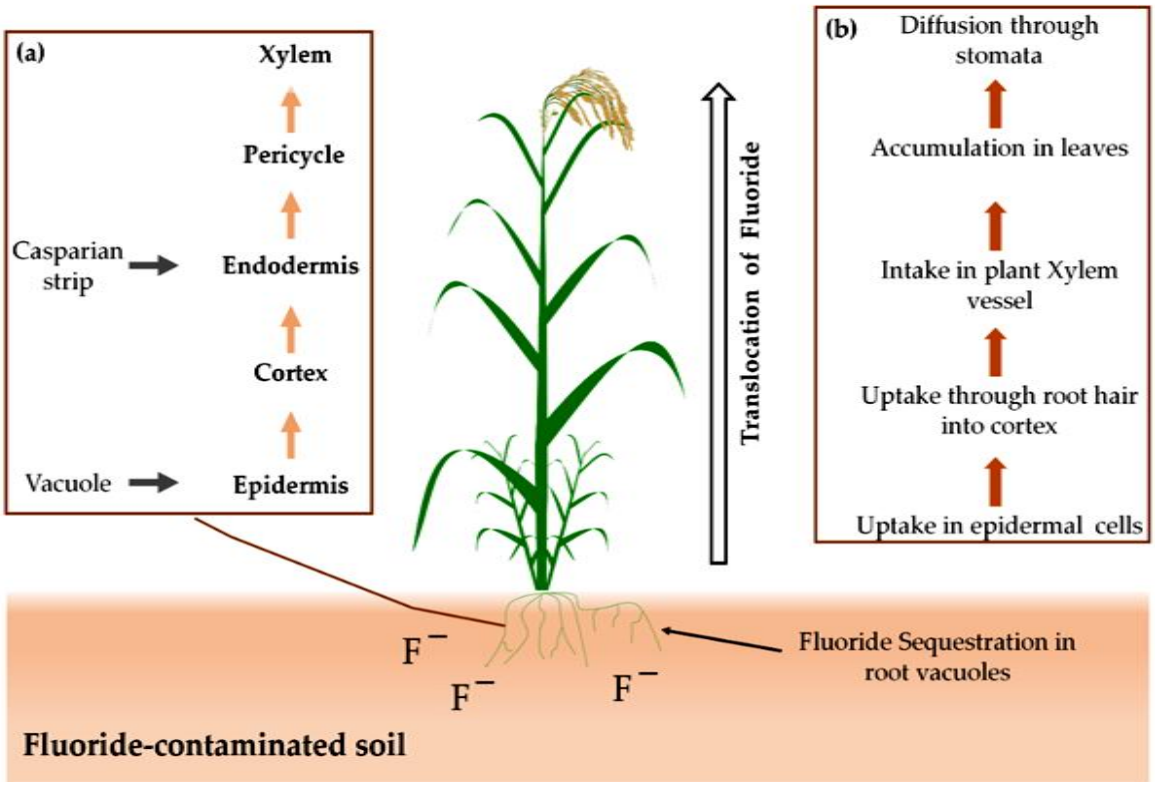
central part of India. Along with anthropogenic activities, various natural sources also contribute toward addition of fluoride in soil and water of Indian subcontinent.



"**Fig. 2.2:** Fluoride level in groundwater of Indian states [*Reprinted with permission from Springer Nature, Environmental Geochemistry and Health, Groundwater fluoride contamination, probable release, and containment mechanisms: a review on Indian context. Mukherjee I and Singh UK, 2018 vide license number 5633551033607*]."

2.4. Uptake and bioaccumulation of fluoride in plants

In recent time, fluoride toxicity has become one of the most common contaminants for plant species. Plants generally absorb fluoride from air via leaves and from soil and water via roots that ultimately leads to higher accumulation of fluoride ions in various plant parts (Anshumali 2014; Smolik 2011). Several factors such as soil properties, concentration of fluoride in soil and plant species determine the amount of fluoride uptake from the soil (Tylenda 2003). In addition, presence of other anions and pH of soil also influence the fluoride accumulation in plants (Ruan et al. 2004).



"Fig. 2.3: Fluoride uptake and accumulation in plants: (**a**) uptake mechanism in roots and (**b**) overall translocation from roots to shoots. [The image has been taken from Kumar et al. (2021) under Creative Commons Attribution License (CC BY)]"

Plants uptake fluoride ions from the soil via apoplastic transport system or anion channel. Due to the presence of positive charges on the cell wall, apoplastic uptake of fluoride is the dominant pathway in the roots of the plants. Fluoride reaches to vascular stele through free space and cell wall of epidermis and cortex. This pathway occurs especially in secondary root as it grows through endodermis and allows the entry of fluoride ions directly to the xylem/phloem, bypassing the casparian strip (Singh et al. 2018). Another way of uptake of

fluoride ions from root to shoot is via anion channels. Due to polarization, fluoride ions cannot freely cross the lipid membrane and carried out by some selective channels or carrier proteins that facilitate the efflux and influx of fluoride ions from the cells (Fig 2.3).

Accumulation of fluoride ions in various parts of plants mostly depend on its translocation from root to shoot and other parts (Agarwal and Chauhan 2014). Deposition of fluoride in leafy vegetables such as spinach and coriander (42.3 and 2.3 mg kg^{-1}, respectively) was found to be more, as compared to that of grains and fruit crops (Gupta and Banerjee 2011). According to Rhimi et al. (2016), deposition of fluoride ions was found to be higher in the leaf lamina of grape vines, as compared to that of leaf stock and internodes. Ruan and Wong (2001) demonstrated higher level of fluoride in old leaves, as compared to that of other parts.

2.5. Effect of fluoride toxicity on plants

Higher bioaccumulation of fluoride in plants from soil, water and air affects almost all the physiological and metabolic processes of plants.

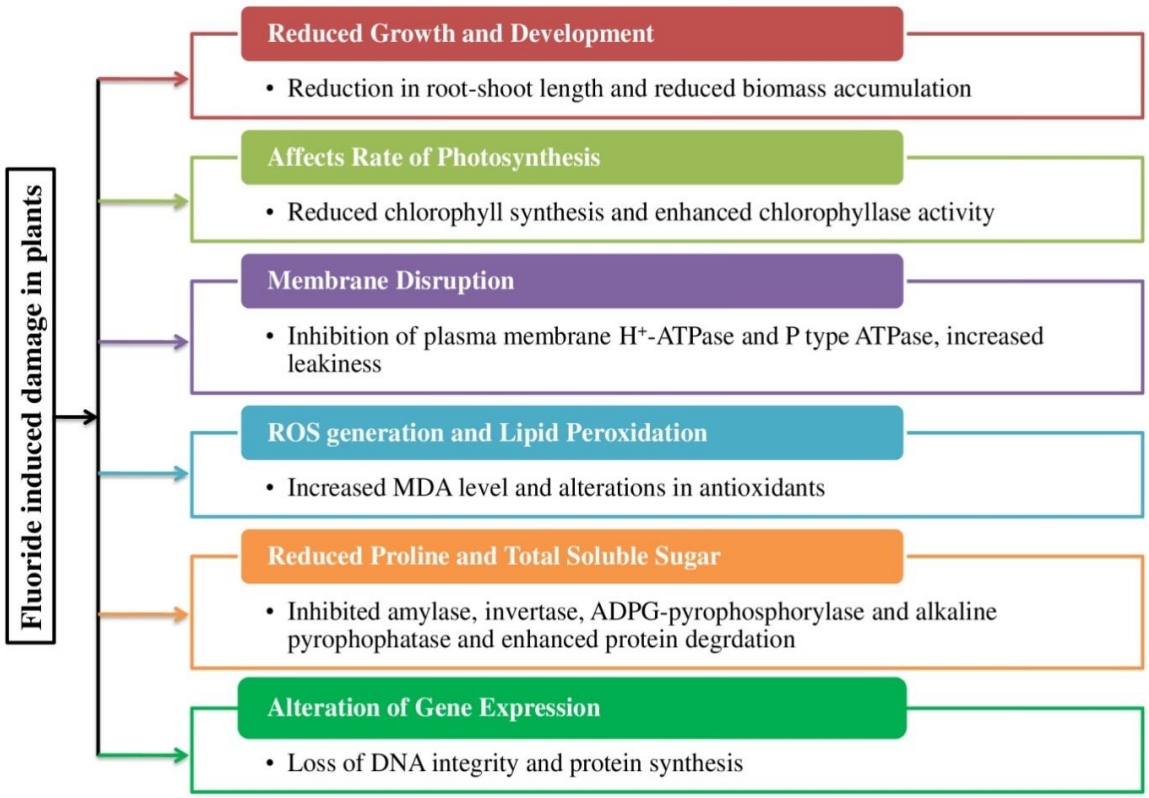
2.5.1. Effect on growth parameters

Accumulation of fluoride above the threshold level significantly lowers the germination and early development of seeds. Reduced germination of seeds might be linked with lower uptake of water and inhibited cell division which result in the enlargement of embryo and overall fall in the level of other metabolic activity that controls this complex process. In addition, Ram et al. (2014) and Panda (2015) showed that fluoride inhibited the activity of phytase enzyme that was responsible for dephosphorylation of phytin compounds, amylase activity and uptake of minerals nutrients that altogether lowered the germination rate of seeds. Fluoride-induced DNA damage also led to reduced RNA and protein synthesis that ultimately inhibited the cell elongation and cell division and consequently inhibited growth (Panda 2015). Fluoride present in atmosphere can be accumulated by stomata that results in leaf tip necrosis and chlorosis which are the primary symptoms of fluoride toxicity (Panda 2015) (Fig. 2.4).

2.5.2. Fluoride induced inhibition of photosynthesis

Fluoride stress significantly affects the rate of photosynthesis mainly by reducing the synthesis of chlorophyll or by enhancing its degradation along with inhibition of Hill reaction and degradation of chloroplast ultra-structure (Baunthiyal and Ranghar 2014). Higher

accumulation of fluoride ions was found to decrease the availability of Fe^{2+} ions that are essential for chlorophyll synthesis and to increase the activity of chlorophyllase enzyme that controls the degradation of chlorophyll and inhibits the process of photosynthesis (Yadu et al. 2016). Fluoride ions chiefly accumulate in the chloroplast of leaves which drastically affects the level of photosynthetic pigments such as chlorophyll a, chlorophyll b, anthocyanins and carotenoids (Kumar and Rao 2008). Fluoride stress affect chloroplast and sub cellular organelles along with inhibited uptake of carbon dioxide from environment that limits the process of photosynthesis in plants. In addition, fluoride ions also affect the activities of sucrose synthase, RuBisCo (ribulose 1,5-biphosphate carboxylase) and other enzymes associated with CO_2 fixation (Banerjee et al. 2019a).



"Fig. 2.4: Fluoride induced physiological and morphological damage in plants"

2.5.3. Generation of ROS

Reactive oxygen species (ROS) are groups of free radicals like superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) that are derived from oxygen (Apel and Hirt 2004). They are strong oxidizing agent that can readily react with any component of cell organelles and causes severe damage to nucleic acid, proteins and lipids (Sharma et al. 2012). Under abiotic stresses including fluoride-exposure, limited CO_2

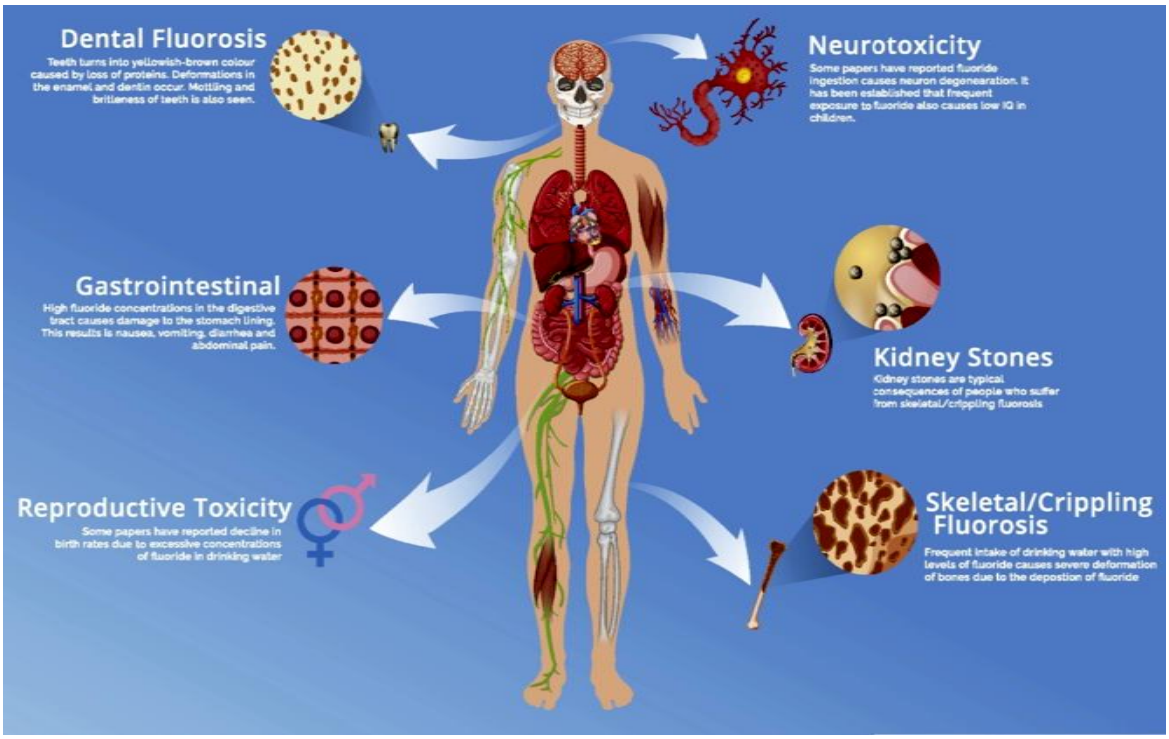
fixation occurs which leads to a decrease in both carbon reduction by the Calvin cycle, and the oxidized $NADP^+$ level. This oxidized form of $NADP^+$ serves as an electron acceptor when ferredoxin is reduced during the transfer of electrons in photosynthesis. Thus, electrons may be transferred from the photosystem I to O_2 to form $O_2^{\bullet-}$ by the process called the Mehler reaction (Gill and Tuteja 2010). Higher fluoride induces the formation of $O_2^{\bullet-}$ via the above mentioned pathways, whereas at lower concentration, fluoride enhances the formation of $OH^{\bullet}$ via Heber-Weiss reaction (Wang et al. 2004) (Fig 2.4).

Enhanced formation of ROS in the cells significantly affects the stability of macromolecules. The most prominent effect noted in the cells is the oxidation of poly unsaturated fatty acids that are important components of cell membrane. Fluoride toxicity also reduces the formation and enhances the degradation of proteins in the seedlings in both time and dose dependent manner (Datta et al. 2012; Gadi et al. 2012). Saleh and Abdel-Kader (2003) demonstrated that fluoride toxicity inhibits the pace of RNA synthesis, and nitrogen metabolism, and enhances the activity of ribonuclease that affects the turnover of protein formation. Similar to that of lipids, proteins are also affected by higher ROS accumulation that causes deleterious modification such as carbonylation, nitrosylation, glutathionylation and formation of disulfide bonds (Parkhey et al. 2014). In addition, proteins with amino acids, containing sulfur such as cysteine and methonine, are highly prone to ROS (Parkhey et al. 2014).

2.6. Effect of fluoride toxicity on humans

In humans, fluoride is important for the integrity of bones and teeth till an optimum level. Sufficient level of fluoride in the body prevents osteoporosis, increase bone density and prevent dental caries (Ericsson 1970; Arlappa et al. 2013). On the other hand, consumption of fluoride above safe limit can be highly toxic. Long term exposure to fluoride might lead to dental fluorosis which is early symptom of fluoride toxicity (Choubisa and Sompura 1996). Choubisa (2001) and Choubisa and Sompura (1996) conducted a survey in Dungarpur, Udaipur and Banswara districts of Southern Rajasthan and found that more than 21% children and 36% adults were suffering with dental fluorosis on consumption of fluoride contaminated water. Other symptoms of fluoride toxicity include limb motor dysfunction, joint pain and reduced joint movement (Choubisa and Sompura 1996). Chiba et al. (2012) stated that acute fluoride toxicity inhibited the secretion of insulin that eventually lowered the level of osteocalcin and further worsened the conditions of patients suffering from skeletal

fluorosis by inducing bone lesions (Thomas et al. 1996). Further study by Trivedi et al. (1993) demonstrated that 40% of the patients suffering from acute fluoride toxicity also show impaired glucose tolerance along with higher serum insulin on ingestion of glucose orally. Irreversible damage to hard tissues (teeth and bones) leading to osteo-dental and non-skeletal fluorosis in domesticated animals due to consumption of fluoridated water released from industries have also been well reported (Choubisa 2017).



"Fig 2.5: Detrimental effects of fluoride exposure on human health. Fluoride intoxication exerts several health hazards in human beings. It causes dental and skeletal fluorosis. In addition, fluoride-induced oxidative stress can be detrimental to renal, intestinal and central nervous system functions. [The figure has been taken from Kashyap et al. (2021) under creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)"

Along with significant effect on hard tissues, fluoride can severely affect the mammalian nervous system due to its capacity to penetrate blood-brain barrier (Zuo et al. 2018). According to Shivarajashankara et al. (2002), higher consumption of fluoride inhibits brain specific metabolism by inhibiting the activity of neuronal enzymes and inhibiting lipid peroxidation. In an interesting study conducted on rats by Cheng et al. (2002), it was shown that fluoride ions directly hamper the function of neurons, neurotransmitters and myelin. Higher fluoride consumption by pregnant mother severely affected the neonatal

neurodevelopment, as compared to that of pregnant women, who were not exposed to fluoride toxicity (Susheela et al. 1993). Similarly, Zhang et al. (2015) showed that the IQ of the children residing in the fluoride contaminated areas was significantly lower than those residing in non-contaminated areas. This proves that consumption of fluoride above threshold level drastically affects the development of nervous system (Fig 2.5).

Soft tissues such as heart, lungs, kidney and liver are very prone to fluoride toxicity. Kumar and Kumari (2011) reported that prolonged dose of fluoride severely affects the function of heart, liver and kidney. Higher risk of chronic kidney disease was noted in people who were continuously exposed to fluoride toxicity through food and water (Dharmaratne 2015). People working in aluminum mines and factories show high signs of asthma (Søyseth and Kongerud 1992). According to Macnee and Rahman (2001), higher intake of fluoride via respiration induces the formation of ROS in lungs, thus leading to oxidative stress and other respiratory diseases. In addition, excess fluoride exposure also induces the rate of cellular apoptosis and arrest cell cycle in G2 phase (Meng et al. 2014).

2.7. Internal protective mechanism of plants

In order to survive under harsh conditions, plants usually influx and synthesize various metabolites and accumulates them for regulating homeostatic status of the cells which in turn maintain the growth and development of plants. The internal protective machineries of the plants can be broadly categorized under three major groups: (i) osmolytes, (ii) non-enzymatic antioxidants and (iii) enzymatic antioxidants

2.7.1. Osmolytes

In plants, the production of osmolytes confers the osmotic adjustment in the cells that eventually protects the cellular organelles and regulates the tolerance capability of the plants. The term osmolytes generally refer to neutrally charged low molecular weight metabolites or compounds such as sugar, secondary metabolites, polyamines and amino acids (Hussain et al. 2008). Synthesis and accumulation of osmolytes is considered as one of the paramount responses of plants for combating against osmotic and oxidative stress due to various stressors. Some of the major osmolytes generated in plants in response to abiotic stresses are proline, polyamines, glycine betaine and sugars.

To protect against osmotic stress, plants induce the formation of proline which serves as antioxidant and ROS scavenger. Higher accumulation of proline in cells is due to its higher production, being catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) or due to its lower degradation (Dos Reis et al. 2012). Delauney et al. (1993) reported that glutamine serves as the precursor of proline in plants; however, in nitrogen deficient condition, orinithine serves for the same. The protective role of proline against fluoride stress is widely reported in various plant species. Recently, Chakraborty et al. (2022) reported that exogenous application of dopamine enhances the formation of proline in rice cultivar which in turn enhances the survival capability seedlings on being exposed to 50 mg L^{-1} NaF solution. Similar report was also published by Farhangi-Abriz and Ghassemi-Golezani (2022) where they also showed that treatment of the mint plant under fluoride stress significantly enhances the level of proline. In an another study conducted by Banerjee and Roychoudhury (2019a), it was shown that treatment of rice seedlings with fluoride solution significantly enhanced proline level in fluoride tolerant cultivar; however, the level was comparable to that of control seedlings in the susceptible variety. The defensive role of proline against other abiotic stress such as drought, salinity and heat has also been widely reported by various researchers in past (Molla et al. 2014; Shahid et al. 2014; Wu et al. 2017; El Sabagh et al. 2015a; El Sabagh et al. 2015b).

Polyamines are nitrogenous compounds that play a significant role in various cellular mechanisms (Kusano et al. 2007; Paschalidis et al. 2005). Several types of polyamines such as the diamine (putrescine), triamine (spermidine) and tetramine (spermine) play a pivotal role in mitigating the negative effects of abiotic stress in plants. Polyamines block the ion channels that help to achieve the osmotic balance in cells by means of inorganic ions and also scavenge ROS that in turn enhances the tolerance level of the plants. Cuevas et al. (2008) reported higher formation of putrescine during chilling stress in *Arabidopsis* that conferred tolerance to the plant. Sadeghipour (2019) and Nahar et al. (2016) reported that spermine, spermidine and putrescine enhances drought and salt stress tolerance, respectively in mung beans by increasing the net photosynthetic rate, relative water content and protein and chlorophyll content in the seedlings. In addition, exogenous application of spermine and spermidine was also shown to curtail the negative impacts of fluoride toxicity in rice plants by inducing the activity of protective machineries in seedlings (Banerjee et al. 2019b; Banerjee et al. 2019c).

In response to abiotic stresses, plants along with the above mentioned metabolites also induce the formation of glycine betaine. Choline and glycine serves as the precursor of glycine betaine (Chen and Murata 2002). By double step oxidation of choline to toxic intermediate betaine aldehyde, catalyzed by the enzymes, viz., choline monooxygenase and betaine aldehyde dehydrogenase (BADH), glycine betaine is formed. Glycine betaine treatment induces the activity of the enzymatic antioxidants which in turn scavenge the ROS generated in *Crataegus monogyna* in response to chilling stress that enhances the survival capability of the plant (Razavi et al. 2018). Al-Taweel et al. (2007) demonstrated that glycine betaine reduces the accumulation of ROS and also enhances the activity of photosystem II in tobacco plants, thus leading to higher tolerance level against drought stress.

It is widely proven that soluble sugar and starch molecules directly take part in carbon storage, ROS scavenging, stabilization of protein structures and osmotic adjustment (Strand et al. 1999; Gill et al. 2001). Starch molecules are the stored form of carbon that is finally stored in grains or fruits, whereas sucrose is mostly involved in the translocation of carbon molecules (Zhou et al. 2002). According to Krasensky and Jonak (2012), under stressed condition, remobilization of starch due to the enhanced activity of starch phosphorylase and α-amylase provides energy that is utilized by the plants to overcome the stressed condition. The degradation of starch release sucrose molecules that serve as osmoprotectant in cells. In addition, sucrose is formed in the tissues by the enzymatic activity of sucrose phosphate synthase on uridine diphosphate glucose and d-fructose 6-phosphate. Acid invertase and sucrose synthase control the accumulation of sucrose in cells by regulating its degradation. The protective role of sucrose and starch is widely reported against various abiotic stresses (López et al. 2008; Cha-Um et al. 2009).

2.7.2. Non-enzymatic antioxidants

Similar to that of osmolytes, non-enzymatic antioxidants are also electrically neutral compounds such as ascorbic acid, glutathione, carotenoids and phenolic compounds that scavenge ROS, maintain homeostasis of cell and structure of proteins and lipids.

Ascorbic acid is one of the most extensively studied antioxidants that is widely present in almost all of the cell organelles and apoplasts of plant (Domanovic et al. 2021). Ascorbic acid is generally synthesized in mitochondria and is transported to cellular compartment either by facilitated diffusion or by electrochemical proton gradient (Gill and Tuteja 2010). Ascorbic

acid plays a pivotal role in the regulation of growth and metabolism of plants along with scavenging of ROS, thus preventing oxidative damage in cells. Ascorbic acid can directly detoxify singlet oxygen, superoxide radical and hydrogen peroxide which in turn generate tocopherol as a by-product (Shao et al. 2007). The role of ascorbic acid against various abiotic stresses such as drought, light and UV-B was extensively studied in past (Eltayeb et al. 2007; Matos et al. 2022). In addition, promising role of ascorbic acid in abrogating negative effect of fluoride toxicity is also known. Gao et al. (2018) reported higher ascorbic acid formation in the tissues of *Hydrilla verticillata* on being exposed to 0, 10, 20, and 40 mg L^{-1} fluoride solution.

Glutathione is a tripeptide (γ-glutamylcysteinylglycine) that is primarily present in reduced form and its concentration is highest in the chloroplast, as compared to other cell organelles (Hajam et al. 2023). In two steps catalyzed by glutathione synthetase and γ-glutamylcysteine synthetase, the amino acids are joined in an ATP-dependent manner to form tripeptide. Glutathione plays a pivotal role in the regeneration of ascorbic acid via ascorbate-glutathione cycle (Millar et al. 2003). The protective role of glutathione against fluoride stress has been widely established by various reports in past. Chakraborty et al. (2022) reported that priming of rice seeds with dopamine enhanced the formation of glutathione in rice seedlings on being treated with NaF solution that in turn regulated the tolerance capability of the seedlings. Earlier, Sharma et al. (2019) also showed that exposure of *Spirodela polyrhiza* L. Schleiden to fluoride toxicity induced the generation of glutathione that in turn scavenged ROS and caused lesser cellular damage, maintaining the integrity of the cell membrane.

Carotenoids are tetraterpene pigments that are widely found in photosynthetic bacteria, algae, plants and animals. Carotenoids can be further categorized into carotenes (α-carotene, β-carotene, lycopene) and xanthophylls (β-cryptoxanthin, fucoxanthin, lutein, astaxanthin, zeaxanthin and peridinin). The primary activity of carotenoids is based on their conjugated double-bonded structure and ability to delocalize unpaired electrons to quench singlet oxygen (Mortensen et al. 2001). Ghassemi-Golezani and Farhangi-Abriz (2019) showed that fluoride stress reduced the level of carotenoids in safflower that in turn reduces the tolerance capability of the plant; however, on application of biochar, the level of carotenoids is restored that in turn enhances the survival ability of the seedlings. In addition, Xu et al. (2020) showed that during drought and heat stress, the level of carotenes and xanthophylls was induced in two varieties of alfalfa (Deqin and Algonguin) that in turn protected the photosynthetic

machineries and maintained the photosynthetic rate of the plant. Similarly, the role of carotenoids in abating the negative effects of other abiotic stresses is also widely accepted (Samanta et al. 2021a; Gupta et al. 2009).

Phenolics are diverse group of secondary metabolites comprising of flavonoids, lignin and tannins that are abundant in plant cells. Phenolics are strong antioxidants due to their property of being highly reactive as hydrogen or electron donors, the ability of forming the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans et al. 1997). Phenolics interfere with the oxidation of lipids and other molecules by the rapid donation of hydrogen atoms to radicals. Similar to that of other antioxidants, the role of phenolics in ameliorating the negative effects of abiotic stresses is also widely analyzed. Parida et al. (2004) reported that the treatment of *Aegiceras corniculatum* with 250 mM NaCl solution enhanced the concentration of polyphenols in cells. Similarly, Phimchan et al. (2004) also observed higher activity of phenylalanine ammonia lyase (PAL, the key enzyme involved in polyphenol biosynthesis) under drought stress led to higher accumulation of phenolics in the fruits of capsicum. The beneficial role of phenolic compounds is also established in plant species exposed to fluoride toxicity. Sharma and Kaur (2019) reported that the level of phenolic compounds was enhanced in *Spirodela polyrhiza* L. Schleiden exposed to 5, 10, 15, 20, 25 and 50 mg L^{-1} fluoride solution to abrogate the negative effects of fluoride-induced damage and conferring tolerance. This proved the beneficial role of phenolic compounds in response to fluoride stress.

2.7.3. Enzymatic antioxidants

Along with non-enzymatic antioxidants, enzymatic antioxidants also play a pivotal role in reducing the effects of oxidative stress in plants by scavenging ROS accumulated in cells. Various enzymes constitute the enzymatic antioxidant defense system. Enzymatic antioxidants either prevent Foyer–Halliwell–Asada pathway or Haber-Weiss reaction that reduces the accumulation of H_2O_2 in plants. The major antioxidants found in plants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR).

Superoxide dismutase catalyzes the detoxification of superoxide radicals to less toxic H_2O_2 molecule and oxygen. Depending on the presence of metallic co-factors, SOD has various

isoforms such as Fe-SOD, Cu/Zn-SOD and Mn-SOD. These cofactors stabilize the transition bond formed during the reaction. Superoxide radicals have one extra electron that is transferred to these cofactors which ultimately passes the extra electron to hydrogen to yield H_2O_2 and release oxygen as the end product. Enhanced SOD activity in rice and *Phaseolus vulgaris* cultivars was detected in response to drought stress (Sharma and Dubey 2005; Zlatev et al. 2006). Eyidogan and Oz (2007) reported higher activity of all the three isoforms of SOD against salinity stress in *Cicer arietinum*. Similarly, higher SOD against fluoride stress was also reported in rice seedlings (Banerjee and Roychoudhury 2019a).

Catalase does not require any reductant for its activity. In a two step reaction, H_2O_2 first oxidize Fe atom present in CAT to produce iron peroxide as intermediate, followed by the second step where another molecule of H_2O_2 reacts with iron peroxide to produce water and oxygen (Oshino et al. 1975). Multiple isoforms of CAT have been reported to be expressed at different developmental phases and abiotic stresses in plants (Anjum et al. 2016). The activity of CAT was differentially regulated in plants in response to abiotic stresses. Fluoride stress inhibited the activity of CAT, attributed to the replacement of hydroxyl groups present in the iron atoms of the CAT by low molecular weight fluoride ions when present in sufficient amount (Kumar et al. 2009). Enhanced activity of CAT was also noted in case of other abiotic stresses like drought, salinity, heat and heavy metal stress (Kukreja et al. 2005; Shi et al. 2018; Zafar et al. 2020).

Ascorbate peroxidase is class I haem-peroxidase and is otherwise known as ascorbate-dependent peroxidase due to its dependency on ascorbate as a cofactor to scavenge H_2O_2. APX has been localized in cytosol, chloroplast, mitochondria and membrane-bound cell organelles such as glyoxisome and peroxisome. According to Teixeira et al. (2005), the expression of *APX* gene varies in tissues, based on developmental stages and abiotic stresses. Salt and drought stress induced the expression of *APX* gene in French bean seedlings (Nageshbabu and Jyothi 2013; Eltelib et al. 2012). In a similar study, Caverzan et al. (2012) reported higher APX activity in the cold tolerant maize cultivar, but not in the sensitive variety. Cadmium chloride significantly boosted the APX activity in salt tolerant rice cultivar, whereas in the salt sensitive variety, the level was comparable to that of control seeding (Roychoudhury and Ghosh 2013). Siddiqui (2013) also reported higher APX activity during lead and salt stress in *Vigna radiata* seedlings.

Glutathione peroxidases are the family of multiple isozymes that detoxify the generated H_2O_2 in plant cell in response to abiotic stresses. A number of previous studies have highlighted the role of GPX in regulating the stress tolerance capability of plants against abiotic stresses (Passaia et al. 2013; Islam et al. 2015). Zhai et al. (2013) showed that overexpression of wheat *GPX* gene in *Arabidopsis* enhanced its tolerance capability against salinity stress. Similarly, Passaia et al. (2013) reported overexpression of five *GPX* genes in rice seedling on exogenous application of H_2O_2 and cold stress. GPX isoform was also reported in rice seedlings on being exposed to arsenic stress (Samanta et al. 2021b).

Glutathione reductase is a flavoprotein oxidoreductase that catalyzes the conversion of glutathione disulfide to its reduced form using NADPH as a reductant. According to Gill et al. (2013), GR is mostly localized in the stroma of chloroplast along with its isoforms found in cytosol, peroxisomes and mitochondria. Saruhan et al. (2009) showed leaf rolling in response to water scarcity accompanied by higher GSH level. Additionally, higher GR activity during drought in plants such as rice, wheat, barley and maize is widely reported (Selote and Chopra 2006; Chinnusamy et al. 2005; Kocsy et al. 2002; Chen et al. 2004). Similarly, the higher GR activity during salt stress was also reported in rice, tomato, pea and wheat (Hasanuzzaman et al. 2013; Repetto et al. 2012; Szalai et al. 2009). Elevated GSH level and activity of GR suggested efficient elimination of H_2O_2 in mustard and apple seedlings that enhanced their thermotolerant capacity (Kuk et al. 2003). Similarly, the promising role of GR during fluoride stress was also reported in past by Banerjee and Roychoudhury (2019a).

2.8. Role of externally applied chemical agents

The above mentioned protective metabolites significantly regulate the tolerance capability of crops against abiotic stresses. In past, researchers have externally applied various chemical agents that further boosted the formation and activity of these internal protective metabolites which was beneficial for growth and maintaining proper yield of the crops against abiotic stresses. Calcium and silicon are two such chemical agents whose promising role against abiotic stresses in plants is widely known.

2.8.1. Calcium

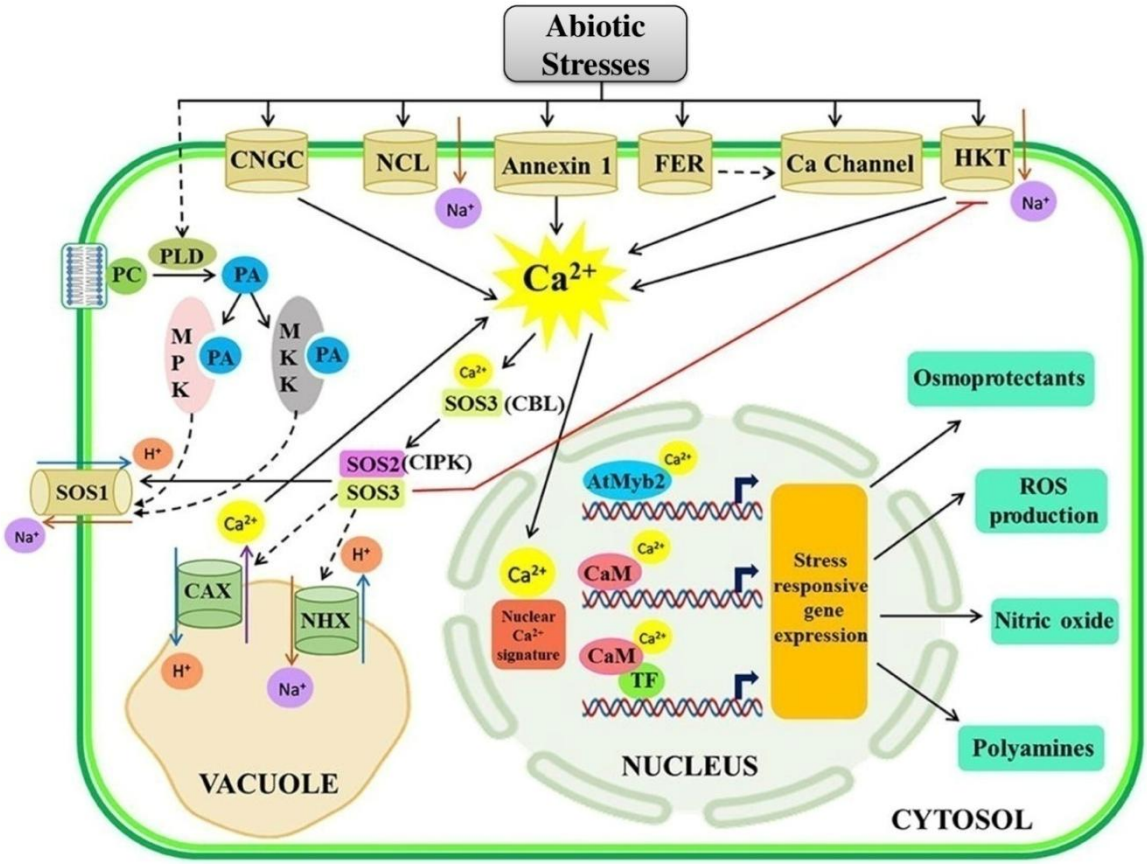
Calcium (Ca) is an important macronutrient for both plants and animals that plays an indispensable role in signaling. Calcium ions also play a pivotal role in maintaining normal metabolism and physiological function of plant cells (Poovaiah et al. 1987). In response to abiotic stresses such as salinity, drought, and low temperature, the cytosolic level of Ca^{2+} ions was enhanced in cells and the signal was transmitted downstream by Ca^{2+}-binding proteins (calmodulin or Ca^{2+} sensors) that regulated plant cell division, elongation, stomata movement and stress response (Gao and Zhang 2019; Zhao et al. 2013; Jing et al. 2019; Iqbal et al. 2020). Higher level of Ca in the cytosol was achieved by Ca^{2+} efflux through pumps and influx through channels. In normal condition, Ca does not remain at high concentration in cytosol as it reacts with phosphoric acid (needed for energy production) to produce by-product that would precipitate and inhibit normal physiological growth of cells and even lead to cell death (Li et al. 2022). To avoid this, Ca^{2+} ions are mostly present in bound form with Ca storing protein in plant cells collectively known as Ca pool (Matthus et al. 2019).

Calcium is also known as secondary messenger in plant cells. Calcium sensors in plants are mainly composed of Ca binding proteins like calmodulins (CaMs), calcineurin B-like (CBL) proteins, Ca dependent protein kinases (CDPKs) and calmodulin like (CML) proteins. The role of Ca signaling in plants cells in response to stimuli of abiotic stresses is widely studied. CBLs interact with CBL-interacting protein kinases (CIPKs) to form a CBL/CIPK signaling network, which plays a key role in the plant response to abiotic stress. These networks may contain many interactions, with CBLs activating CIPKs and CIPKs phosphorylating CBLs. Phosphorylation is the major mechanism affecting downstream proteins (Ma et al. 2020).

2.8.1.1. Ca as a signaling molecule

In resting stage, Ca ions remain in the bound form and Ca channels usually remain in closed state. On receiving external stimuli, Ca channels opens, that lead to rapid dissociation of Ca^{2+} ions from the bonded proteins due to their low affinity for the Ca^{2+} ions and influx of Ca^{2+} ions in the cytosol locally occurs (Matthus et al. 2019). Local influx of Ca^{2+} ions may affect other Ca channels that in turn significantly enhance the cytoplasmic Ca level or they may only activate local Ca channel in the vicinity to influence cellular processes (Medvedev 2005). Coelho et al. (2002) reported that in response to hyperosmotic shock, local Ca^{2+} signal was generated in the embryo rhizoids of *Fucus* sp. They further reported that this local rise in

Ca^{2+} in the cytoplasm was noted near the nucleus region which lasted for about 15-30 miliseconds. According to Dawson (1997), the process of Ca signal propagation in the cytoplasm is achieved by sequential activation of Ca^{2+} channels and presence of Ca^{2+} pumps in endoplasmic reticulum (Fig 2.6).



"Fig 2.6: A generic pathway for calcium regulated gene expression and the stress response. The calcium level increases in response to the stimuli. The increased level of Ca^{2+} is recognised by some Ca^{2+}-sensors or calcium-binding proteins, which can activate many calcium dependent protein kinases. These kinases regulate the function of stress responsive genes, resulted in the phenotypic response of stress tolerance. CNGC: Cyclic nucleotide gated channel; NCL: Na^{+}/Ca^{2+}exchanger like protein; FER: FERONIA; HKT: High-affinity K^{+} transport; PC: phosphatidylcholine; SOS: salt overlay sensitive; PLD: Phospholipase D; PA: Phosphatidic acid; CaM; Calmodulin; TF: Transcription factors; CBL: Calcineurin B-like; CIPK: CBL-interacting protein kinases; CAX: Cation exchanger; NHX: Na^{+}/H^{+} transporter; MPK: Mitogen-activated protein kinase; MKK: Mitogen-activated protein kinase kinase. [The image has been taken from Patra et al. (2021) the Creative Commons Attribution License (CC BY)]"

The amplification of Ca signal is perceived by other secondary messengers IP_3, binding with Ca^{2+}-binding sensor proteins or by modification of cytoskeleton elements. Upon binding with Ca^{2+}-binding sensor proteins such as CaMs, CDPKs, CMLs and CBLs, the calcium signal is transmitted to the effectors (ionic channels, enzymes, cytoskeleton proteins and transcription factors) via reversible phosphorylation of proteins (Sanders et al. 1999). Upon completion of the inevitable process, Ca^{2+} signal is stopped by the excess removal of Ca^{2+} by Ca pumps from the cytoplasm or by its sequestration in the cell organelles, accompanied by the dephosphorylation of proteins that return cells to their initial stage (Trewavas and Malho 1998; Sanders et al. 2002)

2.8.1.2. Role of Ca as protective agent

2.8.1.2.1. Drought Stress

Drought stress or water scarcity is one of most common environmental factors that significantly inhibit the growth and development of plants. Additionally, it also enhances the formation of ROS that promotes peroxidation of lipid membrane (Abid et al. 2018). Kuromori et al. (2022) monitored the water potential of root vascular system and observed that plants relay stress signals to the leaves that lead to stomatal closure, thus improving water use efficiency of plants by reducing the rate of transpiration flux. Damage caused due to drought stress is reduced by the activation of Ca channel mediated closure of stomata that reduced transpiration rate to avoid dehydration (de Carvalho 2008). Feng et al. (2016) demonstrated that drought stress up regulated the efflux of Ca^{2+} ions in mesophyll and epidermal cells in roots of barley seedlings. Additionally, treatment of *Arabidopsis* plants with exogenous Ca lowered the generation of ROS which protected the plasma membrane, maintained normal photosynthetic and metabolism rate that in turn regulated the tolerance capability of the drought stressed seedlings (Zhu et al. 2013). The adverse effect of drought stress is also curtailed by the Ca induced activation of SOD, APX, CAT, GR and peroxidases (POD) that scavenge the ROS generated and thus reduces the impairment of lipid membranes (Hasanuzzaman et al. 2012; Jiang and Hunag 2001) (Fig 2.7).

2.8.1.2.2. Salinity stress

Salt stress limited the growth of plants by causing osmotic balance, ionic toxicity and oxidative stress that affects the sustainability of crops. Under normal condition, presence of

salt content in the soil above the threshold level led to higher uptake of sodium ions (Na^+) by the roots, thus causing hypersaline stress. Ca plays a pivotal role in regulating plant tolerance against salt stress. During salt stress, exogenous application of Ca inhibits the uptake of Na^+ from the soil by regulating nonselective cation channels. Moreover, Ca inhibits the efflux of potassium ions (K^+) from the cells that initiates salt overly sensitive (SOS) signaling pathway and maintains the plasticity of roots (Hryvusevich et al. 2021; Wegner and de Boer 1997; Mahajan et al. 2008). Ma et al. (2019) reported that during salt stress, the cytoplasmic level of Ca in the roots enhances that causes activation of SOS signaling pathways, transduced by SOS3-SOS2-SOS1. Initially, SOS3 acts as a Ca binding protein that interacts with SOS2 to form complex that finally activates SOS1 via phosphorylation (Ma et al. 2014). Vaghela et al. (2009) reported that exogenous application of Ca reversed the effects of saline stress by maintaining the level of protective metabolites, increasing the uptake of nutrients (potassium, nitrogen and phosphorus) and reducing the accumulation of Na^+ ions in the leaves. Similarly, Shariat Jafari et al. (2009) reported that application of Ca and potassium diminished the negative effects of saline stress in sorghum plants. Thus, various mechanisms have been proposed to establish the beneficial role of Ca against salinity stress. Firstly, Ca reduces the uptake of Na^+ ions and enhances the concentration of Ca^{2+} and K^+ ions in the cytoplasm that maintains membrane integrity and secondly it enhances the activity of various enzymatic antioxidants and formation of protective metabolites (osmolytes and non-enzymatic antioxidants) that scavenge the ROS and maintain osmotic balance of the cells (Bolat et al. 2006; Shoresh et al. 2011) (Fig. 2.7).

2.8.1.2.3. Heat stress

Similar to that of drought and salinity stress, heat stress also generates ROS that cause membrane damage and osmotic imbalance, inhibited photosynthetic rate, cell ageing and cell death (Suzuki and Katano 2018). Exogenous application of Ca has shown promising role in abrogating negative impact of heat stress in plants like chrysanthemum, tomato and laver (Hu et al. 2021; Li et al. 2009b; Xing et al. 2021). Dou et al. (2015) reported that exogenous spraying of calcium chloride ($CaCl_2$) in heat stressed tomato seedlings improved stomatal conduction, photosynthetic rate and transpiration rate. Similarly, Hu et al. (2021) also reported that exogenous application of $CaCl_2$ increases the activity of protective enzymes, enhances soluble protein content and reduces the concentration of malonic acid in leaf of tomato seedlings, thus inducing adaptability against heat stress. In an interesting study, Li et

al. (2012) and Zheng et al. (2020) reported that under heat stress, the expression of *CAM1* and *CAM2* was enhanced in *Pyropia haitanensis* that enhanced the survival capability of seedlings. However, in presence of EDTA (ethylene diamine tetraacetic acid; Ca chelator), the expression of both the genes was not significantly up regulated that drastically reduced the tolerance level of the algae. Pretreatment of wheat seedlings with Ca enhanced the formation of Ca based heat shock proteins (Kolupaev et al. 2005) during heat stress. Similarly, Larkindale and Knight (2002) also reported higher formation of Ca dependent heat shock proteins in *Arabidopsis* that induced thermotolerance of the plants (Fig 2.7).

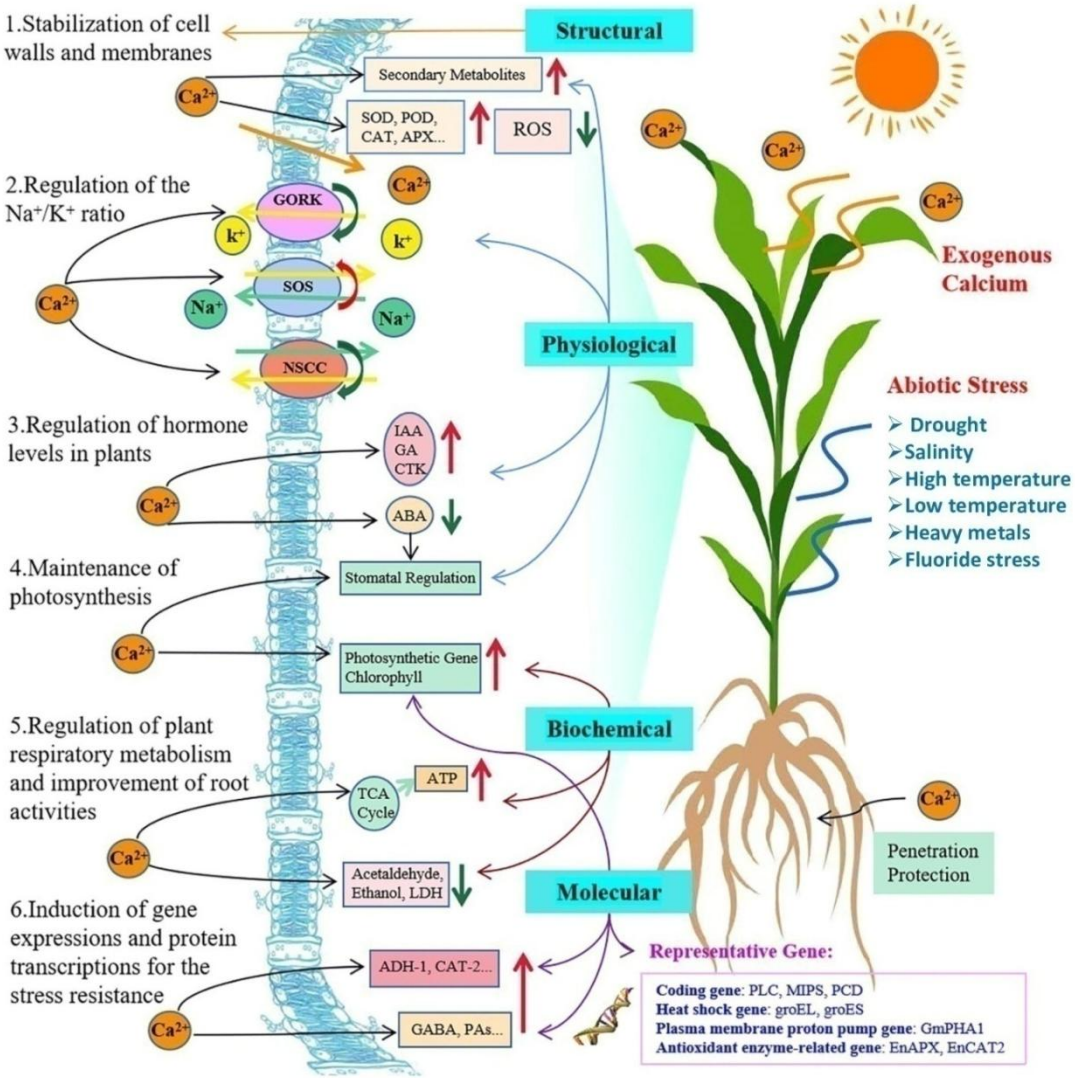
2.8.1.2.4. Cold stress

Cold stress induces the production of ROS in plants, thereby damaging the membrane system and photosynthetic machineries (Suzuki and Mittler 2006). In comparison to that of drought, salinity and heat stress, the role of Ca in cold stress is not well studied and only a few reports are available. Shi et al. (2014a) reported that in response to cold stress, the level of Ca was enhanced in leaves and roots of Bermuda grass that was associated with higher cold tolerance level due to higher formation of antioxidants. Ca treatment in cold stressed tobacco seedlings increased the endogenous Ca level that improved the activity of enzymatic antioxidants such as CAT, SOD and POD and reduced the accumulation of ROS and cytotoxic metabolites like malondialdehyde (Luo et al. 2018). In citrus plants, Ca and CaM could regulate freezing tolerance, while treatment with exogenous Ca chelator like EDTA or CaM blocker could inhibit the freezing tolerance of the plants (Li et al. 1997) (Fig. 2.7).

2.8.1.2.5. Heavy metal toxicity

Rapid industrialization and various anthropogenic activities have drastically increased the content of heavy metals in soil (Hasanuzzaman and Fujita 2012). Under heavy metal stress, the most common response in plants are higher ROS formation, reduced nutrient uptake from soil, higher membrane dysfunction, inhibited photosynthetic rate and reduced water potential (Roychoudhury et al. 2012; Emamverdian et al. 2015). Exogenous application of Ca countered the toxicity of heavy metals in various plants species. Suzuki (2005) reported that exogenous application of $CaCl_2$ restored root elongation and growth of the cadmium stressed seedlings. Exogenous application of 5 mM calcium nitrate $Ca(NO_3)_2$ curtailed the harmful effects of cadmium toxicity (40 µM) in *Lens culinaris* (Talukdar 2012). Application of Ca could also resist the uptake of heavy metal such as chromium, lead and copper from

contaminated soil that improved the tolerance ability of the plants (Kinraide 1998; Huang and Huang 2008). Maksymiec and Baszynski (1999) reported that presence of copper in soil above threshold level led to higher accumulation of Ca in plant roots that might be regarded as a protective measure. Addition of Ca in combination with other metabolites was also highly effective in ameliorating the symptoms of abiotic stresses. Fang et al. (2014) reported that chromium stress enhanced the formation of hydrogen sulfide (H_2S) and Ca signal transduction in *Setaria italica*. They further reported that Ca in combination with H_2S can drastically reduce the effects of chromium toxicity; however, when Ca chelating agent along with H_2S synthesis inhibitor was used, the effect of chromium toxicity was restored in seedlings. In addition, Ca also plays a significant role in the prevention of electrolyte leakage from cells, membrane stabilization and in maintenance of cellular pH (Hirschi 2004) (Fig 2.7).



"**Fig. 2.7:** Plant self-responses to abiotic stresses and mechanisms of exogenous calcium involved in enhancement of plant stress tolerance. The red and green arrows indicate a promotion/increase or an inhibition/decrease, respectively. GORK: Guard cell outward rectifying potassium channels; SOS: Salt overly sensitive; NSCC: Non-selective cation channel; IAA: Indole-3-acetic acid; GA: Gibberellic acid; CTK: Cytokinin; ABA: Abscisic acid; SOD: Superoxide dismutase; POD: Peroxidase; CAT: Catalase; APX: Ascorbate peroxidise; ROS: Reactive oxygen species; ATP: Adenosine-triphosphate; TCA: Tricarboxylic acid; LDH: Lactate dehydrogenase; ADH: Alcohol dehydrogenase; GABA: Gamma-aminobutyric acid; PA: Polyamines; PLC: Phospholipase C; MIPS: Myo-inositol-3-phosphate synthase. [The image has been taken from Feng et al. (2023) the Creative Commons Attribution License (CC BY)]"

2.8.1.2.6. Fluoride toxicity

Sulzbach and Pack (1972) reported that the presence of divalent metals like Ca^{2+} in the soil can combine with fluoride ions and thereby significantly reduce their uptake in the plant tissues which lowered fluoride induced damage in the cells. However, the protective role of Ca against fluoride toxicity in plants is less studied and very few works have been done to showcase the beneficial role of Ca in response to fluoride stress. Ruan et al. (2004) reported that exogenous application of $Ca(NO_3)_2$ could drastically reduce the uptake and accumulation of fluoride ions in tea plants. Similarly, Dey et al. (2012a) demonstrated that exogenous application of 10 mM $CaCl_2$ alleviated the fluoride (20 mM NaF) induced damage in Bengal gram seedlings by reducing the uptake of fluoride ions and maintaining the formation of protective metabolites. The role of Ca compounds in the removal of fluoride ions from water has also been reported earlier which showed that treatment of ground water before field application might also reduce the accumulation of fluoride ion in the agricultural fields (Margaretha et al. 2018) (Fig. 2.7).

2.8.2. Silicon

Earlier, the role of silicon in growth, development and protection of plants against stressed environment was not fully known and thus silicon was considered as non-essential element for plants. However, with time, the importance of silicon and its versatile role in providing protection to the plants was deciphered by various researchers which ultimately make it a quasi-essential "multi-talented" micronutrient for plant growth (Zargar et al. 2012). Some of the pivotal roles of silicon in plants are protection against abiotic stresses, enhancing

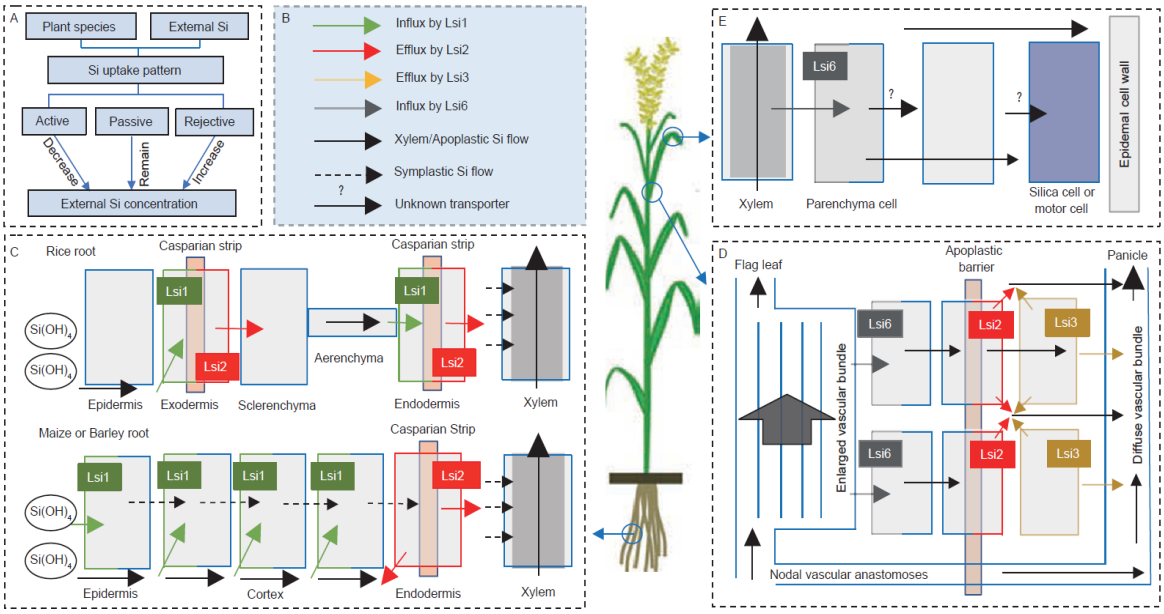
photosynthetic rate and nitrogen fixation and enhancing yield and crop quality (Zargar et al. 2019). Ample amount of silicon is present in earth's crust and is considered as the second most abundant element present in soil after oxygen; however, most of it is present in silicon dioxide forms that cannot be taken up by the plants. According to Marxen et al. (2015), silicon on being taken up by the roots gets deposited in the tissues, thus making them rigid and stronger. Based on silicon accumulation ability, plants can be classified into low silicon accumulator (accumulates silicon less than 0.2% of their dry weight) and high silicon accumulator (deposits silicon more than 1.5% of their dry weight) (Tubana and Heckman 2015). Graminaceous plants such as barley, rice, wheat and sugarcane absorb silicon in much higher quantity, as compared to that of plants like beans and tomatoes (Nikolic et al. 2007). After uptake of silicon from the soil, graminaceous plants deposit silicon in the epidermal tissues where in presence of pectin and calcium, silicon forms a protective barrier that provides protection to the plants against abiotic and biotic stresses (Belanger et al. 2003; Rodrigues et al. 2003). Silicon also interacts with phytohormones such as jasmonic acid and ethylene that provides protection to the tomato plants against *Ralstonia solanacearum* (Ghareeb et al. 2011). Thus, the protective role of silicon against abiotic stress in plants cannot be neglected which makes it an agronomically essential element.

2.8.2.1. Uptake and transportation of silicon in plants from soil

Silicon is mostly absorbed by the roots as mono silicic acid [$Si(OH)_4$)]. According to Liang et al. (2015), available form of silicon that can be taken up by the plants from the soil ranges in between 10 to 100 mg kg^{-1} soil. However most of the silicon present in soil is in insoluble form and is of no use in agronomy. Natural source of silicon in soil is quartz, diopside, calcite, idocrase and garnet (Zargar et al. 2019).

Monocot plants such as rice can deposit silicon in their tissues up to 10% of their weight. The high accumulation of silicon in plants can be attributed to the transporter present in the roots that can be classified in two types: silicon influx transporter [Lsi (Low silicon) 1 and Lsi6] and silicon efflux transporter (Lsi2 and Lsi3). Lsi1 is mainly involved in the uptake of silicon from the soil. Ma et al. (2006) first reported the presence of silicon transporter in rice plants. After being absorbed from the soil, the deposited silicon in the cells is effluxed out by Lsi2 into the apoplast from where it is again translocated upward to the shoot via transpiration system. Along with transpiration system, plants have other pathways to control silicon

transportation. Ma et al. (2011) and Yamaji et al. (2015) demonstrated that during developmental phase, the distribution of silicon is mainly regulated by vascular system near the nodes. According to Yamaji et al. (2015), Lsi1 along with Lsi6, uptake silicon from the soil via roots and transfer it to xylem transfer cells from where it is again symplastically transported by Lsi2 through bundle sheath cells. Finally, before the accumulation of silicon in the grains, silicon is transported by Lsi3 via parenchyma cell layers (Fig. 2.8).



"Fig. 2.8: A schematic model of silicon (Si) transport in plants modified from Ma and Yamaji (2015) and Yamaji et al. (2015). **(A)** Three types of Si uptake. **(B)** Arrows with different colors or symbols showing different Si flow in C–E. **(C)** Two possible Si uptake and radical transport pathways in root. **(D)** Possible Si transport and distribution model in node. **(E)** Possible Si unloading and deposition pathway in leaf."

2.8.2.2. Role of silicon during abiotic stress

2.8.2.2.1. Drought stress

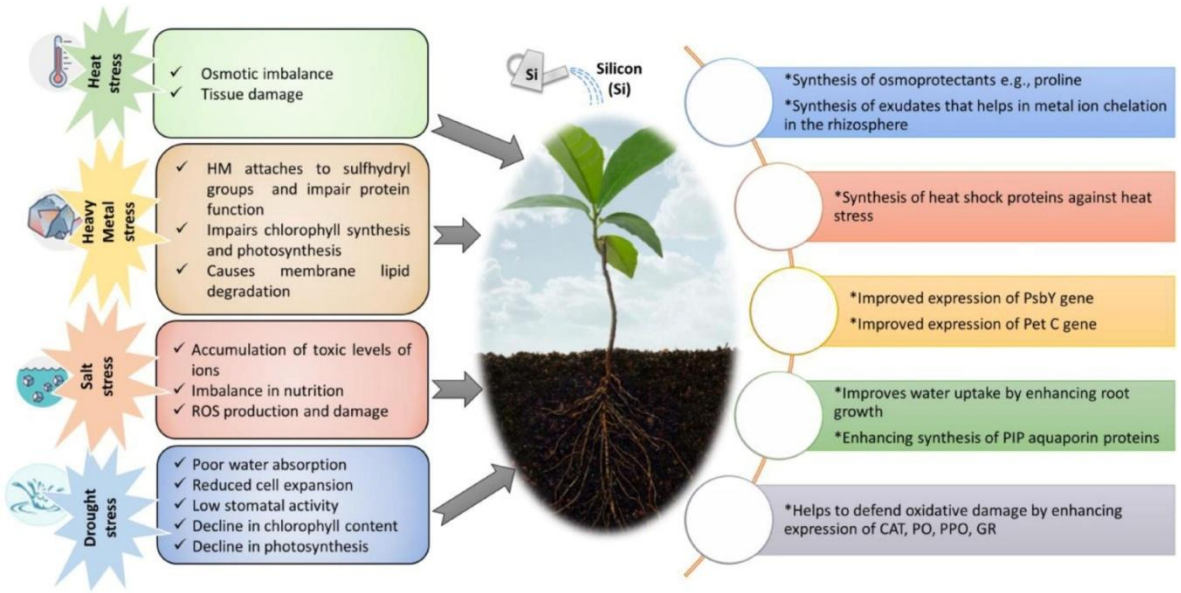
Silicon curtails the negative symptoms of drought by various mechanisms such as up regulation of aquaporin gene and mitigation of ROS induced inhibition of aquaporin activity (Zargar et al. 2019). Chen et al. (2018) reported that application of silicon improved the root hydraulic conductance by enhancing root/shoot ratio, modifying root growth and enhancing the activity of aquaporin channels. In presence of silicon, higher uptake and transportation of

water help to maintain higher photosynthetic rate and thus tolerance to drought stress in plants (Luyckx et al. 2017). Similarly, Zargar and Agnihotri (2013) reported that in presence of calcium silicate, the germination of seeds of maize plants exposed to drought stress was enhanced. They further reported that silicon application also enhanced the biochemical processes and protected the seedlings from ROS induced oxidative damage by enhancing the formation of antioxidants. Another major effect of drought stress is impaired uptake of essential nutrients by plants (Emam et al. 2014). Earlier, Gunes et al. (2008) reported that exogenous application of silicon in soil significantly enhanced the uptake of macronutrients i.e., Ca, Mg, K and P and micronutrients like Mn, Cu and Fe in crops exposed to drought stress. Additionally, Emam et al. (2014) also reported that treatment of rice plants with silicon enhanced the deposition of P and K in the straw of drought stressed plants. Moreover, application of silicon also reduced the effects of drought induced oxidative stress by inducing the activity of enzymatic antioxidants in plant species such as sunflower, chickpea, tomato and wheat (Gunes et al. 2008; Gunes et al. 2007a; Shi et al. 2014b; Tale Ahmad and Haddad 2011) (Fig. 2.9).

2.8.2.2.2. Salinity stress

Similar to that of drought stress, silicon mediated tolerance in plants is regulated by maintenance of water balance, lower ionic accumulation in cells, higher mineral uptake from soil and biosynthesis of compatible solutes. Garg and Bhandari (2015) reported that exogenous application of silicon fertilizers lowered the absorption of Na^+ ions from the soil and also maintained K^+/Na^+ ratio in salt stressed *Cicer arietinum* that effectively abrogated the negative effects of salinity stress. Presence of excess salt in soil also hampered the uptake of macro and micronutrients from the soil in plants. Abdalla (2011) reported that in presence of silicon, the absorption of K, Mg, Ca and P was restored in salt stressed Egyptian clover plants. Likewise, the same was also reported in case of tomato plants where salt stress inhibited the absorption of Mg and Ca by roots (Gupta and Huang 2014). Silicon application also maintained transpiration rate, stomatal conductance, photosynthetic rate, leaf water status, water use efficiency and root hydraulic conductance in various crops like wheat, rice, cucumber, okra and maize (Chen et al. 2014; Mahdieh et al. 2015; Amirossadat et al. 2012; Abbas et al. 2015; Rohanipoor et al. 2013). Moreover, exogenous application of silicon further enhanced the formation of osmoprotectants like proline, glycine betaine and amino acids in salt stressed okra seedlings (Abbas et al. 2015). Silicon application also enhanced the

activity of enzymatic antioxidants that reduce the effects of oxidative damage by maintaining plasma membrane H^+-ATPase, membrane fluidity and reduced ROS generation (Al-aghabary et al. 2005). In addition, silicon application also activated the genes related to salt stress (*DREB1*, *DREB2* and *DREB3*), antioxidants (*APX*, *SOD* and *CAT*) and silicon transporters (*Lsi1*, *Lsi2* and *Lsi3*) that up regulated the tolerance level of tomato seedlings (Muneer and Jeong 2015) (Fig. 2.9).



"**Fig. 2.9:** Impact of abiotic stress on various aspects of plant growth and defense mechanisms evoked by application of silicon. [The image has been taken from Mir et al. (2022) under the Creative Commons Attribution License (CC BY)]"

2.8.2.2.3. Heat stress

Plants evolved a diverse array of tolerance mechanism that includes formation of heat shock proteins, induction of phytohormones and ROS detoxification. Khan et al. (2020) demonstrated that heat stress stimulated the formation of antioxidants and heat shock proteins (HSPs) in tomato seedlings on application of silicon. Similarly, exogenous administration of silicon was also effective in improving biomass production and shoot length in heat stressed cucumber, sword fern and rice (Liu et al. 2009; Sivanesan et al. 2014). According to Chalanika De Silva and Asaeda (2017), higher thermotolerance in submerged macrophytes could be due to silicon induced higher synthesis of photosynthetic pigments like chlorophyll a, chlorophyll b and carotenoids. Saha et al. (2021) also reported that exogenous application of silicon up regulated the expression of heat tolerance genes such as *DREB2*, *HSFA1a*,

HSFA1b, *HSFA2*, *HSFA3*, *HSFA7* and *MAPK1* in tomatoes. Most remarkably, the heat shock factors (HSFs) and HSPs interact with diverse signaling cascades triggered by Ca^{2+}, phospholipids, salicylic acid, ethylene and ABA to circumvent the negative impacts of heat stress on crop plants (Sharma et al. 2020). Hence, these reports strongly suggest that Si-induced HSPs are at the forefront of establishing proper folding and preventing denaturation and aggregation of cellular proteins through phytohormone regulation under heat-stressed conditions (Abdelrahman et al., 2017) (Fig 2.9).

2.8.2.2.4. Cold stress

According to Habibi (2015), temperature below 15°C can be considered as chilling stress, whereas that below 0°C led to freezing stress in plants that severely influenced their development and growth. Foliar application of silicon effectively maintained the integrity of cellular membrane and reduced photoinhibition in grapevine plants exposed to cold stress. Various reports also suggested that silicon treatment can modulate the level of phytohormones such as jasmonic acid, ABA and salicylic acid that in turn induced cold tolerance in plants (Rastogi et al. 2021; Eremina et al. 2016). Moradtalab et al. (2018) reported that silicon enhance cold tolerance by affecting hormonal balance and micronutrient uptake from soil in the early growth phase of maize plants. Silicon alleviates cold stress by modulating the formation of protective metabolites and activity of apoplastic enzymes in leaf apoplasm in barely seedlings (Joudmand and Hajiboland 2019). Silicon in combination with other elements such as selenium was also shown to be beneficial to circumvent the negative effects of cold stress in cucumber plants under field conditions (Hu et al. 2022).

2.8.2.2.5. Heavy metal toxicity

Silicon mediated detoxification of heavy metal toxicity includes various mechanisms such as immobilization of toxic metal in the soil and co-precipitation of heavy metal with silicon that lowers heavy metal concentration in plant tissues (Gu et al, 2011). Additionally, Zhang et al. (2008) reported that exogenous application of silicon stimulated the activity of enzymatic antioxidants and non-enzymatic antioxidant formation and led to the compartmentalization of cadmium in the metabolically inactive part of root cell walls that ultimately lowered their translocation into the shoot of rice plants. Application of silicon based fertilizer can also immobilize toxic metals in soil either by changing metal speciation that led to the formation of silicate complexes or by changing the pH of the soil (Adrees et al. 2015). Sahebi et al.

(2015) reported the importance of silicon in mitigating Mn and Al toxicity in crops such as beans, cucumber, maize, rice, barley, tomato, pumpkin and cowpea. Silicon supplement lowered peroxidation of lipid membrane by inducing the activity of SOD, GR and APX and formation of non-enzymatic antioxidants in cucumber and cowpea (Shi et al. 2005; Iwasaki et al. 2002). In another study, Kim et al. (2014) reported that silicon application significantly down regulated the expression of genes encoding the transporter of heavy metals such as *HMA2* and *HMA3* and up regulated the expression of silicon transporters like *Lsi1* and *Lsi2* in rice plants. It has been observed that silicon treatment accelerated root vascular tissue development, casparian bands and suberin lamellae in maize under cadmium stress (Lukacova et al. 2013; Vaculík et al. 2012). Siliocn application in hydroponic solution also enhanced the uptake and translocation of macro-elements such as Ca, P, K and Mg and micro-elements like Zn and Mn in wheat plants under chromium, copper and cadmium stresses (Rizwan et al. 2012; Tripathi et al. 2015; Keller et al. 2015) (Fig. 2.9).

2.8.2.2.6. Fluoride toxicity

Protective role of silicon in above mentioned abiotic stresses is widely known, however its importance in fluoride stress is not significantly deciphered. Recently, Sogarwal et al. (2023) reported that mixing of silicon fertilizer (200 and 300 mg kg^{-1} soil) in soil alleviated the toxic effects of fluoride stress, improved plant growth and antioxidant defense mechanism, and restricted fluoride uptake and translocation from roots to shoot in wheat cultivars. However, reports are quite limited in this area and in coming time, the importance of silicon in plants exposed to fluoride stress needs to be analyzed.

2.9. Role of metabolic cycle in amelioration of fluoride stress

Plants produce a wide array of different metabolites in environmental or spatiotemporal dependent manner (Fang et al. 2019). This large diversity of metabolites can be divided into two types such as primary metabolites that are directly required for plant growth such as tricarboxylic acid (TCA) cycle intermediates and secondary metabolites that are involved in regulating plant-environment interaction such as nitrogen containing compounds and sulfur containing compounds (Erb and Kliebenstein 2020).

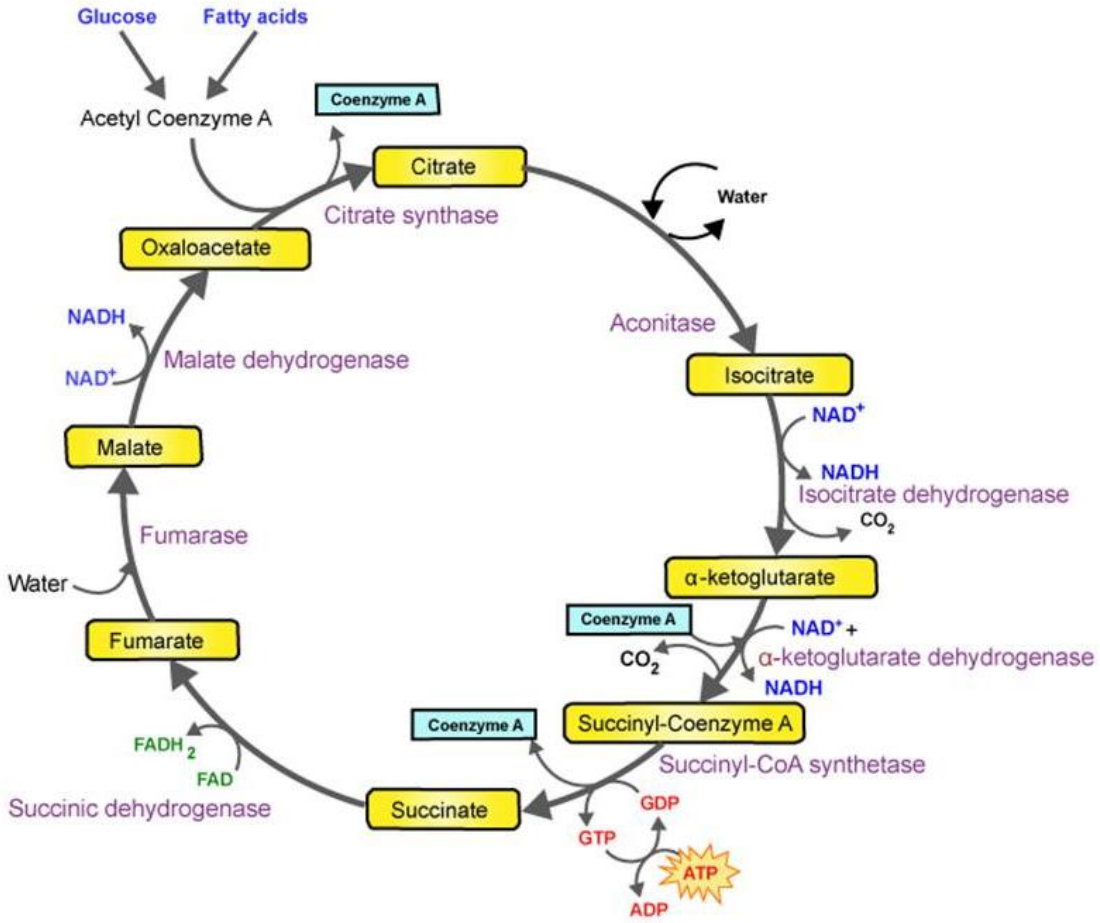
2.9.1. Tricarboxylic acid (TCA) cycle

The TCA cycle or Kreb's cycle metabolites have long been recognized as vital biomolecules needed in cells to produce macromolecules and cellular energy. It is consider as the central metabolic hub for energy production in cells which constantly oxidize carbon to produce energy rich molecules like NADH and $FADH_2$ and also connects glycolysis with electron transport chain (Zhang et al. 2023). Additionally, organic acids such as pyruvate, citrate, fumarate and malate produced as intermediate of TCA cycle serves as osmoprotectants that mitigate the negative impact of abiotic stresses by maintaining the osmotic balance of the cells and scavenging the ROS formed (López-Bucio et al. 2000).

TCA cycle is composed of a series of reactions catalyzed by several enzymes which in turn produce energy rich molecules as a by-product. Pyruvate, the end product of glycolysis, is initially converted into acetyl coenzyme A by the catalytic action of pyruvate dehydrogenase complex, accompanied with the release of NADH molecule. Acetyl coenzyme A thus produced serves as a precursor molecule for TCA cycle which is utilized by citrate synthase to produce isocitrate. Isocitrate is further converted into α-ketoglutarate by the reaction catalysed by isocitrate dehydrogenase along with the release of high energy NADH molecule. Other major step of TCA cycle is the catalysis of α-ketoglutarate into succinate by α-ketoglutarate dehydrogenase which again releases a NADH molecule. Succinate is converted into fumarate by succinate dehydrogenase along with the formation of $FADH_2$ molecule. Fumarate is again converted into malate in presence of water molecule by fumarate dehydrogenase which is finally converted into oxaloacetate by malate dehydrogenase along with production of NADH molecule (Fig. 2.10).

The TCA cycle is regarded as the major pathway of carbon metabolism in cells (Schnarrenberger and Martin 2002). Nunes-Nesi et al. (2013) and Jacoby et al. (2013) stated that TCA cycle connects carbon and nitrogen metabolism along with the release of energy molecules that makes it a central point of metabolism in plants cells. In a recent study, Peng et al. (2023) reported that tea which is a natural hyperaccumulation of fluoride showed higher formation of organic acid such as citrate and malate along with the activity of PyDH in the roots of plants, that efficiently regulated the carbon metabolism in cells and provided sufficient energy to overcome the toxic effects of fluoride stress. Similarly, Banerjee et al. (2019a) also reported that fluoride stress significantly affected the activity of enzymes of

TCA cycle in susceptible rice cultivar, i.e., IR64 which lowered its survival capability due to reduced carbon assimilation and energy formation on being compared to that of tolerant cultivar (Gobindobhog) where in presence of fluoride solution, the activity of the above mentioned enzymes was significantly induced. In addition, the beneficial role of TCA cycle in mitigating the negative effects of other abiotic stress such as drought, salinity, heat and heavy metal stress is also widely demonstrated (Guo et al. 2018; Krasensky and Jonak 2012; Nunes-Nesi et al. 2013; Sil et al. 2018; Sanchez et al. 2008).

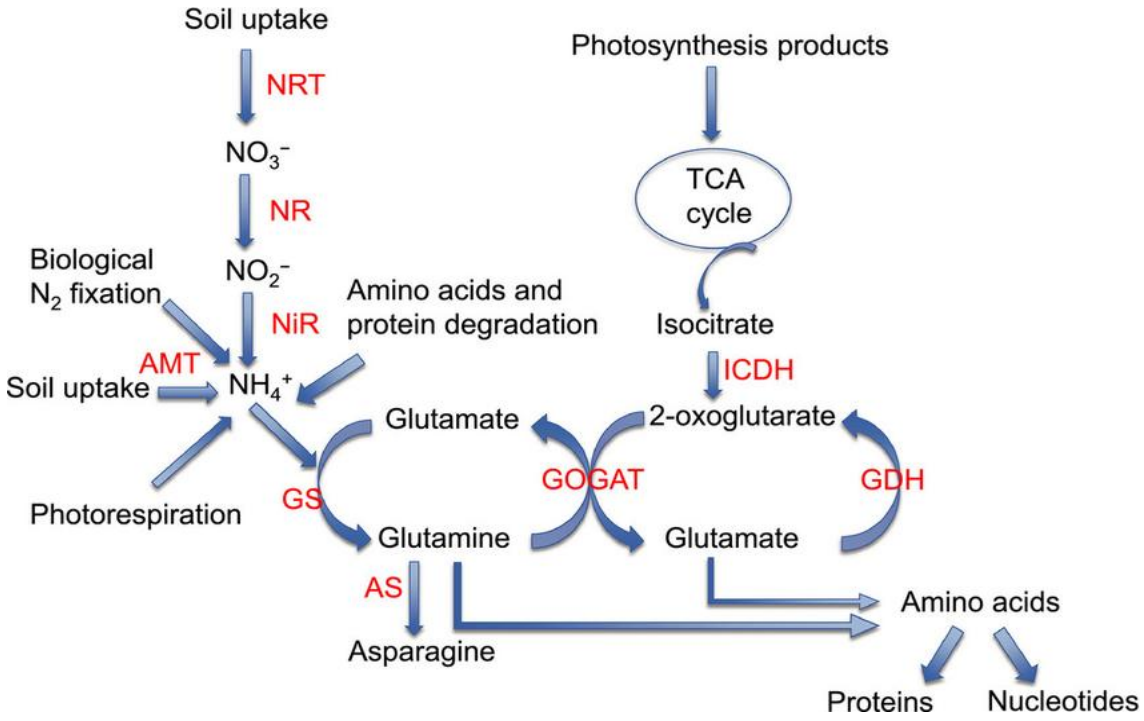


"Fig. 2.10: Overview of TCA cycle. TCA Cycle is a cyclic reaction system consisting of a series of enzymatic reactions, starting with the formation of citric acid from acetyl coenzyme A and oxaloacetate, followed by four dehydrogenations, a horizontal phosphorylation of the substrate, and finally the production of two molecules of carbon dioxide and the re-formation of oxaloacetate."

2.9.2. Nitrogen metabolism

Nitrogen is one of most essential macronutrients for general plant function as it is essential for the synthesis of amino acids that is essential unit of enzymes and proteins. In addition, nitrogen is also one of the fundamental elements found in nucleic acids, polyamines, hormones and chlorophyll (Zhang et al. 2013). In soil, nitrogen is present in both inorganic (nitrate and ammonia) and organic (urea, short peptide and free amino acids) forms. In aerobic soil, nitrate (NO_3^-) is the most abundant form of nitrogen which is readily taken up by the transporters present in the roots of the plants. NO_3^- thus absorbed is converted into nitrite (NO_2^-) by the catalytic action of nitrate reductase in the cytoplasm. Again, NO_2^- is converted into ammonium ions (NH_4^+) by the action of nitrite reductase, which is rapidly converted into glutamate or glutamine by glutamate synthase and glutamine synthetase, respectively due to its toxic nature (Gangwar and Singh 2011). Glutamate synthase and glutamine synthetase together forms the glutamate synthase/glutamine synthetase cycle. The amino acids thus produced serves as a precursor of other amino acids, proteins, nucleic acids, variety of metabolites and photosynthetic pigments that are required for normal growth and development of the plants (Singh et al. 2009). Glutamate is also catabolised by the enzymatic action of glutamate dehydrogenase (GDH) to release 2-oxoglutarate which serves as a substrate for TCA cycle and thus acting as a connector between carbon and nitrogen metabolism in plants (Fig 2.11).

In past, various studies have shown the importance of nitrogen metabolism in plants exposed to abiotic stresses. Banerjee et al. (2019a) demonstrated that the activity of nitrate reductase was enhanced that maintained the assimilation of nitrogen in fluoride tolerant rice cultivar, whereas the same was reduced in the susceptible variety. Asthir and Tak (2017) reported that upon treatment of two wheat cultivars, i.e., HD 3086 (fluoride-tolerant) and WH 1105 (fluoride-sensitive), higher glutamate oxaloacetate transaminase and glutamate pyruvate transaminase activity was noted in HD 3086 which marked the efficient conversion of glutamate and glutamine into other amino acids and thus higher protein, as compared to that of WH1105. Better nitrogen utilization by HD 3086 cultivar can be related to its higher tolerance level as to that of WH1105. Along with this, the beneficial role of nitrogen metabolism in various crops against other abiotic stresses is also widely known (Sil et al. 2020; Ghosh et al. 2013; Gou et al. 2020; El-shora and Ali 2011; Kusano et al. 2011).

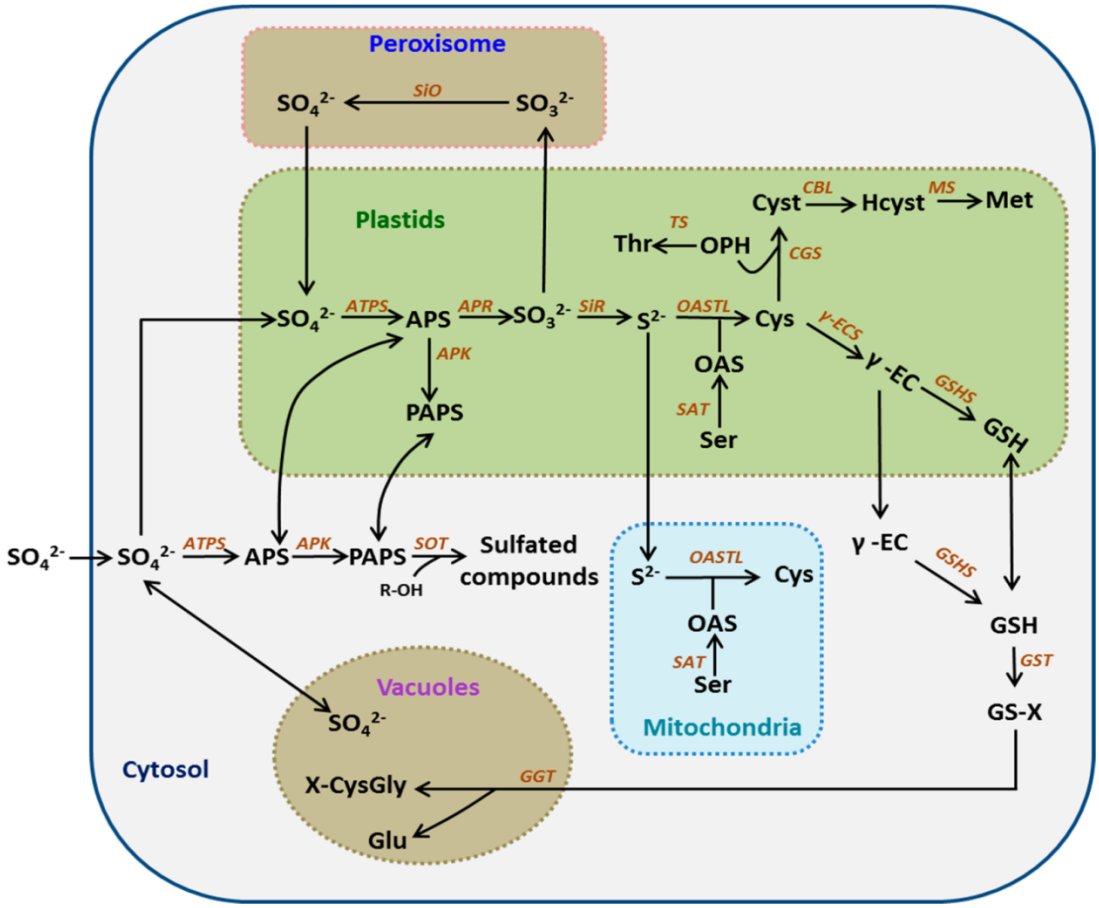


"Fig. 2.11: Nitrogen-assimilation pathway in higher plants. Inorganic nitrogen in the form of nitrate or ammonia becomes incorporated into amino acids and other organic molecules as depicted. The specific steps shown include enzymes: nitrate transporters (NRT), nitrate reductase (NR), nitrite reductase (NiR), ammonium transporters (AMT), glutamine synthetase (GS), glutamate synthase (GOGAT), asparagine synthetase (AS) and glutamate dehydrogenase (GDH). Link between nitrogen metabolism and TCA cycle is also shown in the figure. [The image has been taken from Lu et al. (2016) under the the Creative Commons (CC BY)]"

2.9.3. Sulfur metabolism

Along with carbon and nitrogen, sulfur is also an essential macronutrient for plant development and growth and response to environmental changes. It is essential for the biosynthesis of sulfur containing amino acids like cysteine, methionine, proteins, vitamins and co-enzymes (Yoshimoto et al. 2003). According to Davidian and Kopriva (2010), sulfur is mostly present as sulfate (SO_4^{2-}) in soil which is taken up by the sulfate transporters present in the roots. Once within the cells, sulfate is rapidly converted into adenosine 5'-phosphosulfate by the catalytic action of ATP-sulfurylase (ATP-S) (first enzyme of sulfur assimilatory pathway) (Leustek et al. 1994). Adenosine 5'-phosphosulfate is finally converted into sulfide (S_2^-) by a series of enzymatic reactions. Reduced sulfide ions react with O-acetyl

serine to give cysteine in reaction catalyzed by O-acetylserine(thiol) lyase (OAS-TL). According to Gill and Tuteja (2011), cysteine is the first organic compound of sulfur assimilatory pathway containing reduced sulfur in cells. Level of cysteine is regulated in the cells by the action of cysteine desulfhydrase (DES) that converts cysteine into hydrogen sulfide (H_2S) (Vojtovič et al. 2020). H_2S also plays a pivotal role in regulating the tolerance capability of plants under stressed conditions by the activation of defense mechanisms and alleviating stress-induced injuries in cells (Hancock 2019; Corpas 2019). Various reports have shown that exogenous application of sulfur can play a pivotal role in abrogating the negative effects of environmental stressors by maintaining usual metabolic process (Hasanuzzaman et al. 2018) (Fig. 2.12).



"Fig 2.12: The sulfate assimilation and metabolism pathway. Enzymes are indicated in orange letters. APS: adenosine 5′-phosphosulfate; Ser: serine; OAS,: *O*-acetylserine; Cys: cysteine; Thr: threonine; Hcyst: homocysteine; Met: methionine; γ-EC: γ-glutamylcysteine; GSH: glutathione; Glu: glutamate; ATPS: ATP sufurylase; APK: APS kinase;; SiR: sulfite reductase; SAT: serine acetyltransferase; OAS-TL: OAS (thiol)lyase. [The image has been taken from Li et al. (2020) under the the Creative Commons (CC BY)]"

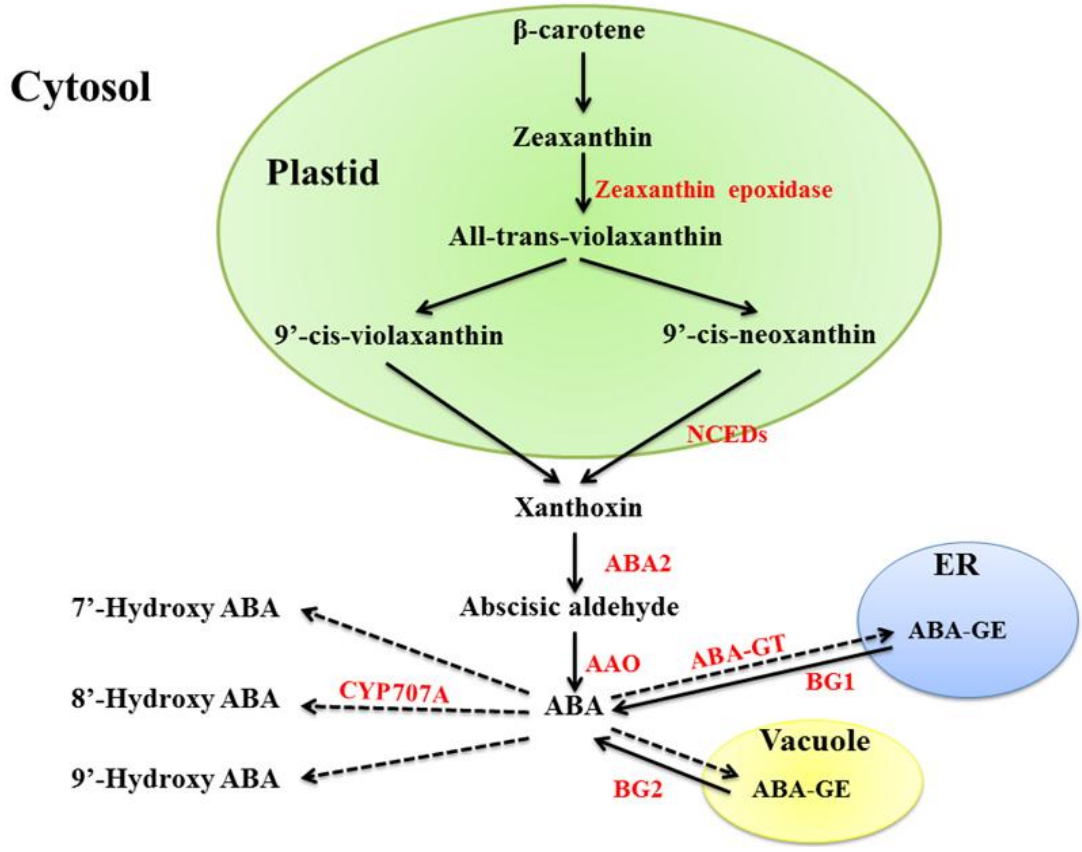
2.10. Abscisic acid: major stress regulator phytohormone

Phytohormones are important for plant growth and development under stressed environment. Among all the phytohormones, abscisic acid (ABA) is the central mediator of abiotic stress responses. ABA is an isoprenoid phytohormone that regulates various physiological processes like bud dormancy, seed germination, stomatal closure, cuticle wax deposition, osmotic regulation and leaf senescence (Roychoudhury et al. 2013). It provides adaptation to salinity, drought and cold stresses and acts as a signaling molecule during plant adaptive response to environmental condition (Benderradji et al. 2021). During stressed condition, the concentration of ABA is induced in the cells that then bind to its receptors to initiate signal transduction, leading to stress response. Therefore, ABA is also known as "universal stress hormone" (Mehrotra et al. 2014). After its discovery, various works have demonstrated the promising role of ABA in abrogating the negative effects of abiotic stress in plants. The ABA-dependent signaling pathway leads to the up regulation of osmotic stress-responsive genes like *TRAB1*, *Responsive to ABA 16A* (*Rab16A*), *Oryza sativa embryonic abundant* (*Osem*) and *dehydration-responsive element binding 2A* (*DREB2A*) that enable the plants to counteract oxidative stress, incurred by salinity (Basu and Roychoudhury 2014). Similarly, Yu et al. (2016) also showed that *Osem* acts as a positive regulator of drought tolerance and its overexpression in rice plants resulted in up regulated expression of *late embryogenesis abundant* (*LEA*) genes like *Rab16A*, *Rab16C*, and other related genes. In addition, ABA also induces the formation of other protective metabolites like polyamines, proline and glycine betaine that in turn further enhances the tolerance capability of plants.

2.10.1. ABA biosynthetic pathway

ABA ($C_{15}H_{20}O_4$) is a type of metabolite known as terpenoid or isoprenoid. Isopentenyl is a five-carbon (C5) molecule that acts as a precursor of ABA. According to Finkelstein (2013), ABA is directly produced from farnesyl diphosphate in fungi, whereas in case of plants, it is indirectly synthesized from β-carotene. Nambara and Marion-Poll (2005) reported that zeaxanthin epoxidase (encoded by *AtABA1*) marks the initiation of ABA formation in *Arabidopsis* by converting zeaxanthin to all-trans-violaxanthin. Further, North et al. (2007) stated that violaxanthin is converted to neoxanthin by *AtABA4*. The cleavage of cis-isomers of neoxanthin and violaxanthin to a C-15 compound is catalyzed by nine-cis-epoxycarotenoid dioxygenase (NCED) enzyme which controls the entire ABA biosynthesis process by

regulating the rate-limiting step. All the above mentioned steps take place in plastids, while the last two steps, i.e., formation of ABA aldehyde from xanthine (catalyzed by AtABA2) and oxidation of ABA aldehyde to ABA [catalyzed by abscisic aldehyde oxidase (AAO)] takes place in cytosol (Seo et al. 2004). Along with de novo synthesis of ABA, Xu et al. (2012) reported that ABA biosynthesis can occur in vacuole and endoplasmic reticulum by hydrolysis of ABA glucosyl ester (ABA-GE) catalyzed by *AtBG2* and *AtBG1*. During stressed condition, ABA formation mostly occurs via degradation of ABA-GE that rapidly induces the concentration of ABA in cells. To regulate the level of ABA, plants mediate the catabolism of ABA via hydroxylation and glucose conjugation. Saito et al. (2004) demonstrated that of all the three methyl groups present at C-9′, C-8′ and C-7’, hydroxylation mostly occurs at C-8′ position catalyzed by the protein encoded by *AtCYP707A* gene. Along with hydroxylation, conjugation of ABA with glucose is catalyzed by ABA-glucosyltransferase (Fig. 2.13).



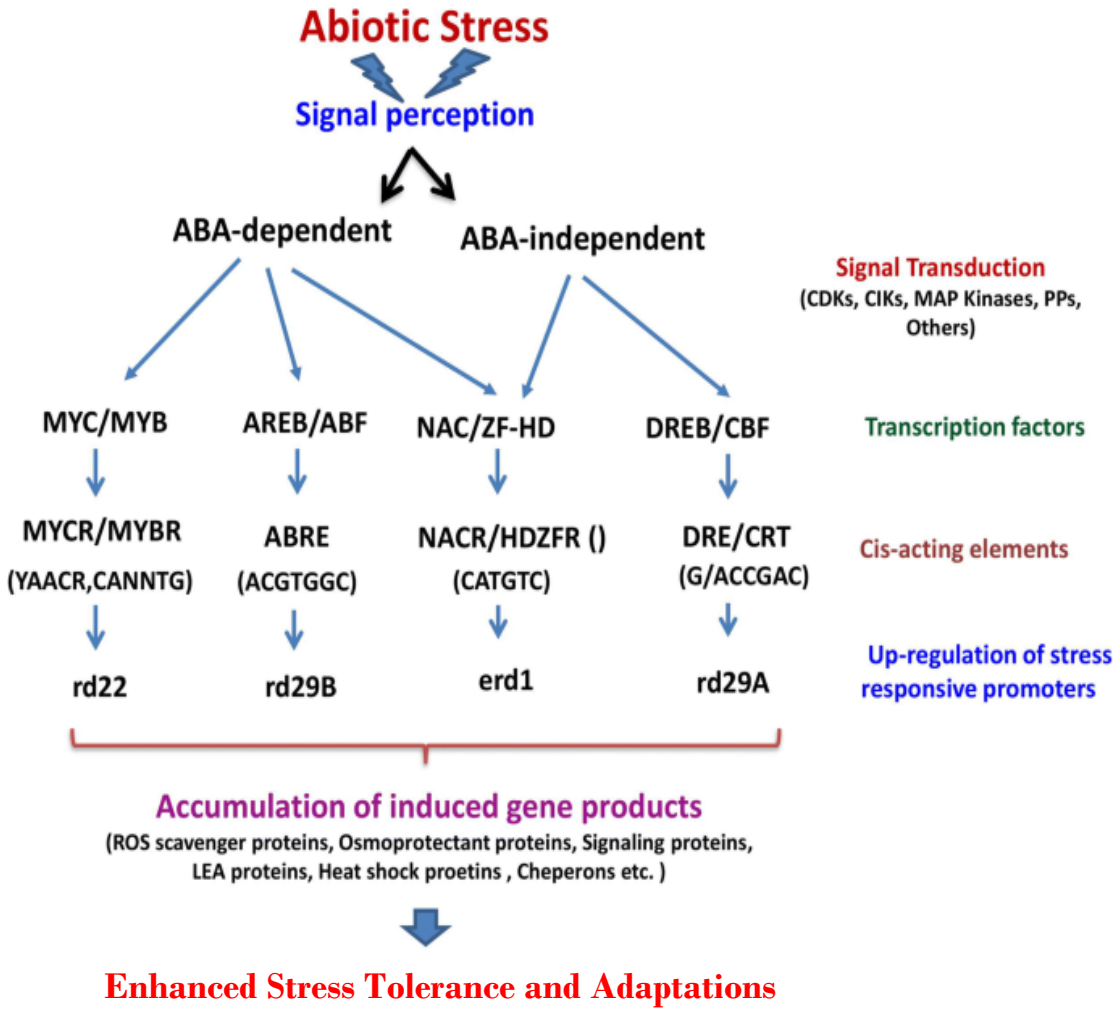
"Fig. 2.13: Metabolism of ABA in higher plants; β-carotene serves as the precursor of ABA biosynthetic pathway. The biosynthesis of ABA begins with the epoxidation of zeaxanthin (formed from β-carotene) to produce all-trans-violaxanthin by the catalytic activity of the enzyme, zeaxanthin epoxidase, which is further converted into 9′-cis violaxanthin and 9′-cis-neoxanthin. The cleavage of cis-isomers of neoxanthin and violaxanthin to a C-15 compound (xanthoxin) is catalyzed by nine-cis-epoxycarotenoid dioxygenase (NCED) enzyme which controls the entire ABA biosynthesis process by regulating the rate-limiting step. Xanthoxin is next converted to abscisic aldehyde by the action of ABA2. Finally, abscisic aldehyde is converted to ABA by abscisic aldehyde oxidase. ABA can also be formed from ABA glucosyl ester present in endoplasmic reticulum and vacuole by BG1 and BG2, respectively {Solid arrow denotes the anabolic pathway and broken arrow denotes the catabolic pathway}. [Reprinted with permission from Springer Nature, Abscisic acid in plants under abiotic stress: crosstalk with major phytohormones, Singh and Roychoudhury, (2023) vide license number 5633630784216]."

2.10.2. Role of ABA in plants during abiotic stress

Under abiotic stresses, the expression levels of numerous genes are up regulated or down regulated via ABA-dependent or independent pathways in plants. Based on their function, stress-responsive genes can be categorized into two groups: firstly genes encoding proteins that are required for stress signaling and secondly genes encoding proteins that are directly involved in stress tolerance.

Genes encoding abiotic stress responsive protein involved in stress signaling are transcription factors, protein kinases, protein phosphatases, and ubiquitination-related proteins involved in protein degradation. Many transcription factors such as MYC, MYB, DREB, bZIP, NAC and ERF are involved in up regulation of stress-inducible genes (Trivedi et al. 2016). The promoter regions of stress induced genes have cis elements such as abscisic-acid-responsive element (ABRE). Gao et al. (2011) reported higher expression of bZIP1 in soybean against abiotic stresses that regulate stomatal closure leading to abiotic stress tolerance. MYC and MYB have significant role in ABA mediated abiotic stress tolerance by inducing the expression of genes such as RD22 (Abe et al. 2003). They further identified some of the MYB genes like *AtMYB15*, *AtMYB44* and *AtMYB60* in *Arabidopsis* that were involved in ABA mediated drought and salt stress tolerance. Moreover, NAC transcription factors also play a significant role in ABA mediated stress tolerance. Nakashima et al. (2009) reported that overexpression of *AtNAC045* and *AtNAC072* from rice and Arabidopsis provides drought

stress tolerance. Additionally, Mao et al. (2014) reported that during multiple abiotic stresses or ABA treatment, the expression of *NAC67* was noticeably induced in wheat cultivar. According to Raghavendra et al. (2010), during stressed environment, plants induce the level of ABA in cells which in turn activates protein kinases such as SnRK, OST1, CPK4 and CPK11 that activates the expression of ABA dependent transcription factors like ABFs, ABI5, ABI4 and ABI3. These activated transcription factors bind with the promoter region of ABA-dependent stress responsive genes that provides tolerance against harsh environmental condition (Trivedi et al. 2016) (Fig. 2.14).



"Fig 2.14: ABA mediated abiotic stress tolerance in plants. [Reprinted with permission from Springer Nature, DREBs-potential transcription factors involve in combating abiotic stress tolerance in plants, Singh and Chandra (2021) vide license number 5633631411469]."

Second group of genes encoding proteins that directly contribute to minimize cellular damage includes enzymes involved in osmolyte formation, heat shock proteins, chaperonins and

antioxidative enzymes. ABA mediates the formation of osmolytes like proline, glycine betaine and polyamines (spermine, spermidine and putrescine). Planchet et al. (2014) demonstrated that application of ABA induces proline accumulation in drought stressed *Medicago truncatula* that might be due to induced activity of proline synthesizing enzymes or reduced activity of proline catabolising enzymes, i.e., proline dehydrogenase which was again proved by Shevyakova et al. (2013) where they showed that ABA treatment lowered the activity of proline dehydrogenase in salt stressed (100 and 300 mM NaCl) *Mesembryanthemum crystallinum*. Similarly, Zhang et al. (2012) also reported that during drought stress, exogenous application of ABA further enhanced the formation of glycine betaine in maize seedlings; however, on application of ABA synthesis inhibitor like fluridone, the level of both ABA and glycine betaine was reduced in the cells. ABA also regulates the level of polyamines during stressed environment. Polyamines are low molecular weight aliphatic nitrogenous compounds carrying two or more amino groups. Overexpression of ADC (arginine decarboxylase), SAMDC (S-adenosyl-methionine decarboxylase) and SPMS (spermine synthase) enhanced the activity of NCED that ultimately triggered the formation of ABA in plants which in turn initiates a positive feedback loop between ABA and polyamine biosynthesis (Pal et al. 2018).

2.10.3. Crosstalk between ABA and major stress regulator phytohormones

2.10.3.1. Crosstalk with melatonin

Melatonin was initially considered just as an antioxidant; however, with time, various recent studies have proved its efficacy as growth and stress regulators in plants. However, melatonin acts antagonist to that of ABA. Tryptophan acts as a precursor of melatonin. In plants, tryptophan is converted into serotonin via tryptamine or 5-hydroxytryptophan. After its synthesis, serotonin is converted into melatonin in a two step reaction catalyzed by serotonin N-acetyltransferase (SNAT), acetylserotonin O-methyltransferase (ASMT) and caffeic acid 3-O-methyltransferase (COMT). SNAT catalyzes an acetylation reaction, whereas ASMT and COMT act as methytransferases (Back et al. 2016). During salt stress, the level of melatonin was enhanced in *Limonium bicolor* which in turn lowered the formation of ABA which can be linked with reduced expression of ABA synthesizing genes (*NCED1* and *NCED2*) and higher expression of ABA degrading genes (*CYP707A1* and *CYP707A2*) (Yuan et al. 2016). Similarly, Tan et al. (2019) also reported that melatonin-mediated seed

germination is controlled by higher H_2O_2 biosynthesis and higher efflux of Ca^{2+} ions that eventually resulted in higher ABA catabolism. During heat stress, application of melatonin suppressed the expression of *ABI5*, *ABI3* and *NCED3* that delayed leaf senescence in *Lolium perenne* (Zhang et al. 2017). In contrast, various studies have also showed a positive interaction between ABA and melatonin. Li et al. (2019) demonstrated that during drought stress, melatonin biosynthesis enhanced the formation of ABA in watermelon seedlings that confers higher tolerance level by wax biosynthesis and deposition on the leaves which ultimately reduced the rate of water loss. Similarly, Fu et al. (2017) also reported that exogenous application of melatonin triggered ABA formation in *Elymus nutans*. Melatonin-ABA synergistic crosstalk in rice seedlings during fluoride stress was also reported by Banerjee and Roychoudhury (2019b). They reported that during fluoride stress, exogenous application of melatonin led to its higher accumulation in the cells which in turn induced the tolerance level of the seedling by inducing ABA formation and up regulating the expression of *NCED3* and reducing its degradation by reducing the expression of ABA catabolizing gene, i.e., *ABA8ox1*. Similar report was also published by Zahedi et al. (2020) in salt-stressed *Fragaria ananassa*. Thus, it can be stated that both positive and negative crosstalk occurs in between ABA and melatonin which varies from plant species to species under harsh conditions.

2.10.3.2. Crosstalk with gibberellin

Gibberellin and ABA is a classic pair of hormone that acts antagonistically during the regulation of various physiological processes in plants such as seed germination, seed dormancy and maturation, flowering time and root growth (Yang et al. 2014). In normal conditions, gibberellin down regulates the ABA signaling pathway by ubiquitination of PSY family of proteins (Vishal and Kumar 2018). Ali et al. (2022) demonstrated that transcription factors containing LEC1, FUS3, AP2 and ABI4 domains negatively regulate the interaction between ABA and gibberellin signaling. Shu et al. (2018) reported that ABI4 enhanced the expression of *NCED6* and *GA2ox7* gene which in turn enhanced the formation of ABA and reduced the level of gibberellin in seed that eventually extended the dormancy period of the seeds. In a similar report, Footitt et al. (2011) demonstrated that during winter season, higher dormancy of the seeds was facilitated by higher ABA formation and gibberellin degradation, whereas contrasting level of both the phytohormones was noted during spring and summer season. During stressed environment, the bioactive form of gibberellin is inactivated due to

enhanced activity of GA2ox7 and GA2ox6 that led to hydroxylation of gibberellin in *Arabidopsis* (Magome and Kamiya 2018). Guilfoyle et al. (2015) demonstrated that RGL2, a DELLA repressor of gibberellin signaling, stimulated the activity of ABI5 and ABI3 which led to the formation of ABA via XERICO protein. Under suitable environmental condition, a negative feedback loop operates between DELLA proteins, ABI2 and *MFT* expression (Vaistij et al. 2018). Toh et al. (2008) reported that high temperature induced ABA formation in *Arabidopsis* seedlings which can be attributed to the up regulated expression of ABA synthetic enzymes genes (*ZEP*, *NCED9*, *NCED5* and *NCED2*). In contrast to ABA level, the formation of gibberellin was drastically reduced in heat stressed seedlings that was largely due to down regulated expression of gibberellin synthesizing genes. Chiu et al. (2012) reported that FUS3 (master regulator of *Arabidopsis* seed development) activated ABA metabolic genes and heat related metabolite formation and inhibited gibberellin metabolism under high temperature. Similar to that of heat stress, cold stress also promotes the expression of gibberellin catabolizing gene (*GA2ox*) which enhanced the level of ABA in *Arabidopsis* seedlings (Achard et al. 2008). Thus, it can be stated that antagonist behavior between gibberellin and ABA widely regulates various physiological processes in plants ranging from seed germination to abiotic stress tolerance.

Chapter 3

Materials and Methods

Data presented in this chapter has already been published in research papers which have been cited at the beginning of each sub-chapter under Results and Discussion

"3.1. Materials

3.1.1. Plant materials

Seeds of the following rice cultivars were used for

Rice cultivar	Source
Khitish (KH)	Rice Research Station, Chinsurah, Hooghly, West Bengal.
MTU1010 (MTU)	Rice Research Station, Chinsurah, Hooghly, West Bengal
Nonabokra (NB)	Central Soil Salinity Research Institute, Canning, South 24 Parganas, West Bengal
Matla (MT)	Rice Research Station, Chinsurah, Hooghly, West Bengal
Jarava (JV)	Rice Research Station, Chinsurah, Hooghly, West Bengal

3.1.2. Chemicals and Stocks

3.1.2.1. Crude chemicals

Chemical/Reagent	Concentration	Purpose
Mercuric chloride ($HgCl_2$)	0.1% (w/v)	Surface sterilization of rice seeds
		Preparation of Nessler's reagent
Deionized water		Several purposes
Liquid nitrogen		Freezing sample for homogenization
Chilled acetone	80% (v/v)	Quantification of total chlorophyll Extraction of xanthophylls and carotenoids
Trichloroacetic acid	0.1% (w/v)	Extraction of H_2O_2 and MDA
	5% (w/v)	Estimation of protease, starch phosphorylase activity Deproteinization of pyruvic, citric and malic acid content
Potassium iodide (KI)	20% (w/v)	Estimation of MDA and protein carbonylation assay
	100% (w/v)	Estimation of protein carbonylation assay
	0.2% (w/v)	Enzyme activity of glycine betaine and α-amylase estimation

		Preparation of Nessler's reagent
	1 M	Estimation of H_2O_2 content
2,4-dintrophenylhydrazine (DNPH)	0.02% (w/v)	Estimation of pyruvic acid content
	0.1% (w/v)	Estimation of malic acid and gibberellic acid content
	0.2% (w/v)	Estimation of protein carbonylation
Casein hydrolysate	20 mg mL^{-1}	Estimation of protease activity
2-thiobarbituric acid (TBA)	0.5% (w/v)	Estimation of MDA content
Linoleic acid	0.5 mM	Estimation of LOX activity
	0.1% (v/v)	Substrate buffer for LOX isozymes
Ethylene diamine tetraacetic acid (EDTA)	0.5 mM	Estimation of AsAO activity
	1 mM	Extraction of Gly I, Gly II, APX, GR, MDH, INV, SPS, SS, PHO, NR and P5CS
	2 mM	Extraction of GST, GOGAT, GS, GDH PyDH, H_2S ATP-S and OAS-TL
	3 mM	Extraction of NOX
	10 mM	Estimation of H_2S
	100 mM	Estimation of SOD activity Substrate buffer for SOD isozymes
Dithiothreitol (DTT)	0.5 mM	Extraction of GPOX and GOGAT
	1 mM	Estimation of NOX and DES activity
	1.25 mM	Extraction of NOX
	2 mM	Extraction of Gly I, Gly II, ATP-S and OAS-TL
	2.5 mM	Extraction of SPS and SS
	500 mM	Gel loading dye for SDS-PAGE
	33.4 mM	Estimation of OAS-TL
Phenyl methyl sulfonyl fluoride (PMSF)	1 mM	Extraction of NOX, INV, PHO Estimation of NOX activity

Sucrose	0.19 M	Extraction and estimation of SDH activity
	250 mM	Extraction and estimation of NOX activity
	0.4 mM	Estimation of INV activity
	0.7 M	Extraction of PyDH
	250 mM	Tris-KCl-$MgCl_2$ buffer
2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT)	0.5 M	Estimation of NOX activity
	0.5 mM	Estimation of superoxide anion
Nicotinamide adenine dinucleotide phosphate reduced (NADH)	100 µM	Estimation of NOX activity
	0.2 mM	Estimation of GPX activity
	3.24 mM	Estimation of GR activity
	0.4 mM	Estimation of P5CS and GDH activity
	2 mM	Estimation of NR activity
L-ascorbic acid	0.1 mM	Estimation of AsAO activity
Sulfanilamide	1% (w/v)	Color developing reagent for NR and NiR assay
N-(1-Naphthyl) Ethylenediamine Dihydrochloride (NEDA)	0.2% (w/v)	Color developing reagent for NR and NiR assay
Perchloric acid	5% (v/v)	Extraction of methylglyoxal and cysteine
	10% (v/v)	Homogenization for polyamine extraction
	52% (v/v)	Measurement of starch content
	concentrated	Estimation of silicon
Zinc acetate	0.1 mM	Estimation of NR activity
	10 mM	Extraction and estimation of H_2S
N-acetyl cysteine	500 mM	Estimation of methylglyoxal content
Potassium nitrate (KNO_3)	10 mM	Estimation of NR activity

Nicotinamide adenine dinucleotide hydrogen (NADPH)	0.4 µM	Estimation of MDH activity
	2.5 mM	Estimation of NR activity
Magnesium sulfate ($MgSO_4$)	15 mM	Estimation of Gly I and GS activity
Reduced glutathione (GSH)	1.7 mM	Estimation of Gly I activity
	4 mM	Estimation of GPX activity
	5 mM	Estimation of GST activity
Methylglyoxal (MG)	3.5 mM	Estimation of Gly I activity
5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)	0.2 mM	Estimation of Gly II activity
	1 mM	Estimation of CS activity
	10 mM	Estimation of reduced glutathione and H_2S
S-lactoyl-D-glutathione (SLG)	1 mM	Estimation of Gly II activity
Sulfosalicylic acid	3% (w/v)	Homogenization of tissue for proline extraction
Ninhydrin		Preparation of acid ninhydrin
Glacial acetic acid		Preparation of acid ninhydrin
Ortho-phosphoric acid	6 M	Preparation of acid ninhydrin
Stannous chloride		Preparation of ninhydrin
Methyl cellosolve		Preparation of ninhydrin
Sodium citrate		Preparation of ninhydrin Homogenization of tissue for PHO extraction
Citric acid		Preparation of ninhydrin Homogenization of tissue for PHO extraction
Sulfuric acid (H_2SO_4)	2 N	Estimation of glycine betaine and ascorbic acid content
	6 N	Preparation of Chen's reagent
	98% (v/v)	Estimation of malic acid, total soluble sugar,

		starch content and total and soluble nitrogen Preparation of anthrone reagent
Ethanol	80% (v/v)	Extraction of total amino acids Extraction and estimation of total soluble sugar
	100% (v/v)	Estimation of protein carbonylation assay
	50% (v/v)	Estimation of ABTS potential
	70% (v/v)	Estimation of OAS-TL
1,2-dichloroethane		Estimation of glycine betaine content
Iodine	1% (w/v)	Preparation of Lugol's iodine solution
Methanol	80% (v/v)	Estimation of gibberellic acid
	90% (v/v)	Estimation of carotene and xanthophyll content
Aluminum chloride ($AlCl_3$)	2% (w/v)	Estimation of flavonoid content
n-Hexane		Estimation of carotene and xanthophyll content
Folin-Ciocalteau reagent		Estimation of total phenolic content
Sodium carbonate (Na_2CO_3)	7.5% (w/v)	Estimation of total phenolic content
Ammonium heptamolybdate	2.5% (w/v)	Preparation of Chen's reagent
	5% (w/v)	Estimation of ascorbic acid
m-phosphoric acid	5% (w/v)	Extraction of ascorbic acid
Sodium phosphate dibasic (Na_2HPO_4)	100 mM	Estimation of methylglyoxal content
	250 mM	Estimation of ascorbic acid
	0.5 M	Estimation of INV activity
L-phenylalanine	40 mM	Estimation of PAL activity
Hydrochloric acid (HCl)	Concentrated	Acid digestion for endogenous total silicon content
	3% (v/v)	Measurement of total silicon content
	1 N	Estimation of malic acid content

	4 M	Estimation of PAL activity
	6 M	Estimation of α-amylase activity
Polyvinylpyrrolidone (PVP)	2% (w/v)	Extraction of CS, P5CS. GOGAT, ATP-S and OAS-TL
	5% (w/v)	Extraction of APX, GR and IDH
	7.5% (w/v)	Extraction of GST
	10% (w/v)	Extraction of SOD
	5 mM	Extraction of GPoX
Nitro blue tetrazolium chloride (NBT)	0.25 mM	Substrate buffer for SOD
	2.45 mM	Substrate buffer for APX
		For staining of Western blot
Tetramethyl ethylene diamine (TEMED)		Estimation of SOD activity
	28 mM	Substrate buffer for APX
Riboflavin	2 mM	Substrate buffer for SOD
	5 mM	Estimation of SOD activity
Hydrogen peroxide (H_2O_2)	4 mM	Substrate buffer for APX isozymes
	5 mM	Estimation of GPoX activity
	12 mM	Estimation of APX activity
	13 mM	Substrate buffer for GPoX
	20 mM	Estimation of CAT activity
	30% (v/v)	Estimation of silicon and total and soluble nitrogen
Bovine serum albumin	0.1% (w/v)	Extraction of GPoX
	0.5% (w/v)	Extraction of PyDH
Guaiacol	0.46 % (v/v)	Substrate buffer for GPoX
	15 mM	Estimation of GPoX activity
β-mercaptoethanol	4 mM	Extraction of APX and GR
	5 mM	Extraction of MDH and PHO
	10 mM	Extraction of IDH and P5CS
	14 mM	Extraction of GST, GS and GDH

	57 mM	Extraction of PyDH
		Tris-KCl-$MgCl_2$ buffer
1-Chloro-2,4-dinitrobenzene (CDNB)	1 mM	Estimation of GST activity
Cumene hydroperoxide	0.5 mM	Estimation of GPX activity
Glutathione reductase	1 U	Estimation of GPX activity
Glycerol	20% (v/v)	Extraction of APX and GR
Sodium ascorbate	2 mM	Substrate buffer for APX isozymes
	4 mM	Substrate buffer for APX isozymes
	5 mM	Estimation of APX activity
Oxidized glutathione	16 mM	Estimation of GR activity
Sodium bicarbonate ($NaHCO_3$)	100% (w/v)	Estimation of polyamines
Potassium hydroxide (KOH)	10% (w/v)	Estimation of gibberellic acid
	30% (w/v)	Estimation of SPS and SS activities
Dansyl chloride		Estimation of polyamines
Toluene		Estimation of polyamines
Sodium hydroxide (NaOH)	0.8 N	Estimation of pyruvic acid content
	10 M	pH adjustment for Na_2HPO_4
	10% (w/v)	Estimation of total and soluble nitrogen
		Preparation of Nessler's reagent
	2 N	Estimation of nitrate
Acetic anhydride	0.5 M	Estimation of citric acid
Pyridine	0.1 M	Estimation of citric acid
Ammonium hydroxide (NH_4OH)	5 N	Estimation of malic acid
	6 mM	Estimation of GS
Orcinol	0.08% (w/v)	Estimation of malic acid
Calcium chloride ($CaCl_2$)	10% (w/v)	Estimation of malic acid
	3 mM	Estimation of α-amylase and GDH activity
Magnesium chloride ($MgCl_2$)	10 µM	Estimation of MDH activity
	20 mM	Extraction and estimation of P5CS activity

	3.3 mM	Estimation of INV activity
	5 mM	Estimation of PyDH activity Extraction of SPS and SS
	15 mM	Estimation of SPS activity
	50 mM	Extraction of MDH
	5 mM	Tris-KCl-$MgCl_2$ buffer
	7 mM	Estimation of ATP-S
Pyruvate	1.5 mM	Estimation of PyDH activity
Cysteine	5 µM	Extraction of α-amylase
	2.6 mM	Estimation of PyDH activity
	25 mM	Extraction of NR
	8 mM	Estimation of DES activity
Coenzyme-A	0.12 mM	Estimation of PyDH activity
Nicotinamide adenine dinucleotide (NAD)	800 µM	Estimation of IDH activity
	1.4 mM	Estimation of PyDH activity
Iso-ascorbic acid	10 mM	Estimation of CS activity
Oxaloacetic acid (OAA)	5 µM	Estimation of MDH activity
	10 mM	Estimation of CS activity
Acetyl-CoA	10 mM	Estimation of CS activity
Sodium isocitrate	2 mM	Estimation of IDH activity
Manganese sulphate ($MnSO_4$)	200 µM	Estimation of IDH activity
Sodium succinate	0.5 M	Estimation of SDH activity
Iodonitrotetrazolium chloride (INT)	8 mM	Estimation of SDH activity
Sodium azide	10 mM	Estimation of SDH activity
Phenol solution	5% (v/v)	Estimation of total soluble sugar and starch content
	1% (v/v)	Estimation of dissolved ammonium ions

Fructose-6-phosphate	25 mM	Estimation of SPS activity
Glucose-6-phosphate	25 mM	Estimation of SPS activity
UDP-glucose	25 mM	Estimation of SPS activity
Fructose	25 mM	Estimation of SS activity
Soluble starch	1% (w/v)	Estimation of α-amylase activity
	5% (w/v)	Estimation of PHO activity
Sodium chloride (NaCl)	0.15 M	Estimation of α-amylase activity
Glucose-1-phosphate	0.1 M	Estimation of PHO activity
Urea	8 M	Estimation of protein carbonylation
Ethyl acetate	100% (v/v)	Estimation of protein carbonylation and endogenous melatonin content
Nitric acid (HNO_3)	Concentrated	Acid digestion for endogenous total silicon content
Tween-20	0.1% (v/v)	Estimation of LOX activity
Dimethyl sulfoxide (DMSO)		Solubilization of XTT for NOX assay
Potassium chloride (KCl)	25 mM	Tris-KCl-$MgCl_2$ buffer
	0.6 M	Extraction of P5CS
Potassium persulfate		Estimation of ABTS potential
ABTS		Estimation of ABTS potential
Ferric chloride ($FeCl_3$)	1% (w/v)	Substrate buffer for CAT
	20 mM	Estimation of FRAP
2,4,6-tris(2-pyridyl)-s-triazine		Estimation of FRAP
Guaiacol	15 mM	Estimation of GPoX activity
Glutathione reductase	1 U	Estimation of GPX activity
Adenosine triphosphate (ATP)	5 mM	Estimation of P5CS and GS activity
	2 mM	Estimation of ATP-S
Glutamic acid	75 mM	Estimation of P5CS activity
	30 mM	Estimation of GS activity

Copper sulfate ($CuSO_4$)	0.5% (w/v)	Preparation of Lowry reagent
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	1.2 mM	Substrate buffer for GPX isozyme
Malate	50 mM	Estimation of fumarase activity
2-oxoglutarate	10 mM	Estimation of GOGAT activity
	20 mM	Estimation of GDH activity
Sodium silicate	10% (w/v)	Estimation of total and soluble nitrogen
Salicylic acid	5% (w/v)	Estimation of nitrate
Sodium nitrite	25 mM	Estimation of NiR
Methyl viologen	3 mM	Estimation of NiR
Sodium hyposulfite	0.025% (w/v)	Estimation of NiR
Sodium nitroprusside	0.05% (w/v)	Estimation of dissolved ammonium ions
Sodium hypochlorite	0.042% (v/v)	Estimation of dissolved ammonium ions
L-glutamine	10 mM	Estimation of GOGAT activity
Casein	1.5% (w/v)	Estimation of GOGAT activity
Aspartate	6 mM	Estimation of GS activity
Triton X-100		Extraction of NOX, CS, Gly I, SPS and GDH
Sulfate-free inorganic pyrophosphatase	0.032 U mL^{-1}	Estimation of ATP-S
O-acetylserine	5 mM	Estimation of OAS-TL
Sodium sulfide	5 mM	Estimation of OAS-TL

3.1.2.2. Fine chemicals/enzymes

Chemical/Enzyme	Purpose	Source
DNase I	Removal of DNA contamination in RNA	Thermo Fisher Scientific
RNase I	Removal of RNA contamination in DNA	Thermo Fisher Scientific

M-MLV Reverse transcriptase	Reverse transcription for semi-quantitative reverse-transcriptase PCR	Thermo Fisher Scientific
Taq DNA polymerase	PCR amplification	Genet Bio
Diethyl pyrocarbonate	Inactivation of RNase enzyme	Invitrogen

3.1.2.3. Stress-imposing agent

Chemical	Concentration
Sodium fluoride (NaF)	25 mg L^{-1} (~ 0.6 mM)
	50 mg L^{-1} (~ 1.2 mM)
Sodium chloride (NaCl)	12 g L^{-1} (~ 200 mM)

3.1.2.4. Chemical used for stress amelioration

Chemical	Concentration
Calcium hydroxide [$Ca(OH)_2$]	0.3 mM
	0.5 mM
Calcium nitrate [$Ca(NO_3)_2$]	0.3 mM
	0.5 mM
Calcium chloride ($CaCl_2$)	0.3 mM
	0.5 mM
Silicon	2 mM
Sodium tungstate (Na_2WO_4)	2 mM

3.1.2.5. Reagents used for biochemical analyses

3.1.2.5.1. Acid ninhydrin

Components	Volume (50 mL)
Ninhydrin	1.25 g
Glacial acetic acid	30 mL

6 M Phosphoric acid	20 mL

3.1.2.5.2. Ninhydrin reagent

Components	Volume	For 25 mL
Stannous chloride	0.02 g	Solution I: Stannous chloride added to 12.5 mL of citrate buffer (pH 5.0)
Citric acid	20.5 mL	
Sodium citrate	29.5 mL	
Ninhydrin	0.5 g	Solution II: 0.5 g ninhydrin in 12.5 mL methyl cellosolve
Methyl Cellosolve	12.5 mL	
12.5 mL of Solution I and 12.5 mL of Solution II mixed to produce 25 mL acid ninhydrin		

3.1.2.5.3. Lugol's iodine solution

Component	Concentration
Potassium iodide	2% (w/v)
Iodine	0.2% (v/v)

3.1.2.5.4. Chen's reagent

Component	Concentration
H_2SO_4	6 N
Ammonium heptamolybdate	2.5% (w/v)
Ascorbic acid	10% (w/v)

3.1.2.5.5. Nitrogen color developing reagent

Component	Concentration
Sulfanilamide	1% (w/v)
HCL	1.5 N
NEDA	0.2% (w/v)

3.1.2.5.6. Anthrone reagent

Component	Concentration
Anthrone	0.2% (w/v)
H_2SO_4	98% (v/v)

3.1.2.5.7. Lowry reagent

Component	Concentration	Volume
Na_2CO_3	2% (w/v)	Solution A (50 mL): 1 g Na_2CO_3 dissolved in 0.2 g NaOH
NaOH	0.1 N	
Copper sulfate ($CuSO_4$)	0.5% (w/v)	Solution B (2 mL): 1 mL $CuSO_4$ mixed with 1 mL sodium potassium tartarate solution
Sodium potassium tartarate	1% (w/v)	
50 mL of solution A and 1 mL of solution B mixed to prepare Lowry reagent or alkaline $CuSO_4$ solution		

3.1.2.5.8. Bradford reagent

Component	Concentration	Volume (1 L)
Coomassie Brilliant Blue G-250		100 mg
Ethanol	95% (v/v)	50 mL
Ortho-phosphoric acid	85% (w/v)	100 mL
Double distilled water		Volume up to 1 L

3.1.2.5.9. 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) reagent

Component	Volume
Potassium persulfate	Solution I: 37.5 mg dissolved in 1 mL distilled water
ABTS	Solution II: 9.7 mg dissolved in 2.5 ml distilled water
44 µL solution I was mixed with solution II to prepare ABTS reagent	

3.1.2.5.10. Ferric-reducing antioxidant potential (FRAP) reagent

Component	Concentration	Volume
Acetate buffer (pH 3.6)	300 mM	10 mL
2,4,6-tris(2-pyridyl)-s-triazine (TPTZ)		1 mL
$FeCl_3$	20 mM	1 mL

3.1.2.5.11. Nessler's reagent

Component	Concentration	Volume (20 mL)
KI		2 g
$HgCl_2$		3 g
KOH	30%	40 g
Water		Volume up to 20 mL

3.1.2.6. Reagents used for molecular analyses

Chemical	Concentration	Purpose
Liquid nitrogen		Freezing the sample for homogenization
TRizol		Extraction of RNA
Chloroform		Extraction of RNA
Ethylenediamine tetraacetic acid	0.5 M	Agarose gel electrophoresis
Acetic acid	10% (v/v)	Agarose gel electrophoresis
Sodium dodecyl sulfate (SDS)	1% (w/v)	Running buffer for SDS-PAGE
Isopropanol		Precipitaion of RNA
Ethanol	70% (v/v)	Washing pellet of RNA
Agarose	1%, 2%, 3% (w/v)	Agarose gel electrophoresis
Ethidium bromide (EtBr)	10 mg mL^{-1}	Visualize the DNA bands
Ammonium persulfate	10% (w/v)	Resolving and stacking gel
Tris (pH 8.8, 6.8)	1 M, 1.5 M	Resolving and stacking gel
Acrylamide	30% (w/v)	Resolving and stacking gel
Bromophenol blue	0.1% (w/v)	Gel-loading dye for native-PAGE and SDS-PAGE
Glycerol	10% (v/v)	Gel-loading dye for native-PAGE and

	SDS-PAGE
Glycine	Running buffer for native-PAGE Running and transfer buffer for SDS-PAGE
Bovine serum albumin	Blocking agent

3.1.2.7. Buffers for biochemical assay

3.1.2.7.1. Sodium phosphate buffers

pH	1 M Na_2HPO_4 (For 100 mL)	1 M NaH_2PO_4 (For 100 mL)
6.6	35.2	64.8
7.0	57.8	42.4
7.2	68.4	31.6
7.4	77.4	22.6
7.8	89.6	10.4

3.1.2.7.2. Potassium phosphate buffers

pH	1 M K_2HPO_4 (For 100 mL)	1 M KH_2PO_4 (For 100 mL)
6.0	13.2	86.8
6.6	38.1	61.9
7.0	61.5	38.5
7.4	77.4	22.6

3.1.2.7.3. Borate buffer

Composition	Concentration
Boric acid	0.1 M
Sodium tetraborate	0.1 M

3.1.2.7.4. Sodium acetate buffers

Concentration	pH	Sodium acetate (For 1 L)	Acetic acid (For 1 L)
10 mM	4.6	405.861 mg	303.4 mg
100 mM	4.8	4.791 g	2.498 g

3.1.2.7.5. Citrate buffer (50 mM, pH 6.0)

Composition	Mass
Citric acid	1.736 g
Sodium citrate dihydrate	12.044 g

3.1.2.7.6. Total ionic strength adjustment buffer (TISAB pH 5.2)

Component	Amount (1 L)
Glacial acetic acid	57 mL
EDTA	4 g
NaCl	45 g
Double distilled water	Up to 1 L

3.1.2.7.7. Tricine buffer

Component	Concentration
Tricine	100 mM
EDTA	1 mM
PVP	5% (w/v)
Glycerol	20% (v/v)
β-mercaptoethanol	4 mM

3.1.2.8. Buffers for molecular analyses

3.1.2.8.1. Buffer for agarose gel-electrophoresis

3.1.2.8.1.1. TAE Buffer (50X, pH 8.0)

Component	Volume (For 1 L)
Tris-base	242 g
EDTA	18.61 g
Glacial acetic acid	57.1 mL
Double distilled water	Volume up to 1 L

3.1.2.8.1.2. 6X DNA loading dye

Component	Concentration	Volume (10 mL)
Bromophenol blue	0.25% (w/v)	25 mg
Xylene cyanol FF	0.25% (w/v)	25 mg
Glycerol	30% (v/v)	3.3 mL
Double distilled water		6.7 mL

3.1.2.8.2. Buffer for isozyme analyses

3.1.2.8.2.1. Running buffer for native-PAGE (5X, pH 8.3)

Component	Volume (1 L)
Tris-base	15.1 g
Glycine	94 g
Double distilled water	Volume up to 1 L

3.1.2.8.2.2. Gel loading dye for native-PAGE

Component	Concentration
Tri-Cl (pH 6.8)	50 mM
Bromophenol blue	0.1% (w/v)
Glycerol	10% (v/v)

3.1.2.8.2.3. Resolving gel buffer for native-PAGE (12%)

Component	Concentration	Volume (10 mL)
Acrylamide mix	30% (w/v)	4.0 mL
Tris-base (pH 8.8)	1.5 M	2.5 mL
Ammonium persulfate	10% (w/v)	100 µL
TEMED		4 µL
Double distilled water		3.4 mL

3.1.2.8.2.4. Stacking gel buffer for native-PAGE (5%)

Component	Concentration	Volume (6 mL)
Acrylamide mix	30% (w/v)	1.0 mL
Tris-base (pH 8.8)	1 M	750 µL
Ammonium persulfate	10% (w/v)	60 µL
TEMED		6 µL
Double distilled water		4.16 mL

3.1.2.8.2.5. Substrate buffer for SOD isozymes

Component	Concentration
Potassium phosphate buffer (pH 7.8)	50 mM
EDTA	100 mM
Riboflavin	2 mM
NBT	0.25 mM
TEMED	28 mM

3.1.2.8.2.6. Substrate buffer for GPoX isozymes

Component	Concentration
Potassium phosphate buffer (pH 7.0)	50 mM
Guaiacol	0.46% (v/v)
H_2O_2	13 mM

3.1.2.8.2.7. Substrate buffer for APX isozymes

Component	Concentration
Sodium phosphate buffer (pH 7.0, 7.8)	50 mM
Sodium ascorbate	2 mM, 4 mM
H_2O_2	4 mM
NBT	2.5 mM
TEMED	28 mM

3.1.2.8.2.8. Substrate buffer for CAT isozymes

Component	Concentration
Sodium acetate buffer (pH 5.5)	50 mM
H_2O_2	4 mM
$FeCl_3$	1% (w/v)
Potassium ferricyanide	1% (w/v)

3.1.2.8.2.9. Substrate buffer for GPX isozymes

Component	Concentration
Tris-Cl buffer (pH 7.9)	50 mM
GSH	13 mM
H_2O_2	0.004% (v/v)
MTT	1.2 mM
Phenazine methosulfate	1.6 mM

3.1.2.8.3. Buffer for immunoblot analyses

3.1.2.8.3.1. Resolving gel buffer for SDS-PAGE (10%)

Component	Concentration	Volume (10 mL)
Acrylamide mix	30% (w/v)	3.3 mL
Tris-base (pH 8.8)	1.5 M	2.5 mL
SDS	10% (w/v)	100 µL
Ammonium persulfate	10% (w/v)	100 µL
TEMED		5 µL
Double distilled water		4.0 mL

3.1.2.8.3.2. Stacking gel buffer for SDS-PAGE (5%)

Component	Concentration	Volume (10 mL)
Acrylamide mix	30% (w/v)	0.83 mL
Tris-base (pH 6.8)	0.5 M	0.63 mL
SDS	10% (w/v)	50 µL
Ammonium persulfate	10% (w/v)	50 µL

TEMED	5 µL
Double distilled water	3.4 mL

3.1.2.8.3.3. Tris-Buffered Saline (10X, pH 7.6)

Component	Volume (For 1 L)
Tris-base	24 g
NaCl	88 g
Double distilled water	Volume up to 1 L

3.1.2.8.3.4. SDS Running buffer (10X)

Component	Volume (1 L)
Tris-base	30.3 g
Glycine	144.4 g
SDS	10 g
Double distilled water	Volume up to 1 L

3.1.2.8.3.5. Transfer buffer (1X)

Component	Volume (1 L)
Tris-base	14 g
Glycine	4 g
Double distilled water	820 mL
Methanol	180 mL

3.1.2.8.3.6. Gel loading dye for SDS-PAGE

Component	Concentration
Tris-Cl	500 mM
Bromophenol blue	0.05%
Glycerol	50%
DTT	500 mM
SDS	10%

3.1.2.9. Kits used for different experiments

Kit	Source
RNAiso plus	Takara, Japan
Maxima First Strand cDNA synthesis kit	Thermo Scientific, USA
Melatonin detection kit	Abcam, UK
ABA immunoassay kit	Sigma-Aldrich, USA

3.1.2.10. List of primers

Genes	Primers	Gene accession ID
PDH	FP: GAACAGGGGATTGCTCTCGT RP: CAGCATTGCAGCCTTGAACC	LOC_Os10g40360
P5CS	FP: CGCAGGATCAATTCGTGAAATCGCA RP: GCAATCTGTACCAAGGCATCAGGA	LOC_Os05g38150
BADH1	FP: TCTTTGGACCGGTCATCTGC RP: CTTGGTGACTTGCTTCACGC	LOC_Os04g39020
CAT	FP: GGAGGAGGAGAGGTTCGACT RP: AGCATCAGGTAGTTTGGCCC	LOC_Os02g02400
SOD	FP: ATGGTGAAGGCTGTTGCTGTG RP: CTAACCCTGGAGTCCGATGAT	LOC_Os07g46990
CBL10	FP: CCAACTCTCTCGATTCGGGG RP: TTCTTTCCAGCCGGTGTTCT	LOC_Os01g51420
CAM1-1	FP: TCACAACCAAGGAGCTGGGA RP: TGGTCTTTGTCGAACACCCT	LOC_Os01g16240
SOS1	FP: GGATCGCTCGAATTTGGCAC RP: ACGGGGTCAGTTGCACTAAG	LOC_Os12g44360
SOS2	FP: GCAGGTCAGTTTTCATGCCC RP: CATCACAAGAGGGCCACCAT	LOC_Os06g40370
SOS3	FP: CGCCCAAACCACCTTTACG RP: CGACCGTACAAACTCCCCAA	LOC_Os05g45810
PyDH	FP: ACCCATCATGTGCTTGTCGT RP: GGATACCTCGCTACATGGGC	LOC_Os03g44300

CS	FP: GATGAGAGGGATGACTGGAATG RP: GGATGAGCAGTTACAGGAAGAG	LOC_Os01g19450
IDH	FP: GTGTCATTGCGGAAGGCAAG RP: CCTGGGAAGCGTCGTACAAA	LOC_Os04g05430
SDH	FP: AATCATTTGGCCCGGAGGAA RP: GATCTTTCCCCACTCGGACC	LOC_Os09g20440
MDH	FP: GGTTCATGGGGGATGACCAG RP: TTCGGGCAGTACTTGGCAAT	LOC_Os10g33800
INV	FP: CGGACGGCTGTCTTCTTCTA RP: AACATTGCTAGAAAGACTTACCAAA	LOC_Os02g01061
SS	FP: CGACACCAAGGGTGCTTTTG RP: GAAGCCAGACACTCCGTTCA	LOC_Os03g28330
SPS	FP: CTCAAGGAGGTCACCAAGGC RP: CACGGGAGAGAACGTGCCAA	LOC_Os01g69030
PHO	FP: TTCACGAGTACGCCAAGGAC RP: CAAAGCGAAACCTTCCTGCC	LOC_Os02g59421
α-amylase	FP: GTCGCCGAGATATGGAGCAA RP: GTAGCCCTGCATGACCTTGT	LOC_Os02g52710
NCED3	FP: CTCACATACAGCGGCAGCAC RP: CGCTCGAGGACATTCGCCAC	LOC_Os03g44380
ABA8ox	FP: AAGTGCTACCTCTGTTCCGC RP: TTCTTGCGGGTGAAGCTCAT	LOC_Os03g32230
Rab16A	FP: ATGAGGGAGGAGCACAAGAC RP: GGGCAGCTTCTCCTTGATCT	LOC_Os02g04680
Osem	FP: ATGGCGTCCGGGCAGCAGCAG RP: CTAGGACTTGGTCTTGTACTTGG	LOC_Os05g28210
TRAB1	FP: TTTCTCACCGGTGGGGATTG RP: GCTGCTGACTCCCTGTTCTT	LOC_Os05g28210
DREB2A	FP: ACCTCATTGGGTCAGGAAGAA RP: TTGGTTCACGGATCTCAGCC	LOC_Os01g07120
CLC1	FP: CGGCAAGTCACACTAACAAGGG	LOC_Os01g0876100

	RP: TGACTACAAAATTTCCATTC	
CLC2	FP: GGCTGATTTTTAAGCGTTTT	LOC_Os02g0558100
	RP: CAAATATGCCTTAGCACAAC	
ADC	FP: AGCGCGCTGGTGTGCGCACCA	LOC_Os06g04070
	RP: TGTCGCAGGTGAGGTCGGAG	
ODC	FP:GCGTCAAGGACAAGAAGGTG	LOC_ Os02g0482400
	RP:GTTGCACTTGACGGCGTAG	
DAO	FP: ACGTGACCTACCACCTC	LOC_Os07g38440
	RP:TACCACAGCACGATGTCCT	
PAO	FP:ACCGAGCTCATGGCGAACAACAGTTCA	LOC_Os09g20260
	RP:CTGAAGCTTTCACAGCCGGGAGATGAG	
SAMDC	FP:TCTTGCTTATGGCGACCTGG	LOC_Os04g42090
	RP:AGCAACAGGTACGTCTTCGG	
SPDS	FP:CCTGGTGAGGCACACTCATT	LOC_Os07g22600
	RP:TTGCTCCACCGAGGAATGTC	
SPMS	FP: ATCCTTTGCTGACACCTGGG	LOC_Os02g14190
	RP:GCGTCTCATTCAACAGCGAC	
TDC	FP: AACCCGGAGTACCTCAAGAA	LOC_Os08g04540
	RP: TGATCCTGAAGCAGACGAGA	
FEX	FP: ATGGAAAGTTCATCAGCTAGGAGCAAC	LOC_Os10g41749
	RP: CAGTAATGCTCTACCCACACCG	
H^+-ATPase	FP: GTCTCGCAGCTGGTCATTTA	LOC_Os01g0711000
	RP: GTGTAGGACTGGTGAATGGTATC	
NR	FP: CAACTGCTGGTTCAGGGTGA	LOC_Os08g36480
	RP: CGTAGGGCTCTCAACGATGG	
NiR	FP: AAACCCTGCTCTCAGCATCT	LOC_Os01g25484
	RP: CATCTTGGCGAGCTCCTTGA	
GS	FP: AGGCGAAGGGAAAAGGCTAC	LOC_Os02g50240
	RP: ATTTCGAGGGAAGGACGCAG	
GOGAT	FP: ACCGATCCATTCATTCGCCA	LOC_Os01g48960
	RP: CCCAGCATCCTTTGTCACCT	

GDH	FP: AGTGTGACGTTGCATTCCCT RP: GTTGAAACCAGGCGGAAACA	LOC_Os01g0558200
β-actin	FP: GAACTGGCATGGTCAAGGCTG RP: ACACGGAGCTCGTTGTAGAAG	LOC_Os11g06390

FP: Forward Primer RP: Reverse Primer

3.2. Methods

3.2.1. Seedling growth condition and stress imposition

- For all the experimental analysis presented in chapter 1 of the results and discussion section, seeds of the rice cultivars, i.e., KH and MTU were initially surface sterilized with $HgCl_2$ for 5 min, followed by extensive washing with double distilled water to remove the traces of $HgCl_2$. The seeds were placed over sterile gauge and were allowed to germinate at 32 ± 2 ºC for three days in dark in presence of 25 mg L^{-1} NaF solution. After three days, uniformly germinated seeds were further divided into two sets: 1st set was further maintained with NaF solution for 7 days and 2nd set was treated with NaF solution for 17 days, Both the sets were allowed to grow in growth chamber (N. R. Scientific, India) with photosynthetically active radiation (PAR), 700 µmol photons m^{-2} s^{-1}, day-night regime: 16:8 h and relative humidity: 65-70%. Thus, the seeds were exposed to fluoride stress for either 10 days (short-term) or 20 days (long-term). Four sets of two varieties were maintained as follows:

  Set 1: Khitish seeds treated with water only for 10 days.
  Set 2: Khitish seeds treated with NaF (25 mg L^{-1}) solution for 10 days.
  Set 3: MTU1010 seeds treated with water only for 10 days.
  Set 4: MTU1010 seeds treated with NaF (25 mg L^{-1}) solution for 10 days.
  Set 5: Khitish seeds treated with water only for 20 days.
  Set 6: Khitish seeds treated with NaF (25 mg L^{-1}) solution for 20 days.
  Set 7: MTU1010 seeds treated with water only for 20 days.
  Set 8 MTU1010 seeds treated with NaF (25 mg L^{-1}) solution for 20 days

  After completion of 10 and 20 days, the seedlings were harvested and washed with milli-Q water and was immediately frozen using liquid nitrogen and were stored at -80°C for

further experiments. For each set, three replicates were maintained (Roychoudhury et al. 2008).

- For all the experimental analysis presented in chapter 2 of the results and discussion section, seeds of the rice cultivar, i.e., KH were initially sterilized (similar to that of above mentioned procedure) followed by priming with two different concentration (0.5 and 0.3 mM) of three calcium salts, i.e., $Ca(OH)_2$, $Ca(NO_3)_2$ and $CaCl_2$ along with distilled water. For priming, the seeds were imbibed with the above mentioned concentrations of calcium salts at 25 °C for 24 h with constant stirring, keeping the seed weight to solution volume in 1:5 ratio (Hussain et al. 2016). The solution was changed after 12 h. After priming, the seeds were air dried to their original moisture content and were allowed to germinate for three days in presence of 25 mg L^{-1} NaF solution. After germination, the seed were further allowed to grow for seven days in presence of NaF solution as mentioned above. Thus, the seeds were maintained in triplicate sets as follows:

  Set 1: Water primed seeds grown for 10 days in presence of water only
  Set 2: water primed seeds grown for 10 days in presence of 25 mg L^{-1} NaF
  Set 3: 0.3 mM $Ca(OH)_2$ primed seeds grown for 10 days in presence of water only
  Set 4: 0.3 mM $Ca(OH)_2$ primed seeds grown for 10 days in presence of 25 mg L^{-1} NaF
  Set 5: 0.5 mM $Ca(OH)_2$ primed seeds grown for 10 days in presence of water only
  Set 6: 0.5 mM $Ca(OH)_2$ primed seeds grown for 10 days in presence of 25 mg L^{-1} NaF
  Set 7: 0.3 mM$Ca(NO_3)_2$ primed seeds grown for 10 days in presence of water only
  Set 8: 0.3 mM $Ca(NO_3)_2$ primed seeds grown for 10 days in presence of 25 mg L^{-1} NaF
  Set 9: 0.5 mM $Ca(NO_3)_2$ primed seeds grown for 10 days in presence of water only
  Set 10: 0.5 mM $Ca(NO_3)_2$ primed seeds grown for 10 days in presence of 25 mg L^{-1} NaF
  Set 11: 0.3 mM $CaCl_2$ primed seeds grown for 10 days in presence of water only
  Set 12: 0.3 mM $CaCl_2$ primed seeds grown for 10 days in presence of 25 mg L^{-1} NaF
  Set 13: 0.5 mM $CaCl_2$ primed seeds grown for 10 days in presence of water only
  Set 14: 0.5 mM $CaCl_2$ primed seeds grown for 10 days in presence of 25 mg L^{-1} NaF

  The seedlings were monitored for their overall growth and after completion of 10 days (3 days + 7 days), they were harvested and stored following the procedure as mentioned above.

- For all the experimental analysis presented in chapter 3 of the results and discussion section, seeds of the rice of rice cultivar, i.e., KH were initially sterilized and were incubated at 32°C in dark for 3 days. After 3 days, the uniformly germinated seeds were allowed to grow for additional 7 days in presence of two different concentrations of NaF solution (25 and 50 mg L^{-1}), either in absence or presence of 2 mM sodium metasilicate (Na_2SiO_3). The six experimental sets included:

  Set 1: KH seeds maintained in water only

  Set 2: KH seeds maintained in presence of 2 mM Na_2SiO_3 solution

  Set 3: KH seeds maintained in presence of 25 mg L^{-1} NaF

  Set 4: KH seeds maintained in presence of 25 mg L^{-1} NaF and 2 mM Na_2SiO_3

  Set 5: KH seeds maintained in presence of 50 mg L^{-1} NaF

  Set 6: KH seeds maintained in presence of 50 mg L^{-1} NaF and 2 mM Na_2SiO_3

  The pH of the Si solution was maintained within the range of 5.5-6.0 with 0.1 M HCl to avoid polymerization of silicates. After completion of 10 days (3 days + 7 days), the seedlings were harvested and were stored for further analysis. The concentration of Na_2SiO_3 was initially standardized by prior exposure of the seedlings to 1 mM, 2 mM and 4 mM Na_2SiO_3 through multiple trial studies. Preliminary standardization of silicon concentration established that the seedlings raised from seeds treated with 2 mM Na_2SiO_3 showed the best performance in terms of percentage of seed germination and overall plant growth, as compared to 1 mM and 4 mM Na_2SiO_3 (Fig. 4.3.1a).

- For all the experimental analysis presented in chapter 4 of the results and discussion section, seeds of the rice cultivars, i.e., NB, MT and JV were selected due to high endogenous ABA level. After sterilization, the seeds of each variety were divided into three sets with each set maintained in either water or 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Thus, altogether, seeds on 9 sets of Petri plates were allowed to germinate for three days, following which the uniformly germinated seeds were again maintained with the above set of treatments for 17 additional days. The nine experimental sets included:

  Set 1: NB seeds maintained with water for 20 days

  Set 2: NB seeds maintained with 12 g L^{-1} NaCl for 20 days

  Set 3: NB seeds maintained with 50 mg L^{-1} NaF for 20 days

  Set 4: MT seeds maintained with water for 20 days

  Set 5: MT seeds maintained with 12 g L^{-1} NaCl for 20 days

Set 6: MT seeds maintained with 50 mg L^{-1} NaF for 20 days

Set 7: JV seeds maintained with water for 20 days

Set 8: JV seeds maintained with 12 g L^{-1} NaCl for 20 days

Set 9: JV seeds maintained with 50 mg L^{-1} NaF for 20 days

After completion of 20 days (3 days + 17 days), all the sets were harvested and the seedlings were stored for further analysis as mentioned above.

In another experiment, the seeds of the three cultivars were again exposed to 50 mg L^{-1} NaF in conjunction with 30 mg L^{-1} ABA, applied exogenously, either in presence or absence of 2 mM Na_2WO_4. Thus, the seeds were maintained with water (control), 50 mg L^{-1} NaF alone, 30 mg L^{-1} ABA solution alone, 50 mg L^{-1} NaF along with 30 mg L^{-1} ABA solution, either in presence or absence of 2 mM Na_2WO_4.The seeds were again grown for 20 days and after that, they were harvested and stored at −80 °C.

3.2.2. Determination of physiological parameters

The number of seeds germinated after 3 days was calculated for each set and germination percentage was obtained. Root length (RL) and shoot length (SL) of 10 seedlings from each set was measured using a centimeter scale. Average RL and SL was expressed in cm. Similarly, biomass of 10 seedlings from each set was measured and average biomass was expressed as mg.

3.2.3. Determination of parameters associated with oxidative damages

3.2.3.1. Estimation of chlorophyll loss

For determination of chlorophyll, 500 mg of freshly harvested seedlings was homogenized in 5 mL of chilled acetone in presence of liquid nitrogen. After homogenization, the mixture was centrifuged at 10,000 revolutions per minute (rpm) for 20 min followed by collection of the supernatant. The absorbance of the supernatant was monitored at 645 and 663 nm and total chlorophyll level present in the tissues was estimated by following the equation of Arnon (1949):

$$Total\ chlorophyll = [\{(8.02 \times A_{663}) + (20.2 \times A_{645})\}/\ 1{,}000 \times W] \times V$$

W = fresh weight of the seedling and V = extraction volume

3.2.3.2. Estimation of electrolyte leakage from tissues

For determination of electrolyte leakage from the tissues, 100 mg of freshly harvested

seedlings was placed in 10 mL of distilled water for 22 h at room temperature. After completion of 22 h, initial conductance of the sample was recorded using digital conductivity meter (Digital Instruments Corporation, D-511, India) following which the samples were boiled for 30 min on water bath and final conductivity recorded (Rady 2011). Electrolyte leakage of the samples was calculated and was expressed in percentage.

3.2.3.3. Quantification of H_2O_2

For estimation of H_2O_2, protocol reported by Velikova et al. (2000) was used. 500 mg of seedlings was homogenized in 5 mL of trichloroacetic acid and was centrifuged for 15 min at 12,000 rpm. After centrifugation, supernatant (100 µL) was collected and mixed with 10 mM potassium phosphate buffer (pH 7.0) and KI. The mixture was kept in dark for 1 h after which the absorbance of the sample was recorded at 395 nm. The concentration of H_2O_2 in samples was calculated using a standard curve.

3.2.3.4. Quantification of superoxide anion

Superoxide anion in tissues was determined following the protocol by Yadu et al. (2017). 200 mg of the freshly harvested tissues was imbibed in 20 mM potassium phosphate buffer (pH 6) containing XTT at 26°C in dark. After 2 h, the absorbance was recorded at 470 nm and content of superoxide radical was calculated using extinction coefficient of 2.16×10^4 M^{-1} cm^{-1}.

3.2.3.5. Quantification of MDA

For determination of MDA content of the seedlings, 500 mg of the tissues was homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing trichloroacetic acid followed by centrifugation at 10,000 rpm for 15 min. After that, the supernatant (0.5 mL) was collected and was mixed with 4 mL of TBA solution [0.5% (w/v) TBA dissolved in 20 (w/v) trichloroacetic acid] and was boiled. After incubating for 30 min, the mixture was quickly placed on ice bath to stop the reaction. The absorbance of reaction mixture was recorded at 532 nm and 600 nm and total MDA concentration was calculated by using the difference in absorbance and using molar extinction co-efficient of 155 mM^{-1} cm^{-1} (Zeb and Ullah 2016).

3.2.3.6. Quantification of methylglyoxal (MG)

2.5 g of the seedlings was homogenized in 5 mL perchloric acid followed by centrifugation for 15 min at 10,000 rpm. After centrifugation, 0.05 mL of the supernatant was collected and mixed with 0.93 mL Na_2HPO_4 (100 mM, pH 7.0) and 0.02 mL N-acetyl cysteine. The rate of

change in absorbance at 288 nm was recorded for 2 min. MG content of the seedlings was estimated using standard curve prepared using different known concentration of MG (Wild et al. 2012).

3.2.3.7. Quantification of protein carbonylation

500 mg of tissues was crushed in 5 mL tricine buffer in presence of liquid nitrogen followed by collection of supernatant after centrifugation at 10,000 rpm for 20 min. The supernatant thus obtained (50 µL) was mixed with DNPH and was allowed to incubate in dark. After 1 h, 100% (w/v) trichloroacetic acid solution was added to stop the reaction and the mixture was placed on ice for 5 min followed by centrifugation at 10,000 rpm for 2 min to obtain the pellet. The pellet was washed in 0.5 mL ethanol:ethyl acetate (1:1) and was finally dissolved in 1 mL guanidine-HCl solution. Absorbance of the mixture thus obtained was recorded at 360 nm and total protein content was measured at 280 nm. Protein carbonyl content was calculated using a known curve of BSA following the protocol of Levine et al. (1994).

3.2.3.8. Estimation of protease activity

For determination of protease activity, 500 mg tissues was homogenized in 5 mL sodium phosphate buffer (100 mM, pH 6.5) followed by centrifugation at 10,000 rpm for 20 min. After centrifugation, Lowry assay was performed following which the protein content of each sample was determined. 200 µg of protein from each sample was incubated with 1 mL of 20 mg ml^{-1} purified casein hydrolysate for 2 h at 30°C. After incubation, trichloroacetic acid was added to stop the reaction and the protein present in the mixture was analyzed to measure protease activity (Basu et al. 2012).

3.2.3.9. Estimation of lipoxygenase (LOX, EC 1.13.2.11) activity

For determination of LOX activity in the tissues, 300 mg seedlings was homogenized in 2 mL sodium phosphate buffer (pH 7.0) followed by centrifugation at 10,000 rpm for 15 min. After centrifugation, 0.5 mL of the supernatant was mixed with 50 mM Tris-Cl buffer (pH 9.0) and 250 mM linoleic acid and change in absorbance of the mixture was recorded at 234 nm for 5 min. Finally, the LOX activity was calculated using molar extinction co-efficient of 25,000 M^{-1} cm^{-1} (Basu et al. 2012).

3.2.3.10. Estimation of NADPH oxidase (NOX, EC 1.6.3.1) activity

500 mg tissue was crushed in 3 mL phosphate buffer (pH 6.8) containing EDTA, DTT, sucrose and PMSF and homogenate was centrifuged at 10,000 rpm for 15 min. The molecular

fraction thus obtained was further resuspended in 50 mM phosphate buffer (pH 6.8) containing Triton X-100, DTT, PMSF and sucrose. The mixture was kept on ice bath for 5 min followed by centrifugation at 13,000 rpm for 45 min. The final supernatant was mixed with 50 mM Tris-Cl (pH 7.5), XTT and NADPH to measure the activity of NOX at 470 nm (Sagi and Fluhr 2001).

3.2.3.11. Estimation of ascorbic acid oxidase (AsAO, EC 1.10.3.3) activity

For determination of AsAO activity, 300 mg seedling was homogenized in 0.1 M phosphate buffer (pH 6.5) and homogenate was centrifuged at 12,000 rpm for 15 min to obtain the supernatant. The reaction mixture, which comprised of supernatant along with 0.1 M phosphate buffer (pH 5.6), EDTA and 0.1 mM L-ascorbic acid, was prepared following the protocol of Pignocchi et al. (2003). The change in absorbance of reaction mixture was recorded at 265 nm.

3.2.4. Determination of elements accumulated in seedlings

3.2.4.1. Estimation of fluoride in tissues

For determination of fluoride accumulated in the tissues, 200 mg seedling was homogenized in 4 mL TISAB buffer using liquid nitrogen. After complete homogenization, the mixture was transferred in test tube and was boiled for 30 min for complete mineralization of the sample after which it was centrifuged at 13,000 rpm for 20 min. The supernatant was carefully collected and fluoride present in the supernatant was measured using fluoride sensitive electrode (Cole Palmer, USA) (Banerjee and Roychoudhury 2019a).

3.2.4.2. Estimation of calcium (Ca) and sodium (Na) ions in tissues

For analysis of Ca and Na, 1 g seedling was freeze dried followed by crushing. After crushing, 200 mg of the sample was pelleted using a tabletop pelletizer by applying 120 kg cm^{-2} pressure for 1 min. Each pellet was analyzed via energy dispersive X-ray fluorescence spectrometry (Horiba Ltd., Japan) following earlier protocol of Attaelmanan (2012).

3.2.4.3. Estimation of silicon in tissues

Silicon accumulated in the tissues was analyzed by following the earlier work of Ang and Lee (2005). Initially, seedlings were dried and 1 g of dried sample was mineralized in concentrated HNO_3 till brown fumes stop appearing. After that, 1 mL H_2O_2 was added to the left over debris along with 1 mL double distilled water. After evaporation, 1 mL concentrated

perchloric acid was added and the whole mixture was mineralized till the formation of viscous yellow complex which was incubated overnight in presence of 1 mL concentrated HNO_3. After incubation, the volume was made up to 50 mL with distilled water. The mixture was finally filtered and the silicon content was measured in flame atomic absorption spectroscopy (Perkin-Elmer, USA).

3.2.5. Estimation of osmolytes synthesized in seedlings

3.2.5.1. Determination of proline

For estimation of proline, 300 mg seedling was crushed in 6 mL sulfosalicylic acid followed by centrifugation at 10.000 rpm for 15 min. After centrifugation, 2 mL supernatant was mixed with 2 mL glacial acetic acid and acid ninhydrin solution and the mixture was boiled in water bath. After completion of 30 min, the mixture was quickly cooled in ice bath for 15 min. The red complex thus formed was analyzed at 520 nm and the level of proline was calculated using standard curve of known concentration of proline (Basu et al. 2012).

3.2.5.2. Determination of amino acids

Supernatant collected for the estimation of proline was also used for the analysis of total amino acid formed in the tissues. 0.2 mL extract was mixed with 1 mL stannous chloride-ninhydrin solution and 0.8 mL of distilled water. The mixture was then kept in boiling water bath for 20 min after which it was quickly cooled on ice. The purple colored complex thus developed was analyzed at 570 nm and the concentration of amino acids was determined using a standard curve of known concentration of proline (Paul and Roychoudhury 2017).

3.2.5.3. Determination of glycine betaine

500 mg seedling was homogenized in 4 mL deionized water followed by centrifugation at 10,000 rpm for 15 min. After centrifugation, the supernatant was collected, mixed with 2N H_2SO_4 and was cooled on ice for 1 h. After 1 h, 0.2 mL chilled Lugol's iodine solution was added and mixture was vortexed followed by overnight incubation at 4°C. Next day, the mixture was centrifuged and periodide crystals thus obtained were dissolved in 7 mL 1,2-dichloroethane. After incubation for 2 h, the absorbance of the mixture was recorded at 365 nm and the amount of glycine betaine present in the seedlings was estimated using standard curve of known concentration of glycine betaine (Grieve and Grattan 1983).

3.2.6. Estimation of polyamines accumulated in seedlings

For determination of polyamines, 3 g seedling was homogenized in 5 mL chilled perchloric acid in presence of liquid nitrogen. After homogenization, the samples were kept on ice for 30 min followed by centrifugation at 10,000 rpm for 30 min. 0.2 mL of the supernatant thus collected was mixed with $NaHCO_3$ until saturation was reached. After complete effervescence, 0.2 mL of dansyl chloride (10 mg mL^{-1}) was added and the mixture was incubated overnight at room temperature in dark. The following day, 0.2 mL toluene was added followed by centrifugation at 10,000 rpm for 10 min. After centrifugation, the upper layer containing dansylated amines were carefully separated and were spotted on a thin layer chromatographic plate of silica gel that was pre-activated at 80°C for 1 h. Similarly, standard of putrescine, spermidine and spermine were also spotted on the silica plate. The plate was placed in glass chamber containing saturated atmosphere with cyclohexane:ethylacetate (3:2) as the mobile phase. After complete migration of the mobile phase, the plate was air dried and spots of the corresponding polyamines were marked. Marked spots were carefully scraped and silica thus obtained was dissolved in 2 mL acetone. The mixture was centrifuged at 10,000 rpm for 10 min and supernatant thus obtained was analyzed using a fluorescence spectrophotometer (Hitachi, Japan) with excitation and emission wavelengths of 360 nm and 506 nm, respectively (Roychoudhury et al. 2011).

3.2.7. Determination of level of non-enzymatic antioxidants in seedlings

3.2.7.1. Level of anthocyanins

For determination of anthocyanin level, 300 mg seedling was homogenized in acidified methanol followed by incubation of the mixture at 4°C overnight. Next day, the mixture was centrifuged at 10,000 rpm for 15 min followed by collection of the supernatant. The absorbance of the extract was measured at 525 nm and total anthocyanin content of the seedling was calculated according to the protocol suggested by Roychoudhury et al. (2007).

3.2.7.2. Level of phenolics

500 mg seedling was homogenized in 3 mL absolute methanol followed by collection of the supernatant by centrifugation of mixture at 10,000 rpm for 10 min. The supernatant (0.1 mL) thus obtained was mixed with 1 mL Folin-Ciocalteau reagent (10 fold diluted) and 2 mL Na_2CO_3 followed by incubation for 30 min in dark at 4°C. After incubation, absorbance of the mixture was recorded at 760 nm and the amount of phenolics was estimated using

standard curve of known concentration of gallic acid (Basu et al. 2012).

3.2.7.3. Level of xanthophylls and carotenoids

500 mg seedling was homogenized in 80% (v/v) acetone followed by collection of supernatant by centrifugation of mixture at 10,000 rpm for 15 min. Next, equal volume of supernatant was mixed with n-hexane followed by centrifugation at 12,000 rpm for 10 min to separate chlorophyll (lower layer) from carotenes and xanthophylls (upper layer). To 1 mL of collected upper layer, 90% (v/v) methanol was added in order to separate carotenes (upper layer) and xanthophylls (lower layer). The amount of carotenes and xanthophylls was estimated by recording the absorbance at 425 nm and 450 nm, respectively (Davies 1965).

3.2.7.4. Level of flavonoids

300 mg seedling was homogenized in 3 mL absolute ethanol and the mixture was centrifuged at 10,000 rpm for 15 min to collect the supernatant. The reaction mixture consists of 0.1 mL extract along with 1 mL $AlCl_3$. After incubating for 1 h in dark, the absorbance of mixture was recorded at 415 nm. The concentration of flavonoids in the tissues was calculated using a standard curve of known concentration of rutin (Basu et al. 2012).

3.2.7.5. Level of ascorbic acid

For estimation of ascorbic acid, freshly harvested seedling (500 mg) was homogenized in 3 mL of m-phosphoric acid followed by collection of supernatant by centrifugation of mixture at 10,000 rpm for 15 min. The reaction mixture consists of 0.1 mL extract, 1 mL ammonium heptamolybdate (dissolved in H_2SO_4) and 1 mL Na_2HPO_4. The mixture was boiled in water bath for 15 min followed by recording the absorbance of mixture at 660 nm. The concentration of ascorbic acid was estimated by using a standard curve of known volume of sodium ascorbate (Ghosh et al. 2008).

3.2.7.6. Level of reduced glutathione

For determination of reduced glutathione, 300 mg seedling was crushed in 5 mL tricine buffer followed by centrifugation of homogenate at 10.000 rpm for 15 min. The supernatant (0.1 mL) thus obtained was mixed with 0.9 mL Na_2HPO_4 and 30 µL DTNB. The reaction was allowed to stand for 2 min followed by measuring the absorbance at 412 nm. The concentration of reduced glutathione was estimated using a standard curve of known concentration of reduced glutathione (Bonifacio et al. 2011).

3.2.7.7. Measure of ABTS potential of seedling

300 mg seedling was homogenized in 3 mL tricine buffer and supernatant was collected by centrifugation of mixture at 10,000 rpm for 15 min. The reaction mixture consists of 1 mL extract and 250 µL ABTS (1 mL ABTS mixed in 88 mL of 50% ethanol) and was allowed to stand for 4 min. The absorbance of mixture was recorded at 734 nm and ABTS potential of seedlings was estimated and expressed as ascorbic acid equivalent (Lalhminghlui and Jagetia 2018).

3.2.7.8. Estimation of FRAP of seedling

For measure of FRAP, the extract obtained for estimation of ABTS potential was used. The reaction mixture composed of 50 µL extract along with 3 mL FRAP reagent. The mixture was incubated at 37°C for 30 min followed by recording the absorbance of mixture at 593 nm; FRAP of the seedling was expressed as milligram ferrous sulfate equivalents per 100 g seedling (Lalhminghlui and Jagetia 2018).

3.2.8. Estimation of activity of enzymatic antioxidants in seedlings

3.2.8.1. Activity of superoxide dismutase (SOD, EC 1.15.1.1)

For estimation of SOD activity, 500 mg seedlings was homogenized in 3 mL Tris-Cl buffer (50 mM, pH 7.5) containing EDTA and PVP at 4°C. The homogenate was centrifuged at 10,000 rpm for 15 min followed by the collection of supernatant. In 3 mL reaction mixture comprising of 50 mM potassium phosphate buffer (pH 7.8) along with NBT, EDTA, TEMED and riboflavin, 0.2 mL extract was added. The reaction mixture was divided into two sets. One set was maintained for 15 min in dark and the other set was exposed to light for same time. After incubation period, absorbance was measured for both set at 560 nm; SOD activity was calculated according to the protocol of Alonso et al. (2001).

3.2.8.2. Activity of catalase (CAT, EC 1.11.1.6)

For estimation of CAT activity, 300 mg seedling was homogenized in 3 mL ice cold sodium phosphate buffer (100 mM, pH 7.0) and was centrifuged at 10,000 rpm for 15 min. The supernatant (20 µL) obtained after centrifugation was mixed with sodium phosphate buffer (50 mM, pH 7.0) and H_2O_2 and change in the absorbance of the mixture at 240 nm due to utilization of H_2O_2 was monitored to estimate the activity of CAT (Banerjee and Roychoudhury 2019a).

3.2.8.3. Activity of guaiacol peroxidase (GPoX, EC 1.11.1.7)

The extract used for estimation of CAT activity was again used for the estimation of GPoX activity. To 0.1 mL extract, H_2O_2 and guaiacol was added in presence of sodium phosphate buffer (50 mM, pH 7.0). The change in absorbance of the mixture was taken at 470 nm for 5 min to calculate the activity of GPoX in seedlings (Awasthi et al. 1975).

3.2.8.4. Activity of ascorbate peroxidase (APX, EC 1.11.1.11)

For estimation of APX activity, the protocol suggested by Nakano and Asada (1981) was followed. 400 mg seedling was homogenized in ice cold tricine buffer (100 mM) containing EDTA, PVP, glycerol and β-mercaptoethanol. The homogenate was centrifuged at 10,000 rpm for 10 min and supernatant obtained was mixed with H_2O_2 and sodium ascorbate in presence of sodium phosphate buffer (50 mM, pH 7.0). The change in absorbance at 290 nm was monitored for 5 min to determine the activity of APX in seedling.

3.2.8.5. Activity of glutathione peroxidase (GPX, EC 1.11.1.9)

GPX activity was estimated using the same extract that was used for determination of APX activity. The reaction mixture comprised of 20 µL extract in presence of potassium phosphate buffer (100 mM, pH 7.0), cumene hydroperoxide, GSH, NADPH and I U of glutathione reductase. The decrease in absorbance was monitored at 340 nm for 5 min to calculate the activity of GPX (Awasthi et al. 1975).

3.2.8.6. Activity of glutathione reductase (GR, EC 1.8.1.7)

GR activity was measured by following the earlier protocol reported by Smirnoff and Colombe (1988). To 20 µL extract (supernatant used for determination of APX activity), tricine buffer (100 mM, pH 7.6), NADPH and oxidized glutathione were added and fall in absorbance (340 nm) was monitored for 5 min to calculate the activity of GR.

3.2.9. Estimation of Δ^1-pyrroline-5-carboxylate synthetase (P5CS, EC 2.7.2.11) and phenylalanine ammonia lyase (PAL, EC 4.3.1.24) activity in seedlings

P5CS activity was estimated following the earlier work of Ghosh and Roychoudhury (2018). 500 mg seedling was homogenized in 5 mL potassium phosphate buffer (100 mM, pH 7.4) containing EDTA, β-mercaptoethanol, PVP, $MgCl_2$ and KCl, followed by collection of supernatant by centrifugation of homogenate at 10,000 rpm for 10 min. The supernatant (100 µL) thus obtained was mixed with Tris-Cl buffer (100 mM, pH 7.2), NADPH, ATP and

$MgCl_2$ to estimate the activity of P5CS. The absorbance of reaction mixture was recorded at 340 nm and decrease in absorbance due to consumption of NADPH was monitored to calculate the activity of P5CS.

Extract of seedlings used earlier for the determination of APX activity was again used for estimation of PAL activity. The reaction mixture consisted of Tris-Cl buffer (100 mM, pH 8.8), L-phenylalanine and 100 µL enzyme extract. The assay mixture was incubated at 37°C for 30 min followed by the addition of 50 µL HCl to terminate the reaction. Finally, rise in absorbance of mixture due formation of *trans*-cinnamic acid was recorded at 290 nm to calculate the activity of PAL (Paul and Roychoudhury 2017).

3.2.10. Estimation of activity of enzymes of glyoxalase cycle

3.2.10.1. Activity of glyoxalase I (Gly I, EC 4.4.1.5)

For estimation of Gly I activity, 500 mg seedling was homogenized in cold phosphate buffer (10 mM, pH 7.0) consisting of EDTA, DTT and Triton X-100. The homogenate was centrifuged at 10,000 rpm for 15 min followed by collection of supernatant. For determination of Gly I activity, 100 µL extract was mixed with phosphate buffer (100 mM, pH 7.0) along with reduced glutathione and $MgSO_4$. The reaction was initiated by adding methylglyoxal followed by monitoring the absorbance at 240 nm for 5 min (Hasanuzzaman et al. 2019).

3.2.10.2. Activity of glyoxalase II (gly II, EC 3.1.2.6)

The extract used for the estimation of Gly I activity was also used for the determination of Gly II activity. The assay mixture comprised of 100 µL enzyme extract, Tris-Cl buffer (100 mM, pH 7.2) and DTNB. The reaction was initiated by adding SLG. Formation of GSH was monitored at 412 nm to determine the activity of Gly II (Hasanuzzaman et al. 2019).

3.2.11. Estimation of phytohormone level of the seedlings

3.2.11.1. Concentration of ABA

Freshly harvested seedling (200 mg) was homogenized in 4 mL potassium phosphate buffer (100 mM, pH 7.4) and was centrifuged at 10,000 rpm for 15 min to collect the supernatant. The extract isolated was diluted using Tris-buffer saline supplied with ABA immunodetection kit (Sigma-Aldrich, USA). ABA content of the seedling was estimated using ABA immunodetection kit at 405 nm following manufacturer's protocol. The actual

ABA content of the seedling was calculated using a standard curve of known concentration of ABA.

3.2.11.2. Concentration of melatonin

For determination of melatonin content, 500 mg seedlings was homogenized in 80% (v/v) methanol followed by incubation of the homogenate at 4°C for overnight. Next day, the supernatant was collected by centrifugation of homogenate at 10,000 rpm for 20 min. Level of melatonin was determined using melatonin immunodetection kit (Abcam, USA) at 405 nm. The concentration of melatonin was calculated using a standard curve of known volume of melatonin.

3.2.11.3. Concentration of gibberellin

200 mg seedling was homogenized in 3 mL absolute methanol and was left overnight at 4°C followed by collection of supernatant by centrifugation of homogenate at 10,000 rpm for 20 min. The supernatant (0.2 mL) was mixed with 1 mL acidified DNPH solution and was incubated at 100°C for 5 min. Following incubation, the mixture was quickly cooled on ice and was diluted using 5 mL potassium hydroxide-methanol solution. The wine red colored (equal volume of mixture mixed with distilled water) thus formed was analyzed at 430 nm to estimate the concentration of gibberellin using standard curve of known concentration of gibberellin (Graham and Thomas 1961).

3.2.12. Estimation of major organic acids and enzymes of TCA cycle

3.2.12.1. Major organic acids of TCA cycle

The level of pyruvate, citrate and malate in the seedling was quantified by crushing 500 mg seedling in 4 mL phosphate buffer (0.2 M, pH 7.4). After centrifugation at 10,000 rpm for 15 min, supernatant was collected and was deproteinized by adding 0.1 mL trichloroacetic acid.

3.2.12.1.1. Level of pyruvate

To 1 mL extract, 1 mL phosphate buffer (0.2 M, pH 7.4) was added along with DNPH followed by incubation for 30 min at 37°C. After incubation, 0.8 N NaOH was added and mixture was allowed to stand for 10 min. The brown color generated after 10 min was measured at 510 nm and the amount of pyruvate in seedlings was quantified by using a standard curve of known concentration of pyruvate (Sadasivam and Manickam 2008).

3.2.12.1.2. Level of citrate

The reaction mixture was prepared by adding 1 mL tissue extract in 0.5 M acetic anhydride and 0.1 M pyridine. The mixture was incubated at 32°C for 30 min in water bath followed by recording of absorbance at 405 nm. The amount of citrate in seedling was calculated by using a standard curve of known concentration of citrate (Saffran and Denstedt 1948).

3.2.12.1.3. Level of malate

To 1 mL supernatant, 0.1% DNPH, 1 N HCl and 10% (w/v) $CaCl_2$ were added followed by incubation at room temperature. After 30 min, 5 N NH_4OH was added to the reaction mixture and again incubated overnight at room temperature. Next day, the mixture was centrifuged at 10,000 rpm for 20 min and supernatant was discarded. The pellet obtained was dried at 105°C for 10 min followed by addition of 0.08% (w/v) orcinol-H_2SO_4 solution. The blue fluorescence of the mixture was quantified by fluorescence spectrophotometry using 340 nm as excitation wavelength and 385 nm as emission wavelength (Hummel 1949).

3.2.12.2. Activity of enzymes of TCA cycle

3.2.12.2.1. Activity of pyruvate dehydrogenase (PyDH, EC 1.2.4.1)

The PyDH activity was assayed by homogenizing 500 mg seedling in 3 mL Tris-Cl buffer (50 mM, pH 7.8) containing EDTA, sucrose, β-mercaptoethanol and BSA. To 0.2 mL supernatant (obtained after centrifugation of homogenate at 10,000 rpm for 15 min), Tris-Cl buffer (50 mM, pH 8.0) was added along with pyruvate, $MgCl_2$, coenzyme A and cysteine-HCl. The reaction was initiated by adding 1.4 mM NAD and change in absorbance was recorded at 340 nm for 2 min to estimate the activity of PyDH (Williams and Randalls 1979).

3.2.12.2.2. Activity of citrate synthase (CS, EC 2.3.3.1)

For the determination of CS activity, 300 mg seedling was homogenized in 3 mL Tris-Cl buffer (0.1 M, pH 8.0) containing iso-ascorbic acid, Triton X-100 and PVP. The homogenate was centrifuged at 10,000 rpm for 15 min to obtain the supernatant. The reaction mixture was prepared by adding 0.1 mL extract to Tris-Cl buffer (100 mM, pH 8.0) along with OAA, DTNB and acetyl CoA. The change in absorbance was noted at 412 nm for 2 min to calculate the activity of CS (Srere 1969).

3.2.12.2.3. Activity of isocitrate dehydrogenase (IDH, EC 1.1.1.41)

For estimation of IDH activity, 300 mg seedling was homogenized in 3 mL HEPES buffer (50 mM, pH 7.5) containing β-mercaptoethanol and PVP followed by centrifugation at

12,000 rpm for 15 min. The reaction mixture comprise of 20 µL enzyme extract, along with HEPES buffer (40 mM, pH 8.2), sodium isocitrate, $MnSO_4$ and NAD. The rise in absorbance was noted for 2 min at 340 nm to estimate the activity of IDH (Zhou et al. 2012).

3.2.12.2.4. Activity of succinate dehydrogenase (SDH, EC 1.3.5.1)

SDH activity was assayed by crushing 300 mg seedling in 4 mL Tris-Cl buffer (50 mM, pH 7.5) containing sucrose followed by centrifugation at 12,000 rpm for 20 min. After centrifugation, the supernatant (200 µL) obtained was mixed with Tris-Cl buffer (100 mM, pH 7.5) containing sucrose, sodium azide, INT and distilled water. The mixture was incubated at 30°C in water bath for 10 min followed by the addition of 95% (v/v) ethanol. The mixture was kept on ice for 15 min and was centrifuged at 8,000 rpm for 10 min. The absorbance of the supernatant thus obtained was recorded at 458 nm and activity of SDH was calculated following the method of Green and Narahara (1980).

3.2.12.2.5. Activity of fumarase (EC 4.2.1.2)

For estimation of fumarase activity, 500 mg seedling was homogenized in potassium phosphate buffer (100 mM, pH 7.4) followed by collection of the supernatant by centrifugation at 10,000 rpm for 20 min. The enzyme extract (0.5 mL) thus obtained was mixed with phosphate buffer (70 mM, pH 7.4) and malate. The rise in absorbance due to fumarase activity was estimated at 240 nm for 2 min and fumarase activity was calculated (Puchegger et al. 1990).

3.2.12.2.6. Activity of malate dehydrogenase (MDH, EC 1.1.1.37)

For estimation of MDH activity, 500 mg seedling was homogenized in 3 mL Tris-Cl buffer (50 mM, pH 8.0) containing EDTA, $MgCl_2$ and β-mercaptoethanol followed by collection of supernatant by centrifugation at 10,000 rpm for 20 min. The reaction mixture comprised of 100 µL enzyme extract along with Tris-Cl buffer (100 mM, pH 7.8), OAA and $MgCl_2$. The reaction was initiated by adding NADH and activity of MDH was estimated by recording the absorbance of reaction mixture at 340 nm for 2 min (Kumar et al. 2000).

3.2.13. Amount of sucrose and activity of sucrose metabolizing enzyme

3.2.13.1. Concentration of sucrose in seedling

300 mg seedling was homogenized in 3 mL 80% (v/v) ethanol followed by collection of supernatant by centrifugation of the homogenate at 10,000 rpm for 15 min. The extract

obtained (1 mL) was mixed with 5% (v/v) phenol and 98% (v/v) H_2SO_4 followed by incubation at 30°C for 20 min. After incubation, the intensity of the yellow-orange complex developed was measured at 490 nm; sucrose content of seedling was calculated using standard curve of glucose (Dubois et al. 1956).

3.2.13.2. Activity of acid invertase (INV, EC 3.2.1.26)

For determination of INV activity, 300 mg seedling was homogenized in 2 mL sodium acetate buffer (10 mM, pH 4.6) containing PMSF, EDTA and $MgCl_2$. The homogenate was centrifuged at 12,000 rpm for 15 min followed by collection of the supernatant. The reaction mixture was composed of sodium acetate buffer (10 mM, pH 4.6), sucrose and 200 µL extract followed by incubation of reaction mixture at 30°C for 30 min. After incubation, the reaction was terminated by adding 0.1 mL Na_2HPO_4 (Borkowaska and Szczerba 1991). The activity of INV was estimated by determining the amount of sucrose hydrolyzed following the above mentioned protocol of sucrose estimation.

3.2.13.3. Activity of sucrose phosphate synthase (SPS, EC 2.4.1.14)

400 mg seedling was homogenized in 3 mL HEPES-NaOH buffer (50 mM, pH 7.5) containing, EDTA, $MgCl_2$, DTT and Triton X-100. The homogenate was centrifuged at 12,000 rpm for 20 min followed by collection of supernatant (Hubbard et al. 1989). Activity of SPS was determined following the earlier protocol of Miron and Schaffer (1991). To 1 mL extract, 1 mL HEPES-NaOH buffer (100 mM, pH 7.5), $MgCl_2$, fructose-6-phosphate, glucose-6-phosphate and UDP-glucose were added followed by incubation at 37°C for 30 min. After incubation the reaction was terminated by adding 0.1 mL KOH and the amount of sucrose formed due to the activity of SPS was estimated using anthrone reagent following the previous work of Vassey et al. (1991).

3.2.13.4. Activity of sucrose synthase (SS, EC 2.4.1.13)

For estimation of SS activity, the plant extract was prepared following the above mentioned protocol similar to that of SPS activity. The reaction mixture comprised of 1 mL extract, 1 mL HEPES-NaOH buffer (100 mM, pH 7.5), $MgCl_2$, fructose and UDP-glucose. The mixture was incubated at 37°C for 30 min followed by reaction termination by adding 0.1 mL KOH (Miron and Schaffer 1991). The amount of sucrose hydrolyzed was estimated by using freshly prepared anthrone reagent following the previous work of Vassey et al. (1991).

3.2.14. Amount of starch and activity of starch metabolizing enzyme

3.2.14.1. Concentration of starch in seedling

The residual mass remaining after collection of supernatant during the estimation of sucrose was again suspended in distilled water followed by the addition of 52% (v/v) perchloric acid. The filtrate obtained after centrifugation at 10,000 rpm for 20 min was mixed with 500 µL, each of H_2SO_4 and phenol solution followed by incubation at 30°C in water bath for 20 min. After incubation, the absorbance was monitored at 490 nm and total starch content was estimated (McCready et al. 1950).

3.2.14.2. Activity of starch phosphorylase (PHO, EC 2.4.1.1)

300 mg seedling was homogenized in 4 mL citrate buffer (50 mM, pH 6.0) containing EDTA, PMSF and β-mercaptoethanol followed by collection of supernatant by centrifugation of homogenate at 12,000 rpm for 10 min. The supernatant (500 µL) was mixed with 1.5 mL citrate buffer (100 mM, pH 6.0), soluble starch and glucose-1-phosphate followed by incubation at room temperature for 20 min. After incubation period, 0.1 mL trichloroacetic acid was added to terminate the reaction followed by estimation of inorganic phosphorus formed in the reaction mixture using freshly prepared Chen's reagent at 800 nm (Fiske and Subbarow 1925).

3.2.14.3. Activity of α-amylase (EC 3.2.1.1)

500 mg seedling was homogenized in 3 mL sodium acetate buffer (0.1 M, pH 4.8) containing cysteine, followed by collection of supernatant by centrifugation of homogenate at 12,000 rpm for 10 min. 2 mL supernatant was mixed with 3 mM $CaCl_2$ and was incubated at 70°C for 5 min in hot water bath. Next to that, the prepared solution was mixed with sodium acetate buffer (0.1 M, pH 4.8) containing soluble starch and NaCl followed by incubation at 30°C for 1 h. After incubation, reaction was terminated by adding 6 M HCl. Finally to 1 mL aliquot, I_2-KI solution [1% (w/v) I_2 in 0.2% (w/v) KI] was added followed by recording the absorbance at 660 nm to estimate the activity of α-amylase (Chrispeels and Varner 1967).

3.2.15. Estimation of metabolites and activity of enzymes involved in nitrogen cycle

3.2.15.1. Estimation of total and soluble nitrogen

For estimation of total nitrogen, 1 g seedling was dried at 80°C for 3 days. Dried seedlings

was powdered followed by digestion in 2 mL concentrated H_2SO_4. After digestion, 1 mL H_2O_2 was added and mixture was again heated until all the solution gets decolorized with constant addition of H_2O_2. The reaction mixture was prepared by adding 1 mL of decolorized extract along with equal amount of NaOH, sodium silicate and Nessler's reagent. After incubation for 30 min in dark, the absorbance of the reaction mixture was recorded at 430 nm and concentration of total nitrogen was estimated using a standard curve of known concentration of $(NH_4)_2SO_4$ (Vogel 1961).

For determination of soluble nitrogen, 1 g seedling was homogenized in 4 mL distilled water followed by collection of the supernatant by centrifugation of the homogenate at 10,000 rpm for 5 min. 5% (w/v) trichloroacetic acid was added to deproteinize the extract. For estimation of soluble nitrogen, above mentioned protocol (used for the estimation of total nitrogen) was again followed with the deproteinized extract (Vogel 1961).

3.2.15.2. Estimation of nitrate and nitrite

500 mg seedling was boiled in 3 mL water for 30 min followed by collection of aqueous phase. To 1 mL aqueous solution, 0.5 mL salicylic acid was added and the mixture was incubated in dark for 20 min. After incubation, 1 mL NaOH wad added and the reaction mixture was vortexed until the yellow color appeared. The absorbance of the reaction mixture was recorded at 430 nm for the determination of nitrate content of seedling (Cataldo et al. 1975).

500 mg seedling was homogenized in 3 mL phosphate buffer (100 mM, pH 7.7) followed by collection of the supernatant by centrifugation of homogenate at 10,000 rpm for 20 min. To 1 mL supernatant, 500 µL color developing reagent (composed of sulfanilamide and NEDA) was added and the mixture was incubated in dark for 25 min. After incubation, the absorption of the red colored complex was monitored at 540 nm to estimate the nitrite level in seedlings (Snell and Snell 1953).

3.2.15.3. Activity of nitrate reductaes (NR, EC 1.6.6.1) and nitrite reductase (NiR, EC 1.7.7.1)

1 g seedling was homogenized in potassium phosphate buffer (25 mM, pH 8.8) containing EDTA and cysteine, followed by collection of the supernatant by centrifugation of homogenate at 10,000 rpm for 20 min. The reaction mixture comprised of potassium phosphate buffer (100 mM, pH 7.5), 0.2 mL potassium nitrate, 0.4 mL NADH and 0.2 mL

extract. The change in absorbance due to the formation of nitrite was monitored at 540 nm for 3 min to estimate the activity of NR (Hageman and Reed 1980).

500 mg seedling was homogenized in 3 mL Tris-Cl buffer (50 mM, pH 7.5). The homogenate was centrifuged at 10,000 rpm for 30 min for collection of the supernatant. The reaction mixture comprised of 1 mL Tris-Cl buffer (100 mM, pH 7.5), 40 µL sodium nitrite, 2 µL methyl viologen, 200 µL sodium hyposulfite and 300 µL enzyme extract. The reaction mixture was incubated at 30°C for 40 min followed by constant shaking until blue color developed. To the above mixture, 1 mL nitrogen color developing reagent was added and absorbance was measured at 540 nm to estimate the amount of nitrite reduced due to activity of NiR (Vega et al. 1980).

3.2.15.4. Estimation of dissolved ammonium ions

200 mg seedling was crushed in 3 mL deionized water followed by collection of supernatant by centrifugation of homogenate at 12,000 rpm for 15 min. To 0.5 mL plant extract, equal amount of phenol reagent (1% (v/v) phenol mixed with 0.05% (w/v) sodium nitroprusside) was added along with 0.3 mL alkaline sodium hypochlorite. The reaction mixture was incubated at 37°C for 20 min followed by recording of the absorbance at 430 nm to estimate the level of dissolved ammonium ions using a standard curve of known concentration of $(NH_4)_2SO_4$ (Hoshida et al. 2000).

3.2.15.5. Activity of glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.1.14)

For estimation of GS and GOGAT activity, 300 mg seedling was homogenized in 2 mL potassium phosphate buffer (50 mM, pH 7.5) containing, EDTA, DTT, soluble casein and PVP. The supernatant obtained after centrifugation of homogenate at 12,000 rpm for 20 min was used in the preparation of assay mixture.

For estimation of GS activity, reaction mixture was prepared by adding 200 µL enzyme extract to Tris-Cl buffer (100 mM, pH 7.6) along with glutamate, aspartate, EDTA, NH_4OH and ATP. The absorbance of the mixture was monitored at 540 nm and the amount of glutamic hydroxamate formed was calculated to determine the activity of GS (Canovas et al. 1991).

For determination of GOGAT activity, the reaction mixture was prepared by adding 100 µL enzyme extract to potassium phosphate buffer (50 mM, pH 7.4) containing L-glutamine, 2-

oxoglutarate and NADH. Decrease in absorbance at 340 nm due to utilization of NADH was monitored for 2 min to estimate the activity of GOGAT (Chen and Cullimore 1988).

3.2.15.6. Activity of glutamate dehydrogenase (GDH, EC 1.4.1.2)

For estimation of GDH activity, 500 mg seedling was homogenized in 3 mL sodium phosphate buffer (0.2 M, pH 7.5) containing EDTA, KCl, β-mercaptoethanol and Triton X-100. The supernatant (100 µL) obtained after centrifugation of homogenate at 10,000 rpm for 10 min was added to Tris-Cl buffer (100 mM, pH 8.2) containing NH_4Cl, $CaCl_2$, NADH and 2-oxoglutarate. The fall in absorbance due to utilization of NADH was monitored at 340 nm for 3 min to estimate the activity of GDH in seedling (Akihiro et al. 2008).

3.2.16. Estimation of metabolites and activity of enzymes involved in sulfur metabolism

3.2.16.1. Estimation of cysteine and hydrogen sulfide (H_2S)

For estimation of cysteine, 300 mg seedling was homogenized in 2 mL perchloric acid followed by collection of the supernatant by centrifugation of homogenate at 10,000 rpm for 15 min. To 200 µL extract, freshly prepared acid ninhydrin reagent was added along with glacial acetic acid. The reaction mixture was boiled for 20 min followed by quick cooling by placing in ice bath. The absorbance of reaction mixture was measured at 560 nm followed by estimation of cysteine concentration in seedling using a standard curve of known volume of cysteine (Gaitonde 1967).

For estimation of H_2S concentration, 1 g seedling was homogenized in 5 mL Tris-Cl buffer (20 mM, pH 8.0) containing EDTA and zinc acetate. The homogenate was centrifuged at 12,000 rpm for 15 min to obtain the tissue extract. Reaction mixture was prepared by adding 1 mL tissue extract to 3 mL Tris-Cl buffer (100 mM, pH 8.0) containing EDTA, zinc acetate and DTNB. Next, the mixture was allowed to stand for 2 min followed by recording of absorbance at 412 nm. The level of H_2S in the seedling was calculated using a standard curve of known concentration of sodium hydrosulfide (Li et al 2015).

3.2.16.2. Activity of ATP sulfurylase (ATP-S, EC 2.7. 7.4), O-acetylserine(thiol)lyase (OAS-TL, EC 2.5.1.47) and L-Cys desulfhydrase (DES, EC 4.4.1.1)

For estimation of ATP-S and OAS-TL activity, 500 mg seedling was crushed in 3 mL Tris-Cl buffer (50 mM, pH 8.0) containing DTT, EDTA and PVP followed by collection of supernatant by centrifugation of homogenate at 10,000 rpm for 20 min.

For determination of ATP-S activity, the reaction mixture was prepared by adding 100 µL extract to 0.5 mL Tris-Cl buffer (100 mM, pH 8.0) containing $MgCl_2$, ATP and 0.032 U/mL of sulfate-free inorganic pyrophosphatase. The reaction mixture was incubated at 37°C for 15 min followed by recording of absorbance at 340 nm. The ATPS activity was determined by subtracting the control (prepared using 100 µL distilled water) from the sample (Santiago et al. 2020).

For determination of OAS-TL activity, 100 µL extract was added to 1 mL Tris-Cl buffer (100 mM, pH 7.5) containing O-acetylserine, DTT and sodium sulfide followed by incubation at 37°C for 30 min. After incubation, the reaction was terminated by adding 1 mL acidic ninhydrin reagent. For development of color, reaction mixture was again heated at 100°C for 10 min followed by adding ethanol (0.1 mL) to stabilize the color formed. The absorbance was recorded at 560 nm and activity of OAS-TL was calculated by determining the amount of cysteine formed due to enzyme activity (Santiago et al. 2020).

For estimation of DES activity, 1 g seedling was homogenized in 3 mL Tris-Cl buffer (50 mM, pH 8.0) followed by collection of supernatant by centrifugation of homogenate at 10,000 rpm for 20 min. The reaction mixture was prepared by adding 100 µL extract with Tris-Cl buffer (100 mM, pH 9.0) containing L-cysteine and DTT. The mixture was incubated at 37°C for 40 min followed by addition of 100 µL HCl to terminate the reaction. To calculate the activity of DES, the amount of H_2S formed due to activity of DES was estimated following the above mentioned protocol (Li et al. 2012).

3.2.17. Total RNA isolation, DNase I treatment and semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analyses

100 mg freshly harvested seedling was used for RNA isolation. Total RNA was isolated using RNAisoplus (Takara, Japan) following the manufacturer's instructions. The purity of obtained RNA was determined from the A_{260}/A_{280} absorbance ratio. Total RNA was treated with DNase I (1 U μL^{-1}, Thermo Scientific, USA) to remove DNA contamination. About 5 µg of total RNA was reverse-transcribed using Maxima First Strand cDNA synthesis kit (Thermo Scientific). The cDNA sequences retrieved from NCBI Nucleotide Database and Rice Genome Annotation Project (TIGR) were used to design primer sequences for semi-quantitative RT-PCR analysis with *β-actin* as internal standard.

3.2.18. Immunoblot analysis

3 g of freshly harvested seedling was homogenized in TKM buffer using liquid nitrogen. The concentration of the protein isolated was estimated using Bradford assay (1976). 100 µg of protein for each treatment was electrophoresed for 90 min followed by transfer on nitrocellulose membrane (GE Healthcare, USA) using Mini Trans-Blot Cell (Bio-Rad, USA). The membrane was blocked using BSA solution followed by extensive washing to remove the excess BSA solution. Next, the membrane was incubated with primary antibody at prescribed dilutions by the manufacturers. After stringent washes, the anti-rabbit anti-Fc secondary antibody conjugated with alkaline phosphatase (Abcam, USA) at 1: 1000 dilutions was used as the secondary antibody. After incubation, the specific protein bands were detected by incubating the membrane in substrate solution containing NBT-BCIP (Sambrook and Russell 2001).

3.2.19. Isozyme analysis of SOD, APX, GPoX, GPX and CAT

For visualization of isozyme profile of enzymatic antioxidants, 3 g seedling was homogenized in 5 mL chilled sodium phosphate buffer (100 mM, pH 7.0) followed by the collection of supernatant by centrifugation of homogenate at 10,000 rpm for 20 min. The protein concentration of each sample was estimated using Bradford assay (1976). 120 µg protein was loaded for each sample and electrophoresis was performed at 4°C by passing 8 mA current in native-PAGE gel.

For visualization of SOD isozymes band, the gel was incubated in freshly prepared SOD staining substrate buffer under dark condition till bands appeared. The bands were visualized by keeping the gel submerged in the substrate buffer till the completion of incubation period (Roychoudhury et al. 2016).

For visualization of APX isozyme profile, the gel was incubated in phosphate buffer (50 mM, pH 7.0) and 2 mM sodium ascorbate for 30 min followed by incubation in phosphate buffer (50 mM, pH 7.0) containing 4 mM sodium ascorbate and 2 mM H_2O_2 for 20 min. Next, the gel was washed with phosphate buffer (50 mM, pH 7.0) followed by incubation in phosphate buffer (50 mM, pH 7.8) containing TEMED and NBT. The APX isozyme band was visualized under white light as achromatic bands on a purple background (Roychoudhury et al. 2016).

GPoX isozyme was visualized by staining the gel in GPox substrate buffer for 20 min in dark along with gentle shaking. GPoX bands appeared as dark brown band on a colorless background (Roychoudhury et al. 2016).

The isozyme of GPX was visualized by staining the gel in GPX substrate buffer for 20 min followed by brief rinse. The band of GPX was developed in dark by submerging the stained gel in 1.2 mM MTT and 1.6 mM PMS for 10 min. The clear zones of GPX activity were found on purple background (Lin et al. 2002).

The isozyme of CAT was visualized by staining the gel in CAT substrate buffer for 40 min in dark along with gentle shaking (Roychoudhury et al. 2016).

3.2.20. Protein estimation and statistical analysis

For estimating the enzyme activity, the total protein was extracted and their concentration was determined following Bradford (1976) where BSA was used as the standard. Equal concentration of total protein was used in all the experiments from each sample set.

Three replicates (n = 3) for each set were maintained in a completely randomized design (CRD). The data which are graphically represented are mean ± standard error (SE) and statistical significance was calculated at $p \leq 0.05$, using analysis of variance (ANOVA). The XLSTAT 2017 was used for performing all the calculations."

Chapter 4

Results and Discussion

Chapter 4.1

Differential Reprogramming of the Protective Metabolites and Nitrogen and Sulfur Metabolism in Two Indica Rice Varieties, Regulates their Pattern of Fluoride Stress Response

Data Published in this chapter has already been published in:
Journal of Plant Growth Regulation (2022)
doi:10.1007/s00344-022-10780-5

"4.1.1. Overview of the chapter

The results obtained in this chapter showed that unregulated uptake of fluoride ions in 10 and 20 day old Khitish (KH) and MTU1010 (MTU) seedlings, respectively occurred due to higher expression of chloride channel (CLC1 and CLC2). Higher deposition of fluoride ions enhanced the formation of cytotoxic metabolites like superoxide ions, H_2O_2, MDA and MG and triggered the activity of LOX and NOX which in turn enhanced the loss of electrolytes form the cells along with chlorophyll loss in the seedlings that eventually retarded the growth of the seedling and lowered the tissue biomass. Additionally, the level of ammonium ions was also enhanced in seedlings, along with inhibited activity of GS and GOGAT, implying that the incorporation of ammonium ions into glutamine and glutamate via GS and GOGAT was hindered. However, in case of stressed 20-day old KH and 10-day old MTU seedlings, plant growth was maintained with lesser oxidative damages due to up regulated expression of H^+-ATPase and FEX. Moreover, higher accumulation of nitrogen-containing compounds such as nitrate, nitrite and total and soluble nitrogen and sulfur-containing compounds like cysteine and H_2S could also be linked with higher tolerance capability of seedlings. Higher accumulation of nitrogen was due to higher activity and expression of NR and NiR. Similarly sulfur assimilation was catalyzed by the higher activity of ATP-S and OAS-TL. Additionally, the activity of the enzymatic antioxidants (SOD, CAT, APX, GPoX and GPX) and the level of non-enzymatic antioxidants (anthocyanins and flavonoids) were also enhanced that strengthened the antioxidative potential of the seedlings. The present chapter clearly demonstrated that differential reprogramming of the protective metabolites and nitrogen and sulfur assimilation pathways was largely responsible for the differential pattern of adaptive strategies against fluoride stress in the two varieties, viz., KH and MTU, the former exhibiting resilience against long-term stress, whilst the latter showing high susceptibility for the same.

4.1.2. Introduction of the chapter

Continuous discharge of fluoride containing waste materials in the biosphere and unplanned exploitation of natural resources have significantly enhanced the level of fluoride in the environment. Fluoride ions are easily taken up by the plants by the chloride channels due to their smaller size as compared to that of chlorine ions (Baunthiyal and Sharma 2014). Excess deposition of fluoride ions in the tissues led to the formation of reactive oxygen species (ROS) such as superoxide ions and hydrogen peroxide (H_2O_2) that in turn causes membrane perturbation by oxidation of lipid moiety present in cell membrane. Additionally, excess fluoride accumulation also induces the activity of oxidative enzymes like lipoxygenase (LOX) and NADPH oxidase (NOX) which intensify the effects of oxidative damage (Hong et al. 2016). Higher membrane damage causes leakage of electrolytes from the cells along with the formation of other cytotoxic metabolites like methyglyoxal (MG) and malondialdehyde (MDA). Deposition of fluoride ions above threshold level also decreases the level of chlorophyll in the tissues by inhibiting its formation or/and enhancing the activity of chlorophyll degrading enzymes (Debska et al. 2012).

Nitrogen and sulfur is an essential element that plays a pivotal role in controlling growth and development of plants. They are regarded as macronutrient due to their wide involvement in the formation of secondary metabolites, polyamines, proteins, nucleic acids and various metabolic enzymes (Zhang et al. 2013). Nitrogen is taken up from the soil and is assimilated in plants by the activity of nitrate reductase (NR) and nitrite reductase (NiR). Another major nitrogen-containing compound formed in plants due to the activity of NiR is ammonium ion which is highly toxic for plants and is rapidly converted to glutamine and glutamate by the enzymatic action of glutamine synthetase (GS) and glutamate synthase (GOGAT) that together constitutes the GS/GOGAT cycle (Gangwar and Singh 2011). Glutamate is catabolised by the enzymatic action of glutamate dehydrogenase (GDH) that releases 2-oxoglutarate that connects nitrogen with carbon metabolism (Singh et al. 2009). Sulfur is taken from the soil as sulfate ions via sulfate transporters (Davidian and Kopriva 2010). Once sulfate is accumulated within the cells, it is converted into cysteine (first organic compound of sulfur assimilatory pathway) via activity of ATP-sulfurylase (ATP-S) and O-acetylserine(thiol) lyase (OAS-TL) (Gill and Tuteja 2011). Another major sulfur-containing compound is hydrogen sulfide (H_2S) that is formed due to the degradation of cysteine catalyzed by cysteine desufhydrase (DES) (Vojtovič et al. 2020). In addition, plants have

internal protective machineries which consist of enzymatic antioxidants (CAT, SOD, APX and GPX) and non-enzymatic antioxidants (anthocyanins and flavonoids) that strengthen the antioxidative potential and thus help in better survival capability (Waskiewicz et al. 2014).

In spite of considerable research contributing towards our perception regarding the effect of fluoride stress in different plants species, detailed study regarding time-dependent effect of fluoride toxicity in rice is still unexplored. The current work was devised to examine the effect of fluoride stress in two rice cultivars, viz., KH and MTU, exposed to short term (10 days) and long term (20 days) fluoride stress. After 10 and 20 days, fluoride accumulation in seedlings was examined along with expression level of the major transporters (CLC1, CLC2, H^+-ATPase and FEX) involved in fluoride translocation. Certain physiological parameters including those associated with oxidative damages were also analyzed in seedlings exposed to fluoride stress along with the level of other protective metabolites like non-enzymatic antioxidants and activity of enzymatic antioxidants. Additionally, nitrogen and sulfur metabolism in seedlings was also determined to decipher the differential regulation of nitrogen and sulfur metabolism during fluoride toxicity.

4.1.3. Result and discussion

4.1.3.1. Analysis of basic physiological parameters

On being exposed to NaF solution (applied for 10 days), the shoot and root length of KH was significantly reduced (1.6 and 1.7 fold, respectively) in comparison to the control seedlings. However, no prominent reduction was noted in the above mentioned parameters in case of MTU variety. On the contrary, long term fluoride exposure enhanced the adaptability of KH by maintaining their growth, whereas the same was drastically affected in MTU variety (1.6 and 1.3 times reduced shoot and root length, respectively). Similar trend was also observed in case of seedling biomass where 1.7 and 1.4 times reduced biomass was noted in 10 and 20 day old KH and MTU seedlings, respectively, whereas 20 and 10 day old stressed KH and MTU seedlings showed no significant change in their biomass (Fig 4.1.1; Table 4.1.1). Thus, this observation pointed out the fact that KH was initially susceptible to fluoride stress; however; with time; it got adapted to fluoride toxicity and thus showed better growth performance. However, MTU which showed higher stress tolerance capability in initial phase was severely affected on being exposed to long term stressed condition.

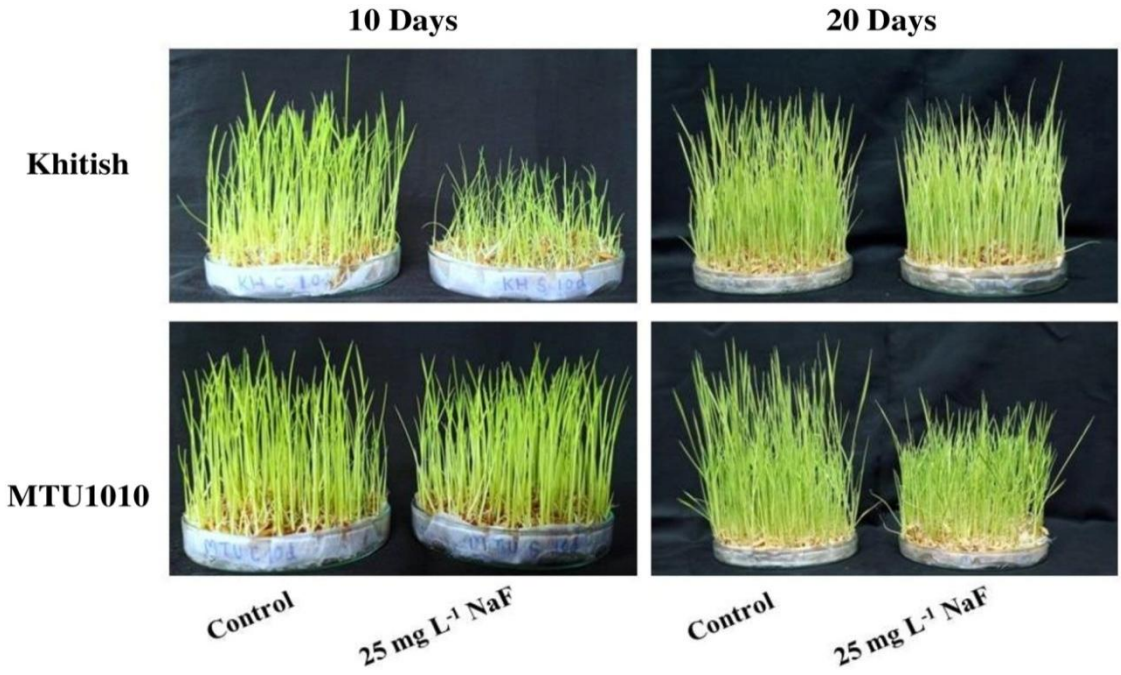


Fig. 4.1.1: Effect of application of 25 mg L^{-1} NaF solution for 10 days and 20 days in KH and MTU seedlings; the untreated seedlings (grown in water) served as experimental control.

Table 4.1.1: Root and shoot length and biomass of 10 day and 20 day old KH and MTU seedlings, grown either in presence or absence of 25 mg L^{-1} NaF solution. Data represented are the mean value (n = 3) ± SE. '*' represents data with significant differences (P ≤ 0.05) between control and stressed seedlings, '#' represents data with significant differences (P ≤ 0.05) between the two cultivars and '+' represents data with significant differences (P ≤ 0.05) between the seedlings grown for 10 days and 20 days.

Variety and Treatment	Period of exposure	Root length (cm)	Shoot length (cm)	Seedling Biomass (mg)
KH (Control)	10 Days	4.98 ± 0.49	10.84 ± 1.14	257.21 ± 13.54
KH (Stress)		2.98 ± 0.74*	6.54 ± 0.98*	149.47 ± 10.54*
MTU (Control)		5.21 ± 1.01	11.24 ± 1.03	297.24 ± 9.84
MTU (Stress)		4.87 ± 0.64#	10.47 ± 0.54#	224.32 ± 14.57#
KH (Control)	20 Days	7.87 ± 1.00	19.64 ± 1.40+	402.12 ± 15.24+
KH (Stress)		7.27 ± 0.98+	16.74 ± 1.54+	354.21 ± 11.42+
MTU (Control)		7.24 ± 0.74	20.54 ± 0.64+	421.21 ± 14.54+
MTU (Stress)		5.34 ± 0.69*#	12.47 ± 1.34*	301.49 ± 12.74*

4.1.3.2. Uptake and accumulation of fluoride ions

Reduced growth of 10 day-old KH and 20 day-old MTU seedlings could be attributed to 27.7 and 40.1 fold higher accumulations of fluoride ions in the respective cultivars during stress (Fig. 4.1.2a). Higher uptake of fluoride ions by the above mentioned cultivars could be linked with the higher expression of *CLC1* and *CLC2* which facilitates the uptake of fluoride ions via roots (Fig. 4.1.2b, 4.1.2c). Our results were in line with the previous work of Banerjee et al. (2019a) where they showed that higher accumulation of fluoride ions in two different rice cultivars (IR-64 and Gobindobhog) was assisted by the above mentioned chloride channels. They further supported their observation by the fact that chloride channels have larger passage diameter as compared to that of size of fluoride ions and thus can facilitate the uptake

of fluoride ions. On the contrary, regulated uptake of fluoride ions, noted in 20 day-old and 10 day-old KH and MTU seedlings, respectively might be due to down regulation of both the chloride channels in the two cultivars. In addition to chloride channel, analysis of the expression pattern (at both gene and protein level) of H^+-ATPase (involved in maintaining the proton gradient across the cells) and FEX (selectively exporting fluoride ions) revealed that both the transporters were induced in expression in the stressed 20-day old and 10 day-old KH and MTU seedlings, respectively, as compared to that of respective 10-day old and 20 day-old counterparts (Fig. 4.1.2d, 4.1.2e, 4.1.2f, 4.1.2g). Higher expression of H^+-ATPase and FEX plays a pivotal role in stressed seedlings in regulating the cellular homeostasis of the seedlings. Earlier, Baunthiyal and Sharma (2014) reported that higher expression of H^+-ATPase was important in regulating the tolerance capability of three semi-arid fluoride-hyperaccumulator plants (*Cassia fistula*, *Acacia tortilis* and *Prosopis juliflora*) on being exposed to fluoride stress. Similarly, in another study, Banerjee et al. (2019a) observed higher expression of H^+-ATPase in the stressed seedlings of the tolerant cultivar, as compared to that of stressed seedlings of the sensitive variety, supporting our observation. Recently, Agarwal et al. (2021) reported that the expression of FEX was highly induced in *Vigna radiata* that assisted in the exclusion of the accumulated fluoride ions from the plasma membrane of plants, imparting higher survival rate to the seedlings. Additionally, Banerjee and Roychoudhury (2021) also demonstrated that overexpression of FEX in *Nicotiana benthamiana* was efficient to promote fluoride tolerance which coincides with our observation. Thus, unregulated uptake of fluoride ions in 10-day old KH seedling via CLC1 and CLC2 was responsible for stunted growth of the seedlings; however, the seedlings were less affected during long term (20 days) exposure to fluoride because of the enhanced expression of H^+-ATPase and FEX transporters which maintained cellular homeostasis. Moreover, down regulated expression of *CLC1* and *CLC2* also controlled the uptake of fluoride ions. However, contrasting behavior was noted in MTU seedlings where the absorption of fluoride ions was initially regulated due to reduced expression of chloride channels; however, with the passage of time, this regulation was lost, resulting in higher accumulation of fluoride ions in seedlings that eventually led to significant damages in the seedlings.

Fig: 4.1.2a

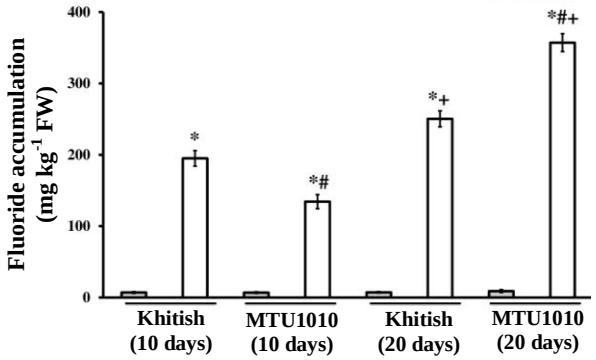


Fig: 4.1.2b

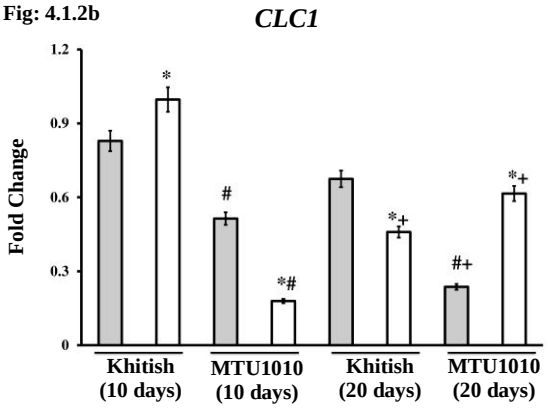


Fig: 4.1.2c

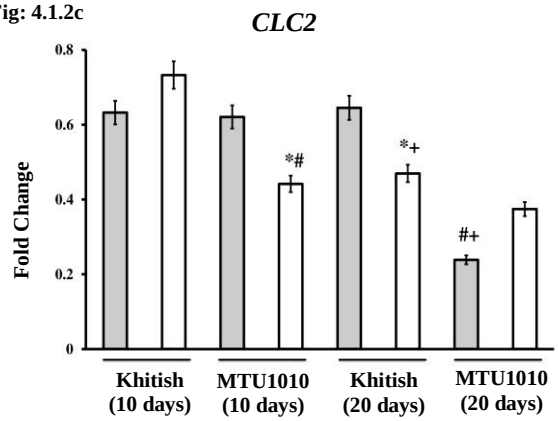


Fig: 4.1.2d

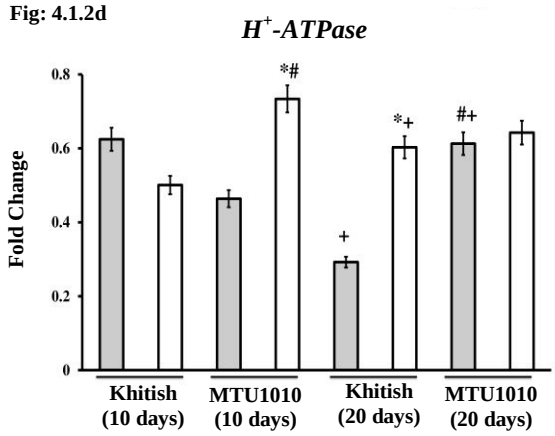
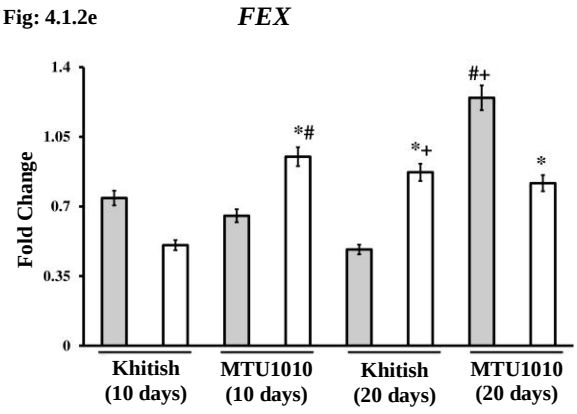


Fig: 4.1.2e



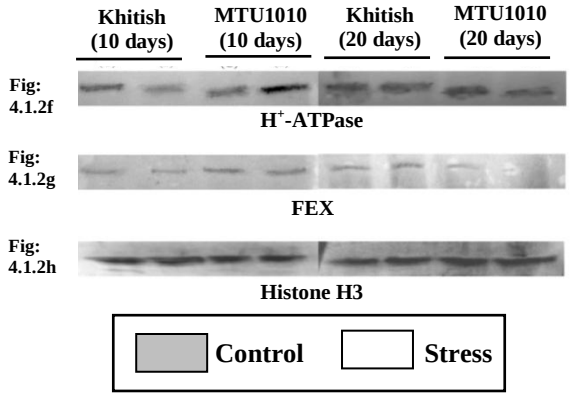


Fig. 4.1.2: Level of fluoride accumulation (**a**), expression pattern of *CLC1* (**b**), *CLC2* (**c**), *H⁺-ATPase* (**d**) and *FEX* (**e**) genes and accumulation of H⁺-ATPase (**f**) and FEX (**g**) proteins in KH and MTU seedlings in presence of 25 mg L^{-1} NaF for 10 days and 20 days. '*' represents data with significant differences ($P \leq 0.05$) between control and stressed seedlings, '#' represents data with significant differences ($P \leq 0.05$) between the two cultivars and '+' represents data with significant differences ($P \leq 0.05$) between the seedlings grown for 10 days and 20 days. Histone H3 (**h**) was used as a control to check equal loading of protein.

4.1.3.3. Higher fluoride accumulation leads to higher oxidative damages

Higher accumulation of fluoride ions in 10 day-old and 20 day-old seedlings of KH and MTU, respectively led to the enhanced formation of ROS such as H_2O_2 (2.7 and 2.4 times, respectively) and superoxide radicals (2.0 and 2.4 fold, respectively) which caused peroxidation of lipid membrane, leading to 2.5 and 2.6 times higher leakage of electrolytes from the cells and also induced the formation of cytotoxic metabolites like MG and MDA in the stressed seedlings, as compared to that of control (non-stressed) seedlings. Additionally, higher accumulation of ROS also led to 1.3 and 1.6 times higher chlorophyll loss in 10 day-old and 20 day-old stressed KH and MTU seedlings, respectively. Similar trend was also noted in case of LOX and NOX activity where exposure to fluoride stress enhanced their activity by 2.2 and 2.0 times, respectively in 10 day-old stressed KH seedlings and by 2.8 and 2.0 fold, respectively in 20 day-old stressed MTU seedlings, as compared to that of control seedlings (Table 4.1.2). Higher activity of LOX and NOX induces the accumulation of ROS in seedlings which further worsened the condition. Thus, it can be concluded that higher fluoride accumulation in NaF-treated 10 day-old and 20 day-old KH and MTU rice cultivar, respectively resulted in higher formation of H_2O_2 in seedlings that hampered the overall integrity of cell membrane, leading to higher electrolyte leakage along with induced formation of MDA and MG, higher chlorophyll loss and LOX and NOX activity. Contrasting trend was noted in case of 20 day-old and 10 day-old KH and MTU seedlings, respectively which might be due to controlled uptake of fluoride ions, restricting the formation of ROS with lower extent of peroxidation of lipid membrane and reduced formation of cytotoxic metabolites (Table 4.1.2). Our observation can be justified by the earlier work of Banerjee and Roychoudhury (2019a) where they reported that fluoride accumulation in the tolerant rice cultivar was significantly lower, as compared to that of susceptible variety upon being treated with 15 and 25 mg L^{-1} NaF solution, causing lower oxidative damage and maintaining the growth of the seedlings.

Table 4.1.2: Chlorophyll level, H_2O_2, superoxide radical, electrolyte leakage, MDA, MG, LOX and NOX activity in 10 and 20 day-old KH and MTU seedlings, grown either in presence or absence of 25 mg L^{-1} NaF solution. Data represented are the mean value (n = 3) ± SE. '*' represents data with significant differences ($P \leq 0.05$) between control and stressed seedlings, '#' represents data with significant differences ($P \leq 0.05$) between

the two cultivars and '+' represents data with significant differences ($P \leq 0.05$) between the seedlings grown for 10 days and 20 days.

Parameters	10 days				20 days			
	KH Control	KH Stress	MTU Control	MTU Stress	KH Control	KH Stress	MTU Control	MTU Stress
Chlorophyll (µg g^{-1} FW)	100.67 ± 10.98	78.98 ± 9.87*	110.87 ± 8.87	89.98 ± 6.87	150.87 ± 11.87^{+}	134.89 ± 12.36^{*+}	165.98 ± 9.87^{+}	100.87 ± 12.98$^{*\#+}$
H_2O_2 (µM g^{-1} FW)	0.13 ± 0.04	0.35 ± 0.01*	0.15 ± 0.02	0.21 ± 0.03$^{\#}$	0.19 ± 0.04	0.29 ± 0.06	0.17 ± 0.05	0.40 ± 0.08$^{*\#+}$
Superoxide anion (µM min^{-1} g^{-1} FW)	2.12 ± 0.12	4.19 ± 0.67*	2.09 ± 0.98	3.16 ± 0.87	2.68 ± 0.57	4.26 ± 0.46*	2.87 ± 0.33	6.78 ± 0.82$^{*\#+}$
Electrolyte leakage (%)	15.31 ± 1.21	39.12 ± 1.56*	14.98 ± 2.12	21.87 ± 1.67$^{\#}$	15.98 ± 1.45	35.65 ± 2.87*	16.90 ± 1.20	43.61 ± 1.43^{*+}
MDA (µM g^{-1} FW)	2.11 ± 0.98	4.89 ± 0.87*	1.97 ± 0.43	2.98 ± 0.57$^{\#}$	2.56 ± 0.52	4.15 ± 0.87*	2.19 ± 0.45	6.86 ± 3.38$^{*\#+}$
MG (mg g^{-1} FW)	24.89 ± 1.56	35.18 ± 1.17*	21.87 ± 0.98	28.19 ± 1.56$^{\#}$	26.92 ± 1.19	39.87 ± 0.59*	24.87 ± 1.67	40.97 ± 1.49$^{*\#+}$
LOX activity (U mg^{-1} leaf protein)	5.87 ± 0.45	12.87 ± 0.98*	4.98 ± 0.87	8.98 ± 0.34$^{\#}$	6.89 ± 1.11	15.98 ± 0.76*	6.98 ± 0.15	19.98 ± 1.78^{*+}
NOX activity (U mg^{-1} leaf protein)	22.27 ± 1.19	43.87 ± 0.95*	24.87 ± 1.67	34.98 ± 0.56$^{\#}$	27.98 ± 0.81	37.98 ± 1.39	29.76 ± 2.67	59.67 ± 1.33$^{*\#+}$

4.1.3.4. Fluoride stress regulated the content of different forms of endogenous nitrogen

The level of total and soluble nitrogen was reduced in fluoride stressed (applied for 10 days) KH cultivars (1.5 and 1.7 times, respectively), whereas in case of MTU, the level of total and soluble nitrogen was not significantly affected in comparison to that of water treated seedlings. However, the level of total and soluble nitrogen was comparable to that of control seedlings in KH cultivars and the same was reduced by 1.5 fold in MTU variety on being exposed to long term fluoride stress. Similar trend was also noted in case of nitrate and nitrite, which was reduced by 2.2 and 2.1 fold, respectively in KH seedlings (10 day-old NaF treated) and 1.3 and 1.4 times in MTU seedlings (exposed to fluoride solution for 20 day) in comparison to that of control seedlings (Table 4.1.3). These observations clearly pointed out that depletion in endogenous nitrogen pool (total and soluble nitrogen, nitrate and nitrite) was one of the prime causes for inhibited growth of KH seedlings in the initial phase of their life cycle. Additionally, with time induction, the restoration of the endogenous nitrogen pool was responsible for overcoming the fluoride-mediated growth inhibition. On the contrary, the depletion of nitrogen-containing metabolites in 20 day-old MTU seedlings led to significant amount of damage which was significantly lesser in 10 day-old seedlings. The present work can be linked with the recent work of Paul et al. (2022), where they showed that exogenous application of nitrogen-containing fertilizers like calcium nitrate and ammonium acetate were highly effective in abrogating the effects of cadmium-mediated oxidative damage in rice cultivar, i.e., Lalat, by differential reprogramming of the nitrogen-containing metabolites. Similarly, Sil et al. (2020) also reported that exogenous application of silicon restored the level of nitrogen-containing metabolites in wheat seedling exposed to arsenic toxicity that eventually enhanced the tolerance capability of the seedlings.

The level of another major nitrogen containing metabolites, formed in plants in response to abiotic stress is amino acids. Amino acids are well known for their ROS-scavenging capability and for maintaining the osmotic balance of the cells. The level of amino acids was enhanced by 1.4 fold in both 10 and 20 day-old fluoride stressed KH seedlings and by 1.7 and 1.3 fold, respectively in 10 and 20 day-old fluoride stressed MTU seedlings (Table 4.1.3). The level of amino acid pool was enhanced in all the treatment; however, the major rise was noted in 20 day-old KH and 10 day-old MTU which accounts for their better growth. The rise in the level of amino acids can be corroborated with the previous work of Azevedo-Neto et al. (2009) and Misra et al. (2006) where they showed higher content of amino acids in roots

and leaves of salt-stressed maize and green gram seedlings, respectively that eventually regulated the survival of the seedlings.

Table 4.1.3: Level of total and soluble nitrogen, nitrate, nitrite, amino acids and ammonium ions in KH and MTU seedlings in presence of 25 mg L^{-1} NaF for 10 days and 20 days. Data represented are the mean value (n = 3) ± SE. '*' represents data with significant differences (P ≤ 0.05) between control and stressed seedlings, '#' represents data with significant differences (P ≤ 0.05) between the two cultivars and '+' represents data with significant differences (P ≤ 0.05) between the seedlings grown for 10 days and 20 days

Variety and treatment	Period of exposure	Total nitrogen (mg g^{-1} DW)	Soluble nitrogen (µg g^{-1} FW)	Nitrate (µg g^{-1} FW)	Nitrite (µg g^{-1} FW)	Amino acid (mg g^{-1} FW)	Ammonium ions (µg g^{-1} FW)
KH (Control)	10 Days	13.83 ± 0.97	76.91 ± 3.24	259.32 ± 7.07	27.91 ± 2.72	335.88 ± 9.09	22.42 ± 2.66
KH (Stress)		9.43 ± 0.15*	44.36 ± 2.61*	119.85 ± 6.41*	13.41 ± 1.19*	467.67 ± 14.94	47.27 ± 2.92*
MTU (Control)		16.52 ± 1.49	70.89 ± 2.33	229.79 ± 6.25	35.05 ± 3.09	347.37 ± 10.60	25.31 ± 3.78
MTU (Stress)		14.00 ± 0.29	67.09 ± 3.92$^{\#}$	201.56 ± 3.99$^{\#}$	31.20 ± 1.88$^{\#}$	593.79 ± 9.12*	34.21 ± 2.22$^{\#}$
KH (Control)	20 Days	21.97 ± 0.47^{+}	130.28 ± 8.02^{+}	354.44 ± 6.59	52.94 ± 3.35^{+}	503.93 ± 8.08^{+}	30.19 ± 3.93
KH (Stress)		28.53 ± 1.56^{+}	111.44 ± 1.27^{+}	327.72 ± 3.88^{+}	57.00 ± 2.17^{+}	705.69 ± 10.29^{*+}	36.67 ± 1.09
MTU (Control)		24.07 ± 0.19^{+}	145.21 ± 2.82^{+}	393.63 ± 4.79^{+}	61.10 ± 1.74^{+}	530.79 ± 9.16^{+}	27.96 ± 2.43
MTU (Stress)		16.28 ± 1.33$^{*\#}$	97.89 ± 3.61*	293.34 ± 5.62^{*+}	42.41 ± 2.68$^{*\#}$	680.41 ± 12.72	52.27 ± 3.81$^{*\#+}$

4.1.3.5. Activity and gene expression of NR and NiR

In 10 day-old KH seedling, the activity of NR and NiR was drastically reduced by 1.7 times and 1.8 times, respectively. However with time, the activity of both the above mentioned enzymes was recovered in fluoride-stressed KH seedlings and became comparable to that of control seedlings. In contrast, the activity of NR and NiR was not significantly reduced in 10

day-old MTU (fluoride treated) seedlings, but with time, the effect of fluoride toxicity became pronounced in MTU seedlings that also inhibited the activity of NR and NiR by 1.5 and 1.7 fold, respectively. Analysis of the expression pattern of *NR* and *NiR* in fluoride stressed KH seedlings showed an up regulation in the expression of both the genes after 10 day, whilst after 20 days, the expression of both the genes were comparable to that of control seedlings. Similarly, no significant change was noticeable in the expression of both the genes in 10 day-old MTU seedling (fluoride-treated); however, after 20 day, their expression was significantly reduced in the stressed seedlings (Fig 4.1.3). Thus, based on the above data, it could be concluded that fluoride stress inhibited the activity of both the enzymes in 10 and 20 day-old KH and MTU seedlings, respectively, which in turn lowered the pool of nitrogen-containing metabolites (total and soluble nitrogen, nitrate and nitrite) as previously observed. The expression of *NR* and *NiR* genes were also lowered in 20 day-old stressed MTU seedlings which also coincided with inhibited activity of both the enzymes. Our findings can be justified by the work of Ghosh et al. (2013), where they demonstrated that arsenic treatment inhibited the activity of NR and NiR which in turn lowered the nitrogen content in wheat cultivars. They further reported that in presence of selenate, the activity of NR and NiR was restored in stressed seedlings which eventually led to higher accumulation of nitrogen containing compounds and helped in overcoming the negative effects of arsenic toxicity. Similarly, the activity and expression of NR was reduced in salt-stressed sensitive rice cultivar, i.e., IR-64, whereas the expression and activity of enzyme was enhanced in salt tolerant rice cultivar (CSR-36) that replenished the nitrogen pool, so as to confer sufficient protection to seedlings (Rohilla and Yadav 2019).

Fig: 4.1.3a

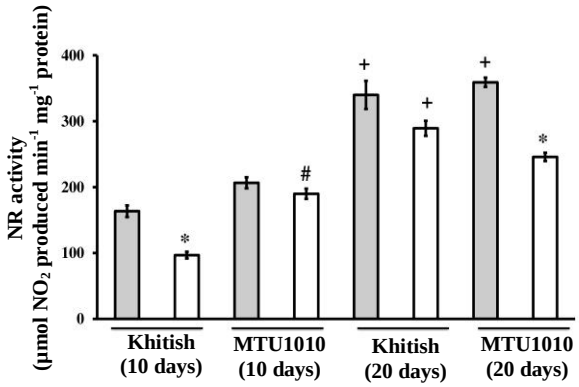


Fig: 4.1.3b

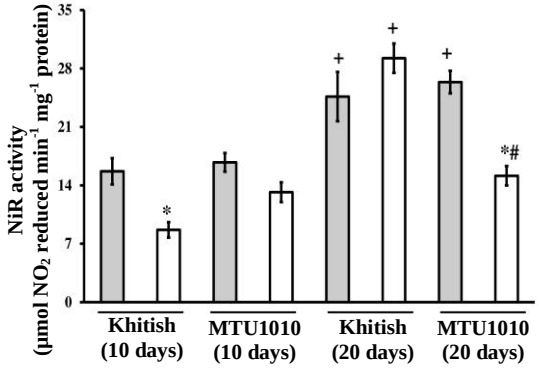


Fig: 4.1.3c

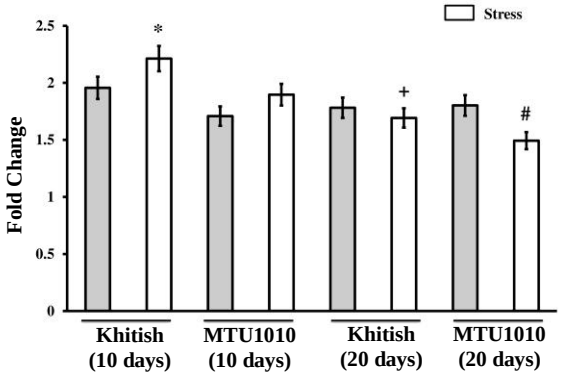
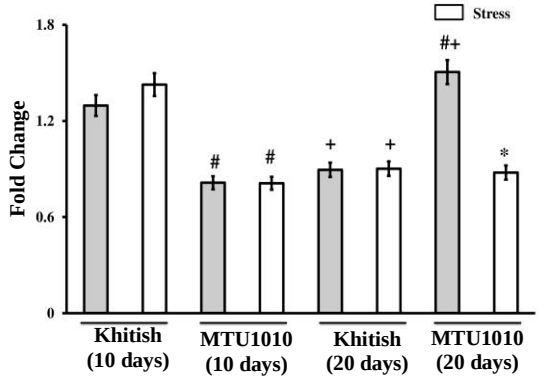


Fig: 4.1.3d



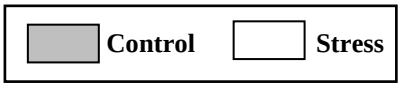


Fig 4.1.3: Activity of NR (**a**) and NiR (**b**) and expression of *NR* (**c**) and *NiR* (**d**) genes in 10 and 20 day-old KH and MTU seedlings, grown either in presence or absence of 25 mg L^{-1} NaF solution. Data represented are the mean value (n = 3) ± SE. '*' represents data with significant differences ($P \leq 0.05$) between control and stressed seedlings, '#' represents data with significant differences ($P \leq 0.05$) between the two cultivars and '+' represents data with significant differences ($P \leq 0.05$) between the seedlings grown for 10 days and 20 days.

4.1.3.6. Level of ammonium ions and activity of GS, GOGAT and GDH

A significant rise was noted in the level of ammonium ions in 10 day-old KH (2.1 fold) and 20 day-old MTU (1.9 fold) stressed seedlings. However, the extent of ammonium ion accumulation in 20 day-old KH and 10 day-old MTU stressed seedlings was comparable to that of water treated seedlings (Table 4.1.3). Additionally, the activity of GS and GOGAT was inhibited by 2.1 and 1.8 times in 10 day-old stressed KH seedlings. In contrast, the activity of both the enzymes was restored in KH seedlings exposed to long term fluoride stress. These results also coincided with the results derived from gene expression analysis, where fluoride stress reduced the expression of both the genes in 10 day-old KH seedlings, whereas the genes were significantly enhanced in seedlings grown for 20 day under NaF solution. In contrast to that of KH, the activity of both the enzymes was inhibited by 1.9 and 1.8 times in fluoride stressed (20 day-old) MTU seedlings, whereas the activity of enzymes was comparable to that of control seedlings in 10 day-old seedlings. Contrasting trend was also noted in the expression patter of *GS* (significantly enhanced) and *GOGAT* (significantly down regulated) in 20 day-old fluoride stressed MTU seedlings, whereas no such difference

was noticeable in 10 day-old stressed seedlings. Another major enzyme involved in nitrogen metabolism is GDH that was enhanced by 1.5 and 2.6 times in 10 and 20 day-old stressed KH seedlings, respectively and by 2.5 and 1.4 times in 10 and 20 day-old stressed MTU seedlings, respectively. Additionally, the expression of *GDH* was significantly induced in all the treatment except in 10 day-old stressed MTU seedlings where no significant rise was noticeable as to that of control seedlings (Fig 4.1.4). Fluoride stress affected the activity of GS and GOGAT in 10 and 20 day-old KH and MTU seedlings, respectively which can be linked with higher accumulation of ammonium ions in the cells. However, higher activity of GS in 20 and 10 day-old KH and MTU seedlings, respectively rapidly convert the toxic ammonium ions into non-toxic glutamine that is further transformed into glutamate by enzymatic action of GOGAT. This rapid incorporation of ammonium ions into glutamate and glutamine was significantly important for the growth of the seedlings, since other amino acids and nitrogenous metabolites are formed from these two amino acids (Kusano et al. 2011). Additionally, Gou et al. (2020) showed that the activity of GS and GOGAT was down regulated in nitrate-stressed cucumber seedlings which in turn elevated the level of ammonium ions. They further reported that in presence of silicon, the activity of both the enzymes was restored which led to higher incorporation of ammonium ions into glutamine and glutamate. Similarly, the activity of both the enzymes was restored in cadmium-stressed rice seedlings in presence of nitrogen supplement that enhanced the tolerance capability of seedlings by reducing the level of ammonium ions in the cells. Furthermore, glutamate and glutamine formed due to higher activity of GS/GOGAT cycle was again utilized by GDH to produce α-ketoglutarate that links the nitrogen metabolism with carbon metabolism and thus providing higher tolerance level to the seedlings. Thus, based on these observations, it can be concluded that induction of GS/GOGAT cycle in 20 and 10 day-old KH and MTU seedlings, respectively rapidly converted toxic ammonium ions into non-toxic amino acids (glutamine and glutamate) which were again utilized by GDH to link nitrogen and carbon metabolism and to produce other amino acids that helped to overcome the detrimental effects of fluoride toxicity.

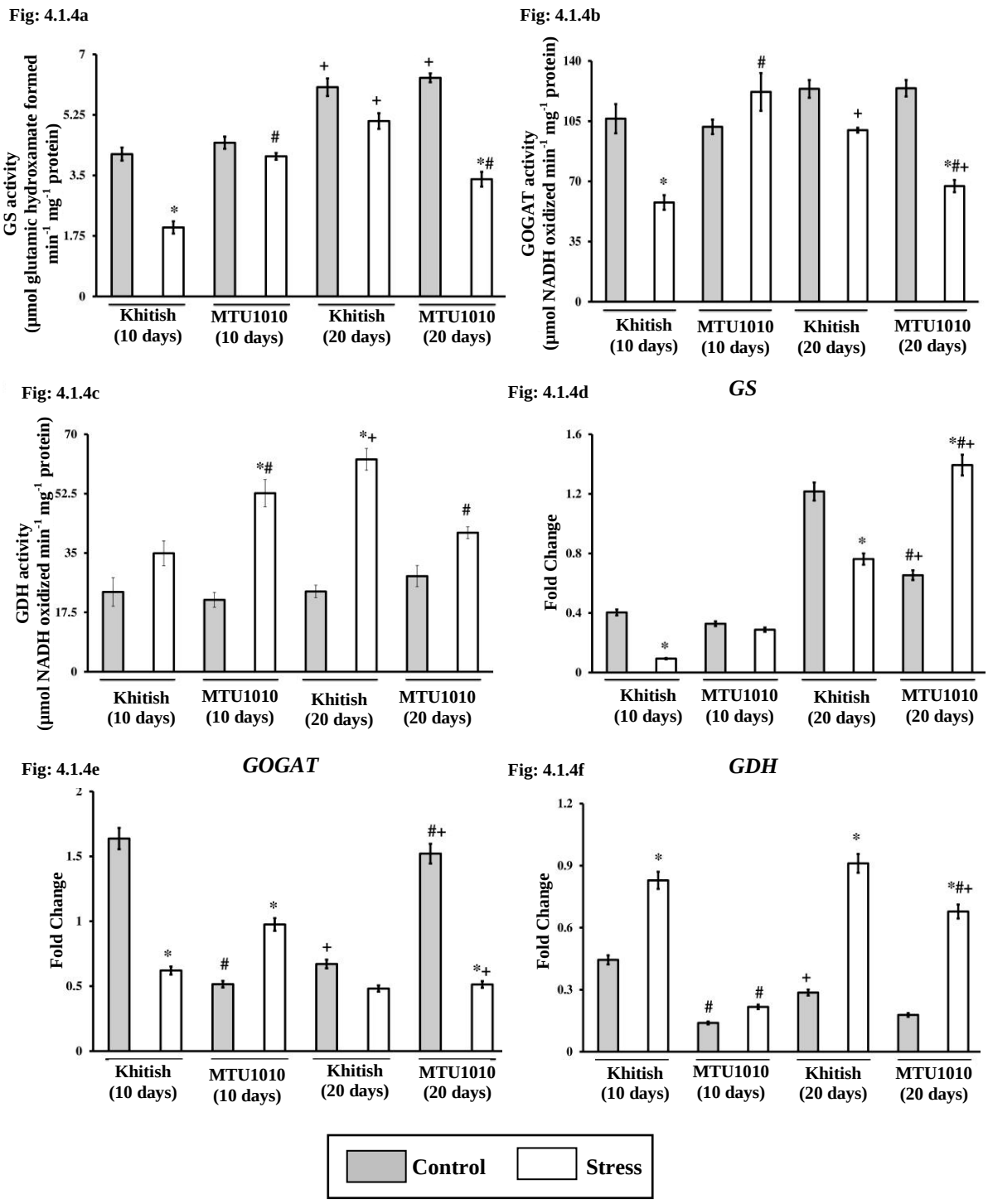


Fig 4.1.4: Activity of GS (**a**), GOGAT (**b**) and GDH (**c**) and expression of genes, *GS* (**d**), *GOGAT* (**e**) and *GDH* (**f**) in 10 and 20 day-old KH and MTU seedlings, grown either in presence or absence of 25 mg L^{-1} NaF solution. Data represented are the mean value (n = 3) ± SE. '*' represents data with significant differences (P ≤ 0.05) between control and stressed seedlings, '#' represents data with significant differences (P ≤ 0.05) between

the two cultivars and '+' represents data with significant differences ($P \leq 0.05$) between the seedlings grown for 10 days and 20 days.

4.1.3.7. Role of sulfur in regulating the tolerance mechanism of seedlings

Upon being challenged with fluoride toxicity, there was lowering in the activity of the major sulfur assimilatory enzymes, viz., ATP-S (1.4 and 1.2 times, respectively) and OASTL (1.6 and 1.3 times, respectively) that led to decrease in the formation of cysteine by 1.6 and 1.3 fold, respectively in 10 day-old and 20 day-old stressed KH and MTU seedlings, thus reducing their tolerance level (Table 4.1.4). Our result can be supported by the previous work of Santiago et al. (2020) where they showed higher ATP-S and OASTL activity in two varieties of arugula, as compared to two cultivars of lettuce that maintained the tolerance level of the seedlings upon being treated with various concentration of selenate (Na_2SeO_4). Contrasting results were noted in case of 20 day-old and 10 day-old KH and MTU seedlings, justifying the efficacy of sulfur as an important protective element against fluoride toxicity. Another important sulfur-containing metabolite that also acts as a major signaling molecule is H_2S. In presence of fluoride stress, the level of H_2S was decreased by 1.5 and 1.3 times in 10 day-old and 20 day-old KH and MTU seedlings, respectively which might be due to reduced formation of cysteine that acts as a precursor of H_2S or it can also be attributed to the reduced activity of DES (the sole enzyme responsible for the generation of H_2S in plant cytosol) by 1.8 and 1.7 fold, respectively in the above mentioned cultivars, as compared to that of water-treated seedlings. On the contrary, higher H_2S level was noted in 20 day-old stressed KH seedlings which might be due to enhanced DES activity, as compared to that of 10 day-old stressed seedlings that eventually contributed towards higher tolerance level in the former. Reduced DES activity and H_2S level in 20 day-old MTU seedlings, as compared to that of 10 day-old seedlings, heightened the fluoride-induced damage in the former (Table 4.1.4). Kushwaha and Singh (2020) also observed that the treatment of tomato, pea and brinjal seedlings with 25 μM potassium dichromate inhibited the formation of cysteine and H_2S in leaf and root. The activity of DES was restored in the stressed seedlings on being treated with potassium sulfate (additional source of sulfur) which resulted in higher formation of H_2S, thereby maintaining the tolerance capability of seedlings.

Table 4.1.4: Level of Cys and H_2S and activity of ATP-S, OAS-TL and DES in KH and MTU seedlings in presence of 25 mg L^{-1} NaF for 10 days and 20 days. Data represented are the mean value (n = 3) ± SE. '*' represents data with significant differences ($P \leq 0.05$) between control and stressed seedlings, '#' represents data with significant differences ($P \leq 0.05$) between the two cultivars and '+' represents data with significant differences ($P \leq 0.05$) between the seedlings grown for 10 days and 20 days.

Parameters	10 days				20 days			
	KH Control	KH Stress	MTU Control	MTU Stress	KH Control	KH Stress	MTU Control	MTU Stress
Cys (µg g^{-1} FW)	1.54 ± 0.12	0.98 ± 0.09*	1.67 ± 0.28	1.78 ± 0.12$^{\#}$	2.78± 0.09	4.77 ± 0.15^{*+}	2.56 ± 0.20	2.01 ± 0.21$^{\#}$
H_2S (µM g^{-1} FW)	8.97 ± 0.54	5.78 ± 0.65*	7.98 ± 0.76	10.98 ± 1.12$^{\#}$	11.94 ± 1.29	17.89 ± 0.98^{*+}	12.76 ± 0.67^{+}	9.67 ± 0.32$^{\#}$
ATP-S activity (µM PPi min^{-1} mg^{-1} leaf protein)	6.78 ± 0.38	4.76 ± 0.87*	7.98 ± 0.37	10.67 ± 0.76$^{\#}$	7.98 ± 1.10	12.87 ± 0.98^{*+}	9.67 ± 0.99	8.14 ± 1.09
OAS-TL activity (µM Cys min^{-1} mg^{-1} leaf protein)	3.12 ± 0.76	1.98 ± 0.16*	4.67 ± 0.18	5.45 ± 0.38$^{\#}$	3.98 ± 0.18	5.76 ± 0.67^{*+}	5.12± 0.17	3.98 ± 0.87^{*+}
DES activity (µM H_2S min^{-1} mg^{-1} leaf protein)	0.98 ± 0.01	0.54± 0.07	1.12 ± 0.12	1.08 ± 0.06$^{*\#}$	1.54 ± 0.09	1.98 ± 0.06^{*+}	1.43 ± 0.03	0.86 ± 0.01$^{*\#}$

4.1.3.8. Enzymatic antioxidants regulating the level of ROS in seedlings

Enzymatic antioxidants play a pivotal role in regulating the stress tolerance capability of plants by scavenging the free radicals formed, thus maintaining cellular homeostasis and redox status. In fluoride-stressed KH and MTU seedlings, the activity of SOD was enhanced by 1.8 and 2.4 fold, respectively in 10 day-old seedlings and by 2.2 and 1.4 times, respectively in 20 day-old seedlings that enabled detoxification of superoxide anions; however, the tolerance in 20 day-old and 10 day-old KH and MTU seedlings was governed by the rise in the activity of the enzyme (Table 4.1.5). Similar trend was noted with regard to

isozyme profiling, where eight different isoforms of SOD were observed. Of all the isoforms, SOD4 and SOD5 were prominently enhanced in 20 day-old KH and 10 day-old MTU seedlings, respectively (Fig. 4.1.5a). SOD converts superoxide anions into H_2O_2 which is again detoxified by CAT, APX, GPoX and GPX into non toxic metabolites. Almost 1.3, 1.4 and 1.5 times higher activity of APX, GPoX and GPX, respectively was noted in 10 day-old KH seedlings, whereas the activity of the above mentioned enzymes was enhanced by 1.4, 1.6 and 1.6 times in 20 day-old KH seedlings, as compared to that of control seedlings which suggested that the enhanced activity of enzymatic antioxidants helped in adaptation of seedlings to fluoride stress. Similarly, in 10 day-old stressed MTU seedlings, the activity of the above protective enzymes was escalated by 1.7, 2.2 and 1.4 fold, respectively, whereas in case of 20 day-old stressed seedlings, the respective rise in the activity of the above enzymes was only 1.1, 1.6, 1.3 times, as compared to that of water-treated seedlings. In case of MTU, the activity of enzymatic antioxidants did not rise significantly, leading to higher accumulation of ROS and increasing the susceptibility of the seedlings (Table 4.1.5). The enhanced activity of the enzymatic antioxidants can be supported by the previous work of Banerjee and Roychoudhury (2019a) where they showed that fluoride stress significantly enhanced the activity of the above mentioned antioxidative enzymes in the tolerant cultivar, viz., Gobindobhog, whereas in case of susceptible variety (IR-64), such induction in the activity of enzymatic antioxidants was insignificant, leading to higher damage in the seedlings. Contrasting results were noted with regard to CAT activity where fluoride stress inhibited its activity by 1.5 and 1.3 fold in 10 day-old KH and 20 day-old MTU seedlings, respectively, whereas no such inhibition was noted in 20 day-old KH and 10 day-old MTU seedlings, respectively (Table 4.1.5). Reduced activity of CAT can be supported by the earlier observation of Kumar et al. (2009) where they showed that fluoride ions replace the hydroxyl moiety present in the ferric group residing at the active site, responsible for maintaining the active conformation of this enzyme. Isozyme analysis of APX revealed four isoforms of APX in seedlings, where APX3 isoform was only detected in 20 day-old seedlings of both the cultivars. In case of GPoX, five isoforms were noted. GPoX3 was observed in only 20 day-old KH seedlings along with higher induction of GPoX5 which was absent in 10 day-old KH seedlings. This suggested that GPoX3 and GPoX5 might contribute toward tolerance level of the seedlings. In case of GPX, eight isoforms were observed and activity of GPX7 was reduced in 10 day-old KH and 20 day-old MTU seedlings, respectively, whereas contrasting effect was noted in case of 20 day-old KH and 10 day-old MTU

seedlings, which suggested the involvement of GPX7 isoform in fluoride tolerance. Upon analysis of CAT isozyme, seven different isoforms of CAT were observed, of which CAT4 was reduced in 10 day-old KH seedlings. Two new isoforms, viz., CAT6 and CAT7 were observed in 20 day-old KH seedlings which suggested the importance of these two isoforms in ameliorating the adverse effects of fluoride stress (Fig. 4.1.5).

Table 4.1.5: Activity of SOD, CAT, APX, GPoX and GPX in KH and MTU seedlings in presence of 25 mg L^{-1} NaF for 10 days and 20 days. Data represented are the mean value (n = 3) ± SE. '*' represents data with significant differences ($P \leq 0.05$) between control and stressed seedlings, '#' represents data with significant differences ($P \leq 0.05$) between the two cultivars and '+' represents data with significant differences ($P \leq 0.05$) between the seedlings grown for 10 days and 20 days

Parameters	10 Days				20 Days			
	KH Control	KH Stress	MTU Control	MTU Stress	KH Control	KH Stress	MTU Control	MTU Stress
SOD activity (unit mg^{-1} protein)	2.12 ± 0.12	3.78 ± 0.34	1.98 ± 0.14	4.67 ± 0.19*	2.67 ± 0.78	5.98 ± 0.98^{*+}	2.76 ± 0.37	3.97 ± 0.67$^{\#}$
CAT activity (µM H_2O_2 min^{-1} mg^{-1} protein)	20.89 ± 1.98	14.09 ± 0.87*	22.98 ± 1.92	30.17 ± 2.87$^{*\#}$	28.17 ± 2.87	35.38 ± 1.54^{*+}	27.89 ± 0.46	21.08 ± 1.74^{*+}
APX activity (µM ascorbate min^{-1} mg^{-1} protein)	168.98 ± 10.27	220.72 ± 12.82	146.83 ± 11.87	250.98 ± 9.76*	219.67 ± 8.8	298.67 ± 12.29^{*+}	200.98 ± 9.46	230.56 ± 12.78$^{\#}$
GPoX activity (µM tetraguaiacol min^{-1} mg^{-1} protein)	15.87 ± 1.53	22.63 ± 1.89	12.37 ± 0.78	26.82 ± 1.01*	19.65 ± 0.67	31.67 ± 1.17^{*+}	13.87 ± 1.98	22.29 ± 0.83$^{*\#}$
GPX activity (µM NADPH min^{-1} mg^{-1} protein)	45.98 ± 2.76	69.18 ± 3.82*	51.72 ± 3.16	72.82 ± 3.91*	61.98 ± 1.87	98.37 ± 2.82^{*+}	57.19 ± 3.28	75.38 ± 2.72$^{*\#}$

Fig. 4.1.5a

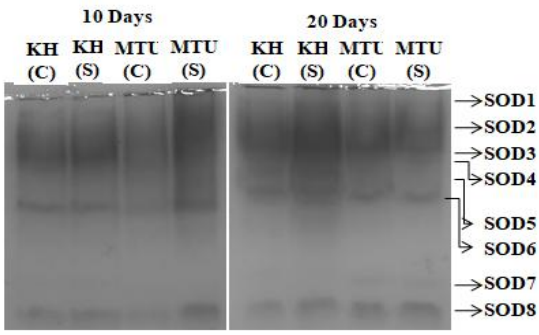


Fig. 4.1.5b

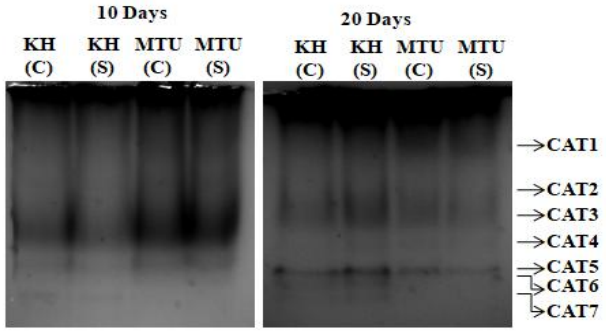


Fig. 4.1.5c

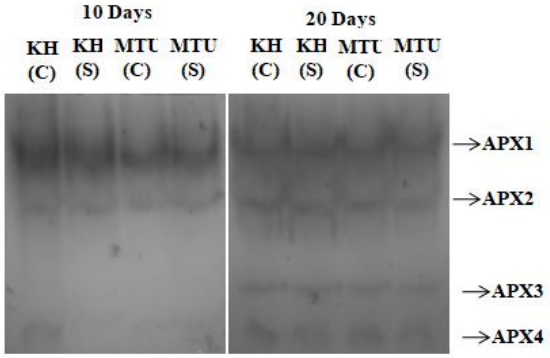


Fig. 4.1.5d

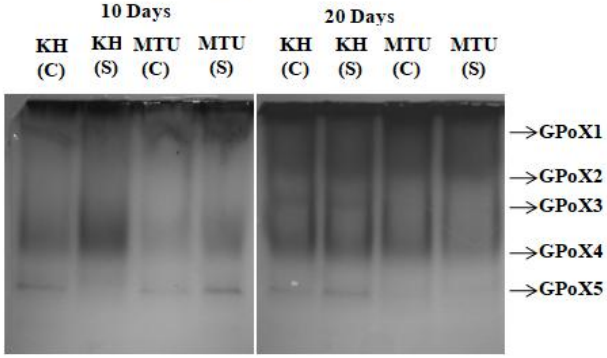


Fig. 4.1.5e

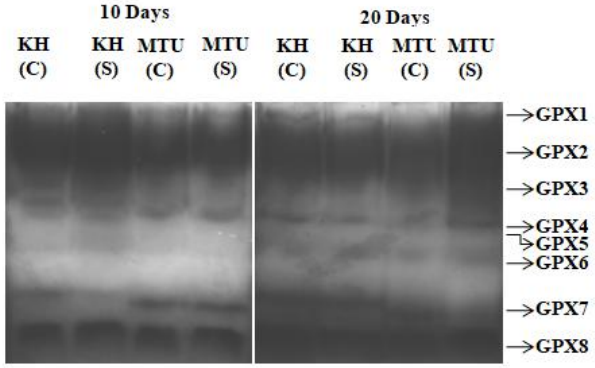


Fig. 4.1.5: In-gel isozyme profile of SOD (**a**), CAT (**b**), APX (**c**), GPoX (**d**) and GPX (**e**) of KH and MTU seedlings in presence of 25 mg L^{-1} NaF for 10 days and 20 days.

4.1.3.9. Fluoride stress regulates the level of non-enzymatic antioxidants in seedlings

Along with enzymatic antioxidants, plants also harbor several endogenous protective metabolites that help in reducing the damaging effects of stress. One such group of protective

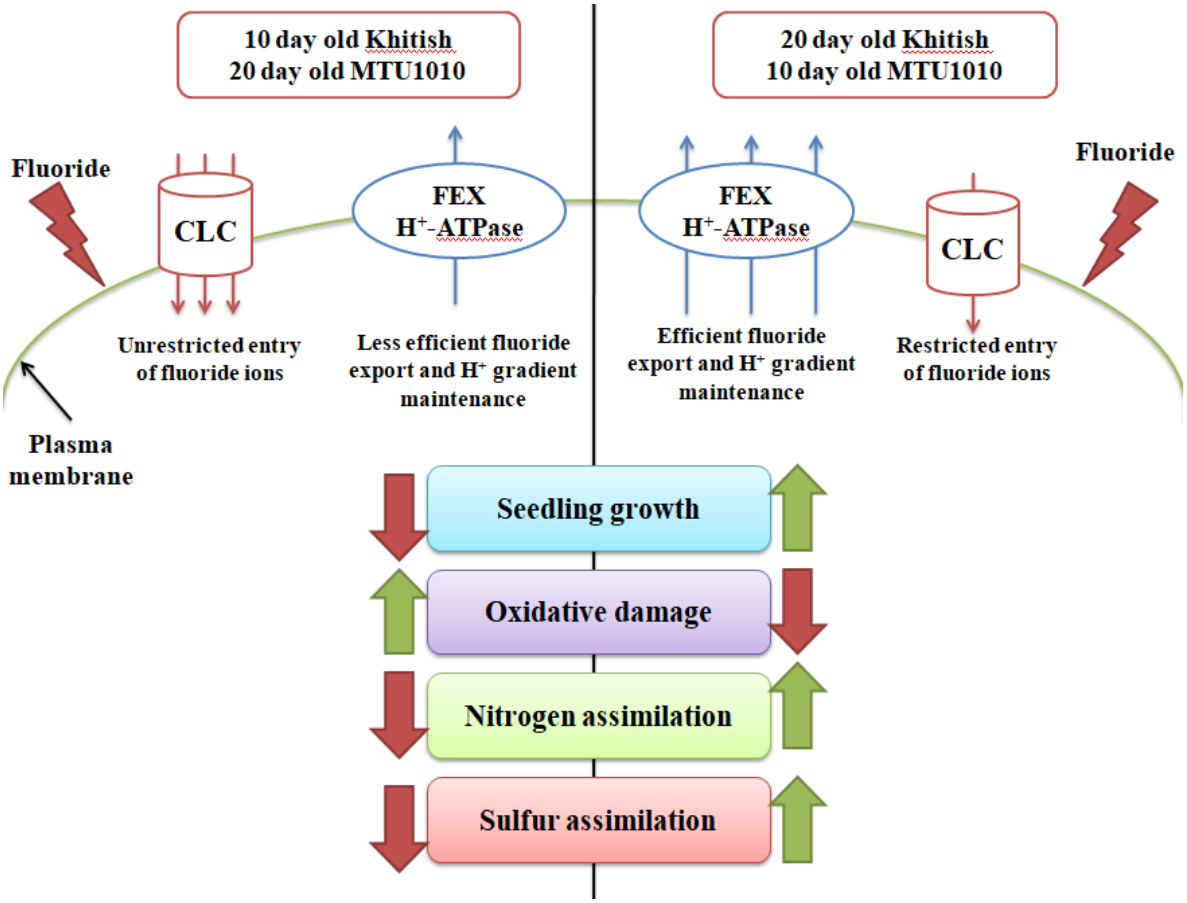
metabolite constitutes the non-enzymatic antioxidants that regulate the expression of genes associated with stress tolerance in plants (Waskiewicz et al. 2014). Almost 1.3 and 1.2 times enhanced level of anthocyanins and flavonoids, respectively was noted in 10 day-old stressed KH seedlings, as compared to that of water-treated seedlings. Exposure of seedlings to fluoride for 20 days in KH seedlings further enhanced the level of the above mentioned metabolites by 1.7 and 1.4 folds, respectively which again suggested that the fluoride induced damages were mitigated by magnifying the amount of such protective metabolites (Table 4.1.6). In case of MTU seedlings, the level of anthocyanins and flavonoids was enhanced by 1.5 and 1.5 folds, respectively in 10 day-old stressed seedling, whereas much lesser increment (1.4 and 1.2 fold) was observed in 20 day-old seedlings, which suggested that with progression in duration of stress, there was reduced accumulation of metabolites, as compared to early response (10 days) that ultimately compromised the tolerance level of this cultivar (Table 4.1.6). The enhancements in the level of these antioxidants also led to increment in ABTS and FRAP in 10 day-old KH seedlings by 1.1 and 1.3 fold, respectively and by 1.7 and 1.3 fold, respectively in 20 day-old KH seedlings which clearly indicated higher antioxidative potential as adaptive strategy against fluoride stress. The reverse trend was noted in case of MTU seedlings where the ABTS and FRAP were induced by 1.7 and 1.3 fold, respectively in 10 day-old stressed seedlings and 1.5 and 1.4 fold, respectively in 20 day-old stressed seedlings. This pointed to the fact that antioxidative potential was severely compromised during long term exposure of MTU seedlings to fluoride stress, with pronounced susceptibility (Table 4.1.6). Similar trend was also earlier reported by Banerjee and Roychoudhury (2019a) where they demonstrated that the radical scavenging potential was reduced in the susceptible variety as compared to that of tolerant cultivar where significant enhancement in antioxidative potential was noted that in turn enhanced the tolerance capability.

Table 4.1.6: Level of anthocyanins, flavonoids, ABTS scavenging activity and FRAP in KH and MTU seedlings in presence of 25 mg L^{-1} NaF for 10 days and 20 days. Data represented are the mean value (n = 3) ± SE. '*' represents data with significant differences ($P \leq 0.05$) between control and stressed seedlings, '#' represents data with significant differences ($P \leq 0.05$) between the two cultivars and '+' represents data with significant differences ($P \leq 0.05$) between the seedlings grown for 10 days and 20 days.

Parameters	10 days				20 days			
	KH Control	KH Stress	MTU Control	MTU Stress	KH Control	KH Stress	MTU Control	MTU Stress
Anthocyanins (µM g^{-1} FW)	20.92 ± 1.28	27.19 ± 2.28	19.65 ± 1.63	30.38 ± 1.83$^{*\#}$	25.28 ± 2.29	42.29 ± 2.98$^{*\#+}$	27.29 ± 1.19	38.29 ± 1.80
Flavonoids (µg g^{-1} FW)	182.28 ± 7.38	220.28 ± 9.82*	167.38 ± 8.34	250.38 ± 9.27*	200.28 ± 10.28	289.29 ± 12.29^{*+}	187.29 ± 12.10	229.29 ± 10.18$^{\#}$
ABTS (mg ascorbic acid equivalent 100 g^{-1} FW)	6.98 ± 0.91	7.78 ± 1.01	5.87 ± 0.87	10.27 ± 1.12$^{*\#}$	7.98 ± 1.01	13.58 ± 0.98^{*+}	8.89 ± 0.67^{+}	11.38 ± 0.76
FRAP (mg ferrous sulfate equivalents 100 g^{-1} FW)	10.23 ± 0.78	12.98 ± 0.67	9.38 ± 0.78	14.29 ± 1.00*	11.92 ± 0.67	15.38 ± 1.21^{*+}	11.27 ± 0.87	15.98 ± 0.71*

4.1.4. Conclusion of the chapter

Overall this chapter was dedicated to reveal the responses occurring in two different rice cultivars, viz., KH and MTU at genetic and metabolic level upon being exposed to fluoride toxicity for two different time periods, viz., 10 days and 20 days. Based on the above data, we can clearly infer that KH exhibited long term fluoride tolerance, as compared to that of MTU which appeared as a susceptible variety. Initially, KH suffered higher oxidative damages due to unregulated uptake of fluoride ions; however, with prolonged stress exposure after 20 days, KH was able to adjust and maintain the cellular homeostasis as well as showing tight regulation of fluoride uptake due to higher expression of H^+-ATPase and FEX, allowing it to behave as the tolerant cultivar. On the contrary, the trend in MTU was totally reverse, since short term stress exposure incited lesser oxidative damages in this cultivar, concomitant with lesser fluoride accumulation, allowing it to maintain growth. However, such regulated uptake of fluoride ions was lost during long-term stress exposure after 20 days that significantly enhanced the accumulation of toxic metabolites, accompanied with higher oxidative damages and reduced growth of the seedlings, making it a highly susceptible variety. The activity of the enzymatic antioxidants was significantly enhanced in the stressed seedlings of KH, as compared to that of MTU exposed to long term fluoride stress, allowing the former to maintain the normal physiology of the seedlings. Similar trend was also noted in case of non-enzymatic antioxidants. Additionally, the nitrogen- (total and soluble nitrogen, nitrate, nitrite and amino acids) and sulfur- containing metabolites (cysteine and H_2S) played a pivotal role for the survival of the seedlings. In 20 day-old KH seedlings, the level of nitrogen- and sulfur- containing metabolites was enhanced, attributed to the higher activity of the enzymes catalyzing nitrogen and sulfur uptake and assimilation in tissues that in turn enabled the seedlings to overcome the detrimental effects of fluoride stress. Overall, a clear-cut varietal difference between KH and MTU was noteworthy from the present investigation. Differential reprogramming of the metabolites and enzymes of nitrogen and sulfur assimilation pathways, along with antioxidative machineries, were synergistically responsible for the differential pattern of adaptive strategies against fluoride stress in the two examined varieties, viz., KH and MTU, the former exhibiting resilience against long-term stress, whilst the latter showing high susceptibility (Fig. 4.1.6)."



"**Fig. 4.1.6:** Model depicting the differential regulation of metabolites and transporters that regulate the tolerance capability of two rice cultivars at different time period."

Chapter 4.2

Calcium modulates the fluoride tolerance level of susceptible rice cultivar

Data Published in this chapter has already been published in:
Protoplasma (2020) 257:767–782,
doi:10.1007/s00709-019-01460-5;
Journal of Plant Growth Regulation (2021) 40:1579–1593,
doi:10.1007/s00344-020-10202-4

“4.2.1. Overview of the chapter

From the experimental results in the previous chapter (Chapter 4.1), it was clearly established that fluoride stress significantly affected the tolerance capability of Khitish seedlings at the initial stages of growth. Thus, the next aim of work was to restore the tolerance level of the seedlings at the early growth stage by using ameliorating agent (calcium) so that the plants become more robust to fluoride stress in later phases of their life. The present chapter demonstrated the promising role of calcium compounds in abrogating the negative effects of fluoride stress and regulating the calcium-mediated signaling in the seedlings. Inhibited uptake of fluoride ions upon priming of seeds with calcium salts effectively lowered the damaging effects of oxidative stress in seedlings and restoring their growth. Calcium-regulated amelioration of damage was mediated by higher proline (due to up regulated *P5CS* and down regulated *PDH* expression) and glycine betaine synthesis (due to higher *BADH1* expression). Additionally, the level of carotenoids and phenolics was enhanced due to calcium priming, whilst stress-enhanced level of flavonoids and ascorbic acid was restored to normal content along with release in inhibition of the activity of AsAO. Calcium priming also regulated the activity of the major enzymatic antioxidants, viz., CAT, SOD and GPoX along with the expression level of *CAT*. Enhanced expression of genes (particularly *CBL10*) related to calcium signaling pathways contributed to the higher tolerance level of seedlings upon calcium priming. In addition, higher endogenous calcium level (due to priming) also enhanced the energy production in seedlings by relieving the fluoride-mediated inhibition of TCA cycle and restored the level of sucrose (by up regulating the activity and expression of SPS and SS) and starch (due to its lower catabolism) which could also be linked with higher survival rate of fluoride-stressed seedlings.

4.2.2. Introduction of the chapter

Higher accumulation of fluoride ions (due to up regulated expression of chloride channels) affects the overall physiology of the seedlings, thereby reducing their growth and development of symptoms like chlorophyll loss and electrolyte leakage. In addition, fluoride toxicity also perturbs the biochemical processes like pigment synthesis, enzyme activity and protein structure. Impaired biochemical processes lead to the overproduction of reactive oxygen species (ROS) that results in oxidative stress, ultimately leading to membrane disruption and overproduction of cytotoxic metabolites like malondialdehyde (MDA) (Kumar and Singh 2015; Yadu et al. 2016).

In order to escape the detrimental effects of fluoride toxicity, rice seedlings have their internal protective machineries that comprise of osmolytes like proline, glycine betaine and amino acids, as well as enzymatic and non-enzymatic antioxidants. Upon exposure to fluoride ions above threshold level, the level of the protective metabolites along with the activity of enzymatic antioxidants are increased that detoxifies ROS and maintain the osmotic balance of the cells (Ahmad et al. 2010).

Along with these protective metabolites and enzymes, tricarboxylic acid (TCA) cycle and sucrose and starch metabolism also help in overcoming the stressed conditions. Organic acids such as pyruvate, malate and citrate collectively help to mitigate stress by participating in energy production, acting as precursors of amino acids and driving other biochemical pathways. Moreover, they also maintain osmotic balance and quench excess ROS (Lopez-Bucio et al. 2000). TCA cycle acts as a major pathway for carbon metabolism and is considered as the central point of plant metabolism. TCA cycle also connects carbon and nitrogen metabolism as shown in the earlier chapter. According to Sanchez et al. (2008) and Usadel et al. (2008), the metabolites of the TCA cycle and organic acids provide protection to the glycophytes against salt, drought and temperature stress. Moreover, sucrose and starch are also two major sources of carbon molecule. According to Zhou et al. (2002), starch is the stored form of carbon which accumulates in the fruits and grains, whereas sucrose takes part in translocation of carbon molecules. Under stressed condition, α-amylase and starch phosphorylase (PHO) degrades starch molecules followed by the release of high energy molecules which is then utilized by the plants to overcome the negative effects of environmental stress. Additionally, sucrose molecules released due to starch remobilization

acts as an osmoregulatory molecule that supports the growth of the plants (Krasensky and Jonak 2012). Plants can also produce sucrose molecules by the enzymatic action of sucrose phosphate synthase (SPS) on uridine diphosphate glucose and d-fructose 6-phosphate. To maintain the optimum level of sucrose in the cells, sucrose synthase (SS) and acid invertase (INV) catalyzes the degradation of sucrose molecules.

Calcium is an important element that plays a pivotal role in maintaining normal physiological functions of plant cells. Calcium functions as a secondary messenger and is involved in plant response to various abiotic stresses (Li et al. 2022). The endogenous calcium level in cells is triggered on being exposed to harsh environment that lead to the activation of calcium sensors such as calmodulins (CaMs), calcineurin B-like proteins (CBLs) and salt overly sensitive (SOSs) which play a pivotal role in regulating the transcriptional expression of multiple anti-stress genes to enhance the adaptability of plants, exposed to extreme environment (Zeng et al. 2020).

Previously, Sulzbach and Pack (1972) showed that the presence of divalent metals like calcium in soil significantly chelates fluoride ions by forming complex like calcium fluoride (CaF_2) which significantly reduces their uptake by roots. Moreover, higher level of calcium also regulates the level of internal protective machineries of plants that in turn lowers the effects of oxidative damages. Earlier, Ruan et al. (2004) showed that the application of calcium nitrate ($Ca(NO_3)_2$) significantly reduced the uptake and assimilation of fluoride ions in tea plants. Similarly, Dey et al. (2012a) showed that the presence of calcium chloride ($CaCl_2$) in the medium chelates fluoride ions that reduce its uptake by the roots of Bengal gram seedlings, helping in combating the symptoms of fluoride toxicity.

In the previous chapter, the detrimental effects of fluoride toxicity have been demonstrated in two different rice cultivars, i.e., Khitish (KH) and MTU1010 (MTU) during short term and prolonged stress. The present chapter validated the promising role of calcium salts (as priming agents) in abating negative effects in 10 day-old KH cultivars by reducing the uptake of fluoride ions from the media and regulating the production of protective metabolites and enzymatic antioxidants. Moreover, the positive role of calcium priming in maintaining the carbon metabolism in seedlings was also demonstrated since carbon metabolism is the major source of energy production in cells.

4.2.3. Results and discussion

4.2.3.1. Calcium mediated regulation of seedling growth

1.8 fold lower germination was noted in the stressed seedlings, whereas priming of seeds with both the concentration, i.e., 0.3 mM and 0.5 mM each of calcium hydroxide ($Ca(OH)_2$), $Ca(NO_3)_2$ and $CaCl_2$ rescued their germination rate appreciably (Table 4.2.1). Similarly, priming of stressed seeds with the above mentioned calcium compounds also markedly restored their root and shoot length and tissue biomass, as compared to that of water primed stressed seedlings (Table 4.2.1). $Ca(OH)_2$ and $Ca(NO_3)_2$ seemed to work better at lower concentration (0.3 mM), whilst $CaCl_2$ seemed to be more potent at higher concentration (0.5 mM) (Fig. 4.2.1). Reduced germination of water primed stressed seedlings could be due to lower water uptake, impairment of metabolic processes or inhibited cell division as reported by Yadu et al. (2016) during fluoride stress in plants. Similarly, Gadi et al. (2012) also reported inhibited growth of *Vigna radiata* on being exposed to fluoride stress. Priming of seeds with calcium rescued the effects of fluoride-induced damages in seedlings, as justified by the previous work of Dey et al. (2012a) where they showed that exogenous application of any calcium source such as $CaCl_2$ was highly effective in reducing the signs of fluoride toxicity.

Table 4.2.1: Percentage of seed germination, seedling biomass, RL and SL of rice seedlings grown hydroponically in presence of NaF (25 mg L^{-1}) for 10 days, either in the absence or presence of seed priming with 0.3 mM and 0.5 mM, each of $Ca(OH)_2$, $Ca(NO_3)_2$, and $CaCl_2$ for 24 h. Data are the mean value (n = 3) ± SE. Data with significant differences obtained against control are labeled with '*' at $P \leq 0.05$.

Sample		Percentage germination (%)	Total seedling biomass (g)	Shoot length (cm)	Root length (cm)
Control		94.48 ± 0.25	140.54 ± 0.25	10.24 ± 0.49	5.74 ± 0.26
NaF		54.47 ± 0.36*	100.25 ± 0.28*	6.44 ± 0.75*	2.45 ± 0.41*
$Ca(OH)_2$	**0.3 mM**	96.25 ± 0.35*	152.01 ± 0.88*	11.28 ± 0.44*	5.54 ± 0.48*
	0.5 mM	97.85 ± 0.48	150.24 ± 0.59	11.55 ± 0.48	5.79 ± 0.41
	0.3 mM +	90.02 ± 0.58	132.01 ± 0.25*	9.41 ± 0.15	4.57 ± 0.38

	NaF				
	0.5 mM + NaF	91.25 ± 0.79	135.04 ± 0.48	9.13 ± 045	4.79 ± 0.60
$Ca(NO_3)_2$	**0.3 mM**	96.45 ± 0.44	148.25 ± 0.55*	10.84 ± 0.33	5.21 ± 0.48
	0.5 mM	95.44 ± 0.46	144.00 ± 0.55	11.04 ± 0.50	6.01 ± 0.49
	0.3 mM + NaF	80.54 ± 0.75	134.05 ± 0.45	9.27 ± 0.50	4.12 ± 0.49*
	0.5 mM + NaF	78.58 ± 0.94	124.02 ± 0.25	8.94 ± 0.32	4.87 ± 0.59
$CaCl_2$	**0.3 mM**	92.41 ± 0.46	139.04 ± 0.54	10.64 ± 0.60	5.94 ± 0.94
	0.5 mM	93.45 ± 0.44	141.47 ± 0.25	11.19 ± 0.60	6.12 ± 0.50
	0.3 mM + NaF	71.05 ± 0.25*	115.05 ± 0.79*	8.15 ± 0.45	3.97 ± 0.88*
	0.5 mM + NaF	82.41 ± 0.49	138.04 ± 0.50*	9.95 ± 0.46	4.21 ± 0.68

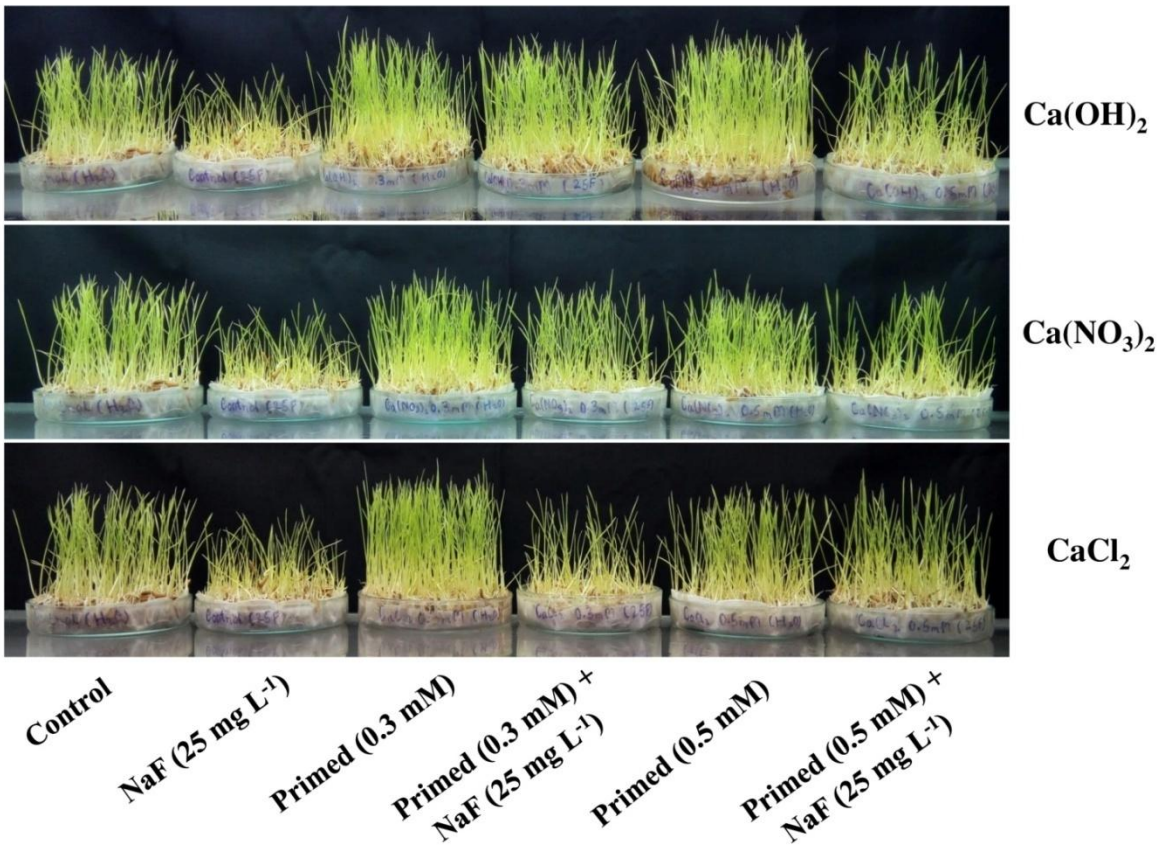


Fig. 4.2.1: Effect of seed priming with calcium compounds, viz., $Ca(OH)_2$, $Ca(NO_3)_2$ and $CaCl_2$ (0.3 mM and 0.5 mM), for 24 h on seedling growth under stressed condition (25 mg L^{-1} NaF) for 10 days; the untreated seedlings (with or without calcium pre-treatment of seeds) served as experimental control.

4.2.3.2. Calcium priming interferes with fluoride uptake

The level of fluoride ions in water primed seedlings was as high as 28 times compared to that of water primed non-stressed seedlings. On the contrary, priming of seeds with 0.3 mM $Ca(OH)_2$, $Ca(NO_3)_2$ and $CaCl_2$ reduced the extent of fluoride accumulation in seedlings by 2.1, 2.6 and 1.4 times, respectively, while in case of seedlings primed with 0.5 mM calcium compounds, 1.8, 1.9 and 2.5 fold, respectively lower fluoride ion accumulation was noted (Table 4.2.2). The endogenous content of calcium in water primed fluoride-stressed seedlings was reduced significantly by 2.6 times, whereas priming of seedling with 0.3 mM calcium compounds restored their endogenous calcium pool by 1.6, 1.5 and 1.2 fold, respectively. Similarly, priming of seeds with 0.5 mM calcium compounds enhanced the endogenous calcium level by 1.5, 1.2 and 1.4 times, respectively (Table 4.2.2). Based on these observations, it was clear that in water primed stressed seedlings, higher accumulation of fluoride ions chelated the endogenous calcium that lowered the calcium content of the seedlings, leading to higher deterioration in the growth parameters of fluoride stressed seedlings. Calcium priming inhibited the deposition of fluoride ions in the metabolically active tissues by forming complex with fluoride ions. These calcium-fluoride complexes could be deposited in inactive tissues of the seedlings as earlier suggested by Afzal et al. (2012) during salt stress in the aromatic rice cultivars. Furthermore, calcium priming restored the endogenous calcium pool which in turn might up regulate calcium signaling pathway of seedlings, leading to better retaliation of fluoride induced damage.

Table 4.2.2: Calcium content and fluoride accumulation of rice seedlings grown hydroponically in presence of NaF (25 mg L^{-1}) for 10 days, either in the absence or presence of seed priming with 0.3 mM and 0.5 mM, each of $Ca(OH)_2$,$Ca(NO_3)_2$, and $CaCl_2$ for 24 h. Data are the mean value (n = 3) ± SE. Data with significant differences obtained against control are labeled with '*'at $P \leq 0.05$.

Sample		Calcium content (mg kg^{-1} FW)	Fluoride accumulation (mg kg^{-1} FW)
Control		618.00 ± 23.69	7.00 ± 2.26
NaF		230.33 ± 14.22*	199.00 ± 1.41*
$Ca(OH)_2$	**0.3 mM**	540.33 ± 14.05*	7.50 ± 0.98
	0.5 mM	638.00 ± 15.87	7.70 ± 1.27
	0.3 mM + NaF	388.67 ± 16.56*	94.00 ± 5.65*
	0.5 mM + NaF	346.67 ± 30.75	113.50 ± 0.70*

$Ca(NO_3)_2$	**0.3 mM**	560.67 ± 12.26*	7.30 ± 0.98
	0.5 mM	527.00 ± 26.29	7.90 ± 2.68
	0.3 mM + NaF	348.33 ± 20.26*	75.30 ± 1.55*
	0.5 mM + NaF	291.67 ± 27.54	104.30 ± 2.12*
$CaCl_2$	**0.3 mM**	521.00 ± 28.93*	7.30 ± 1.27
	0.5 mM	506.67 ± 27.75	6.60 ± 1.69
	0.3 mM + NaF	283.00 ± 31.19*	142.80 ± 1.97*
	0.5 mM + NaF	322.67 ± 23.69	79.10 ± 1.83*

4.2.3.3. Calcium mediated lowering of oxidative damage

The extent of oxidative damage in water primed stressed seedlings was highly enhanced as compared to that of non-stressed seedlings. Fluoride stress triggered the formation of hydrogen peroxide (H_2O_2) by 2.2 fold that caused higher formation (1.8 fold) of MDA. Moreover, higher content of fluoride ions also led to higher degradation of chlorophyll molecules (1.9 times), electrolyte leakage (2.7 fold) and inhibits the activity of ascorbic acid oxidase (3.2 fold) (Table 4.2.3). Priming of seeds with 0.3 and 0.5 mM $Ca(OH)_2$, $Ca(NO_3)_2$ and $CaCl_2$ reduced the extent of fluoride accumulation in the metabolically active tissues that relieved the symptoms of fluoride toxicity. Cell membranes are the prime targets for damage due to the formation of ROS like H_2O_2 during abiotic stresses. In the present study, it was found that fluoride ions significantly induced the generation of H_2O_2 following Mehler pathway that led to peroxidation of lipid membrane, thus causing higher leakiness of cell as shown by Ghassemi-Golezani and Farhangi-Abriz (2019) in safflower during fluoride stress. Additionally, fluoride ions also triggered the degradation of chlorophyll molecules or reduced their synthesis (Banerjee and Roychoudhury 2019a). Priming of seeds with calcium compounds lowered the magnitude of oxidative damage by reducing the availability of fluoride ions as discussed above and also enhanced the calcium mediated detoxification of ROS. Fluoride mediated inhibition of AsAO activity was relieved due to calcium priming that also highlighted the efficacy of calcium priming of seeds during fluoride contamination.

Based on all the above analyses, it was evident that $Ca(OH)_2$ and $Ca(NO_3)_2$, each at 0.3 mM concentration, while $CaCl_2$ at 0.5 mM concentration were more effective in increasing the endogenous calcium content, which in turn, participated in ameliorating the toxic effects of fluoride stress in rice seedlings. Therefore, further analyses on defence mechanism in terms

of osmolyte and antioxidant activation were undergone only at the above concentrations of the respective calcium compounds.

Table 4.2.3: Chlorophyll content, electrolyte leakage, MDA and H_2O_2 content, and AsAO activity in rice seedlings grown hydroponically in presence of NaF (25 mg L^{-1}) for 10 d, either in absence or presence of seed priming with 0.3 mM and 0.5 mM, each of $Ca(OH)_2$, $Ca(NO_3)_2$ and $CaCl_2$ for 24 h. Data are the mean value (n = 3) ± SE. Data with significant differences obtained against control are labelled with star '*' at P ≤ 0.05.

Sample		Chlorophyll (µg g^{-1} FW)	Electrolyte leakage (%)	MDA (µM g^{-1} FW)	H_2O_2 (µM g^{-1} FW)	AsAO activity (µM min^{-1} mg $protein^{-1}$)
Control		100.26 ± 4.88	15.15 ± 1.23	2.80 ± 0.13	0.13 ± 0.04	51.43 ± 1.53
NaF		52.20 ± 1.72*	41.46 ± 0.82*	5.11 ± 0.08*	0.29 ± 0.03*	15.92 ± 1.55*
$Ca(OH)_2$	**0.3 mM**	112.03 ± 1.25	11.47 ± 2.07*	2.44 ± 0.10	0.10 ± 0.04	46.19 ± 1.49*
	0.5 mM	107.05 ± 1.53	23.47 ± 1.65*	2.94 ± 0.13	0.12 ± 0.06	43.85 ± 2.04
	0.3 mM + NaF	91.71 ± 2.54	13.98 ± 0.58	3.34 ± 0.07	0.13 ± 0.06	37.45 ± 0.99
	0.5 mM + NaF	85.25 ± 3.69	29.48 ± 0.82	4.45 ± 0.16*	0.15 ± 0.04	29.27 ± 1.63*
$Ca(NO_3)_2$	**0.3 mM**	93.07 ± 5.06	17.12 ± 0.55	2.41 ± 0.27*	0.09 ± 0.02	37.08 ± 0.76*
	0.5 mM	94.75 ± 1.92	23.30 ± 1.15	2.87 ± 0.01	0.14 ± 0.03	36.81 ± 1.49
	0.3 mM + NaF	95.45 ± 1.81	23.46 ± 0.78	3.28 ± 0.10	0.15 ± 0.07	33.02 ± 1.38
	0.5 mM + NaF	83.02 ± 1.39	30.92 ± 0.52	3.65 ± 0.17	0.18 ± 0.06	22.66 ± 1.70*
$CaCl_2$	**0.3 mM**	92.77 ± 3.03	32.69 ± 2.15	4.58 ± 0.19*	0.15 ± 0.04	29.78 ± 1.52
	0.5 mM	84.02 ± 3.08	22.24 ±0.48*	2.81 ± 0.17*	0.10 ± 0.06	36.24 ± 2.24
	0.3 mM + NaF	60.36 ± 3.99*	38.51 ± 0.70*	4.74 ± 0.20*	0.17 ± 0.06*	20.16 ± 2.07*
	0.5 mM + NaF	79.81 ± 3.66*	26.96 ± 1.19*	3.33 ± 0.21*	0.15 ± 0.04	32.47 ± 1.21

4.2.3.4. Calcium regulates the formation of osmolytes in seedlings

The level of osmolytes like proline, glycine betaine and amino acids was enhanced by 1.7, 1.7 and 2.0 fold, respectively in water primed stressed seedlings (Table 4.2.4). The expression of *proline dehydrogenase* (*PDH*) was considerably reduced which suggested that seedlings are trying to recover from fluoride damage by conserving endogenous proline content. Along with *PDH*, the expression level of Δ^1-pyrroline 5-carboxylate synthetase (*P5CS*) was also reduced. The level of *betaine aldehyde dehydrogenase 1* (*BADH1*) was comparable to that of control seedling which showed that glycine betaine level was maintained in the stressed seedlings (Fig 4.2.2). During fluoride stress, the enhanced level of osmolytes can be supported by the earlier work of Tak and Asthir (2017) where they showed that wheat seedlings grown in presence of NaF solution maintained higher level of the above mentioned osmolytes that helped in detoxification of ROS and maintained the osmotic balance of cells. Higher level of proline can be attributed to its higher formation by P5CS or its lower degradation catalyzed by PDH. In this study, higher proline content of the seedlings was due to reduced expression of *PDH*. Similarly, higher level of glycine betaine (due to *BADH1* expression) and amino acids in water primed stressed seedlings can be justified by the earlier work of Hasthanasombut et al. (2011) and Azevedo-Neto et al. (2009) during salt stress in rice and maize seedlings, respectively. On priming of seeds with calcium compounds, the level of osmolytes, i.e., proline, glycine betaine and amino acids was reduced which highlighted the efficacy of all the three calcium compounds in ameliorating the negative effects of fluoride toxicity. In addition, the expression of *P5CS* was significantly up regulated in all the calcium primed stressed seedlings which suggested efficient utilization of the synthesized proline molecules in abating the signs of fluoride toxicity. Priming of seedlings with calcium also lowered the expression of *BADH1* linked with the lower level of glycine betaine in the seedlings (Fig 4.2.2; Table 4.2.4).

Table 4.2.4: Level of proline, glycine betaine and total amino acids in rice seedlings grown hydroponically in presence of NaF (25 mg L^{-1}) for 10 days, either in absence or presence of seed priming with 0.3 mM, each of $Ca(OH)_2$ and $Ca(NO_3)_2$ and 0.5 mM $CaCl_2$ for 24 h. Data are the mean value (n = 3) ± SE. Data with significant differences obtained against control are labeled with '*' at $P \leq 0.05$.

Sample		Proline (µg g^{-1} FW)	Glycine betaine (mg g^{-1} FW)	Total amino acids (µg g^{-1} FW)
Control		0.04 ± 0.02	14.92 ± 0.52	0.26 ± 0.01
NaF		0.07 ± 0.03*	25.32 ± 0.52*	0.51 ± 0.02*
$Ca(OH)_2$	**0.3 mM**	0.04 ± 0.08	16.06 ± 0.47	0.30 ± 0.04
	0.3 mM + NaF	0.05 ± 0.02	19.55 ± 0.36	0.36 ± 0.07
$Ca(NO_3)_2$	**0.3 mM**	0.04 ± 0.06	16.21 ± 0.36	0.31 ± 0.03
	0.3 mM + NaF	0.06 ± 0.01*	23.23 ± 0.36*	0.39 ± 0.06*
$CaCl_2$	**0.5 mM**	0.04 ± 0.07	19.43 ± 0.57	0.33 ± 0.02
	0.5 mM + NaF	0.06 ± 0.09*	23.96 ± 0.36*	0.47 ± 0.01*

Fig: 4.2.2a

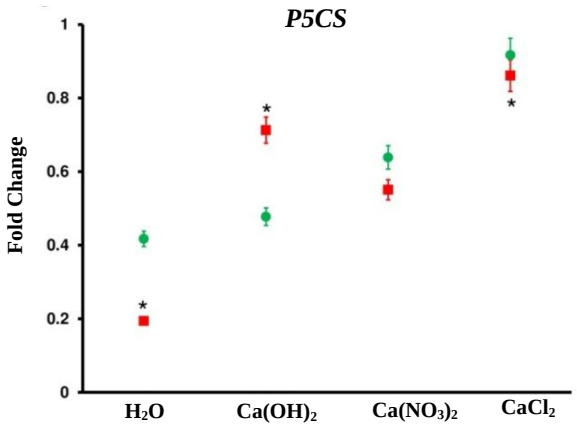


Fig: 4.2.2b

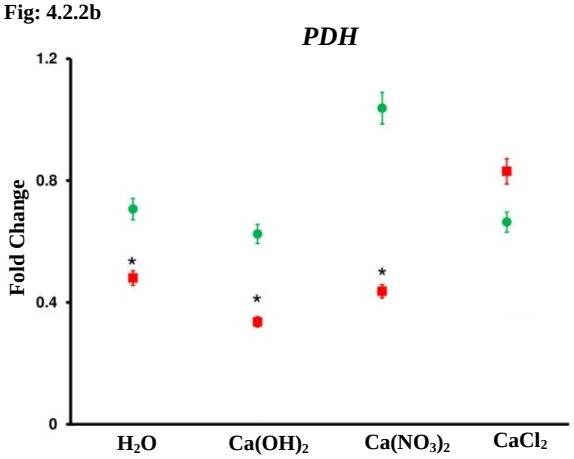


Fig: 4.2.2c

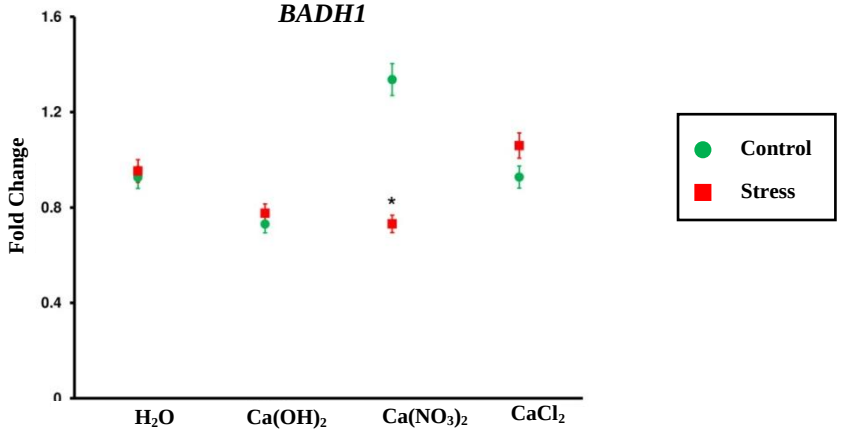


Fig. 4.2.2: Effect of seed priming with calcium compounds, viz., $Ca(OH)_2$ and $Ca(NO_3)_2$ (0.3 mM) and $CaCl_2$ (0.5 mM), for 24 h on the expression level of genes, viz., *P5CS* (**a**), *PDH* (**b**) and *BADH1* (**c**) in seedlings under stressed condition (25 mg L^{-1} NaF) for 10 days; the untreated seedlings (with or without calcium pre-treatment of seeds) served

as experimental control. Data are the mean value (n = 3) ± SE. Data with significant differences are labeled with '*' at P ≤ 0.05, as compared with non-primed control set.

4.2.3.5. Calcium mediates the level of non-enzymatic antioxidants in seedlings

The level of flavonoids and ascorbic acid was enhanced by 1.5 and 1.4 fold, respectively in water primed stressed seedlings, whilst the level of the other two non-enzymatic antioxidants, i.e., carotenoids and phenolics was lowered by 1.6 and 1.5 fold, respectively as compared to that of water treated non-stressed seedlings (Table 4.2.5). Priming of seeds with $Ca(OH)_2$, $Ca(NO_3)_2$, and $CaCl_2$ lowered the level of flavonoids and ascorbic acid. In contrast, the level of carotenoids and phenolics was restored in calcium primed stressed seedlings and was comparable to that of control set (Table 4.2.5). During fluoride stress, the enhanced level of ascorbic acid can be supported by the inhibited activity of AsAO as observed earlier which suggested that seedlings managed to conserve the endogenous ascorbate pool; in presence of calcium, the activity of AsAO was restored that led to higher oxidation of ascorbic acid suggesting its considerable utilization as an antioxidant to overcome the stressed condition. Higher ascorbic acid during fluoride stress can also be supported by the work of Bhargava and Bhardwaj (2010) where they demonstrated higher ascorbate formation in fluoride stressed wheat seedlings. Similarly, induced level of flavonoids can be supported by the work of Sarker and Oba (2018) and Quan et al. (2016) where they observed higher flavonoid content in *Amaranthus* leafy vegetables and rice, respectively under drought stress. In contrast, fluoride stress deteriorated the level of carotenoids and phenolics in seedlings which was in accordance with the work of Sachan and Lal (2018) that demonstrated lower carotenoid level in barley upon treatment with fluoride solution. However, on priming with calcium compounds, the level of phenolics and carotenoids was restored which again proved the intrinsic potential of calcium compounds in abrogating the fluoride stress in rice seedlings.

Table 4.2.5: Carotenoids, ascorbic acid, phenolics and flavonoids in rice seedlings grown hydroponically in presence of NaF (25 mg L^{-1}) for 10 days, either in absence or presence of seed priming with 0.3 mM, each of $Ca(OH)_2$ and $Ca(NO_3)_2$ and 0.5 mM $CaCl_2$ for 24 h. Data are the mean value (n = 3) ± SE. Data with significant differences obtained against control are labeled with '*' at P ≤ 0.05.

Sample		Carotenoids (µM g^{-1} FW)	Ascorbic acid (µg g^{-1} FW)	Phenolics (mg g^{-1} FW)	Flavonoids (mg g^{-1} FW)
Control		29.64 ± 0.41	6.26 ± 0.50	0.74 ± 0.02	2.71 ± 0.63
NaF		18.60 ± 0.47*	9.08 ± 0.84*	0.47 ± 0.02*	3.98 ± 0.45*
$Ca(OH)_2$	**0.3 mM**	29.88 ± 0.47	6.15 ± 0.48	0.71 ± 0.01	2.87 ± 0.33
	0.3 mM + NaF	25.66 ± 0.47	7.15 ± 0.67	0.66 ± 0.01	3.16 ± 0.41
$Ca(NO_3)_2$	**0.3 mM**	28.30 ± 0.54	6.22 ± 0.49	0.70 ± 0.02	2.84 ± 0.48
	0.3 mM + NaF	23.65 ± 0.61*	7.27 ± 0.71	0.63 ± 0.01	3.68 ± 0.07*
$CaCl_2$	**0.5 mM**	27.57 ± 0.47	6.44 ± 0.54	0.67 ± 0.01	2.89 ± 0.45
	0.5 mM + NaF	21.73 ± 0.61*	8.52 ± 0.74*	0.59 ± 0.01*	3.77 ± 0.37*

4.2.3.6. Activity and gene expression analysis of enzymatic antioxidants

During fluoride stress, the activity of catalase (CAT) was reduced by 1.9 fold, as compared to that of control seedlings which can be explained by the fact that fluoride ions replace the hydroxyl ions from the iron moiety present in the active site of the enzymes. Thus, the enzyme structure is distorted, compromising its activity (Yadu et al. 2016). In contrast, calcium priming scavenged the fluoride ions which lowered its bioavailability in tissues that led to the structural integrity of CAT (Table 4.2.6). Additionally, the above observation can also be supported by *CAT* gene expression in water primed stressed seedlings where the expression of *CAT* gene was down regulated, whilst on calcium priming, the expression was comparable to that of water primed stressed seedlings, showing uninterrupted enzyme function in presence of calcium (Fig. 4.2.3a). The activity of the other enzymatic antioxidants i.e., superoxide dismutase (SOD) and guaiacol peroxidase (GPoX) was enhanced in stressed seedlings which suggested that in absence of CAT activity, the major task of detoxification of ROS was carried out by these two enzymes (Table 4.2.6). Enhanced activity of GPoX as observed during this work coincided with the earlier work of Kumar et al. (2009) in fluoride stressed mulberry leaves. The enhanced activity of SOD might be due to its higher gene expression in water primed stressed seedlings as observed here (Fig 4.2.3b). Additionally, higher SOD activity was also reported by Chakrabarti and Patra (2013) in fluoride stressed spinach, radish, coriander and mustard seedlings. Moreover, calcium priming lowered the activity of SOD and GPoX which showed the efficacy of calcium compounds in detoxifying ROS without much need of the involvement of antioxidative enzymes.

Table 4.2.6: Activity of CAT, SOD and GPoX in rice seedlings grown hydroponically in presence of NaF (25 mg L^{-1}) for 10 d, either in absence or presence of seed priming with 0.3 mM, each of $Ca(OH)_2$ and $Ca(NO_3)_2$ and 0.5 mM $CaCl_2$ for 24 h. Data are the mean value (n = 3) ± SE. Data with significant differences obtained against control are labeled with '*' at P ≤ 0.05.

Sample		CAT activity (µM min^{-1} mg^{-1} leaf protein)	SOD activity (unit mg^{-1} leaf protein)	GPoX activity (µM min^{-1} mg^{-1} leaf protein)
Control		30.01 ± 1.09	1.72 ± 0.05	8.16 ± 0.24
NaF		16.00 ± 0.86*	3.57 ± 0.07*	13.82 ± 0.31*
$Ca(OH)_2$	0.3 mM	28.93 ± 1.18	1.74 ± 0.09	8.96 ± 0.24
	0.3 mM + NaF	24.34 ± 0.73	2.49 ± 0.06	10.07 ± 0.16
$Ca(NO_3)_2$	0.3 mM	31.12 ± 1.09	1.88 ± 0.06	9.12 ± 0.21
	0.3 mM + NaF	24.28 ± 0.75	3.10 ± 0.06*	11.09 ± 0.16*
$CaCl_2$	0.5 mM	31.92 ± 0.59	1.88 ± 0.08	9.33 ± 0.24
	0.5 mM + NaF	23.60 ± 0.80*	3.27 ± 0.09*	10.09 ± 0.19

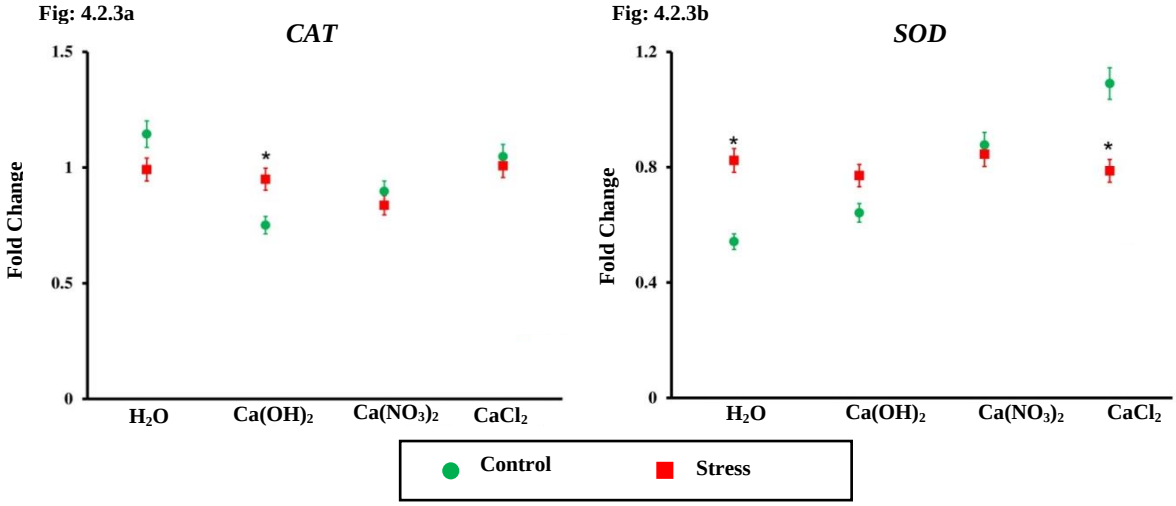


Fig. 4.2.3: Effect of seed priming with calcium compounds, viz., $Ca(OH)_2$ and $Ca(NO_3)_2$ (0.3 mM) and $CaCl_2$ (0.5 mM), for 24 h on expression level of genes encoding antioxidant enzymes, CAT (**a**) and SOD (**b**), in seedlings under stressed condition (25 mg L^{-1} NaF) for 10 days; the untreated seedlings (with or without calcium pre-treatment of seeds) served as experimental control. Data are the mean value (n = 3) ± SE. Data with

significant differences are labeled with '*' at P ≤ 0.05 as compared with non-primed control set.

4.2.3.7. Expression analysis of genes linked with calcium signaling pathway

The expression of genes involved in calcium signaling pathway, i.e., *CaM1-1*, *CBL10*, *SOS1*, *SOS2* and *SOS3* was analyzed under all the experimental conditions. Data recorded showed strong inhibition in calcium signaling pathways due to down regulated expression of *CaM1-1*, *CBL10*, *SOS2* and *SOS3* in water primed stressed seedlings (Fig 4.2.4). In water primed stressed seedlings, the availability of calcium ions was reduced probably due to complex formation with fluoride, thus ultimately causing lower expression of the calcium sensors in seedlings. Our observation also coincided with the previous work of Asad et al. (2019) where they showed that lower level of calcium ions reduced the expression of calcium responsive genes in *Arabidopsis* and rice seedlings. However, in contrast to *SOS2* and *SOS3*, the expression of *SOS1* was induced in fluoride stressed water primed seedlings; however, this induction was insufficient to overcome the detrimental effects of fluoride toxicity as the activation of SOS1 is directly controlled by SOS2 and SOS3. On priming the seeds with calcium compounds, the expression of all the above mentioned genes was restored which might be due to induced endogenous calcium content of the seedlings. The most remarkable change was noted in case of *CBL10* whose expression was drastically enhanced in calcium primed fluoride stressed seedlings which can be supported by the earlier work of Cheong et al. (2003). They reported higher expression of *CBL* genes in calcium rich environment. Additionally, higher expression of CaM and SOS pathway genes can be supported by the previous report of Tuteja and Mahajan (2007) where they stated that higher calcium content in the cells can be sensed by the calcium sensor that lead to the up regulation of calcium signaling genes. Overall, the most noteworthy change was observed in the expression of *CBL10* genes in calcium primed fluoride stressed seedlings which proved that it acts as a nodal regulator of calcium signaling pathways involved in fluoride tolerance of seedlings.

Based on all the above analyses, it was evident that of all the calcium compounds, $Ca(OH)_2$ and $Ca(NO_3)_2$, at lower (0.3 mM) concentration were more effective in increasing the tolerance capability of seedlings, as compared to that of $CaCl_2$ at higher concentration (0.5 mM). Therefore, further analyses on TCA cycle and sucrose and starch metabolism was undergone only at the above concentrations of $Ca(OH)_2$ and $Ca(NO_3)_2$.

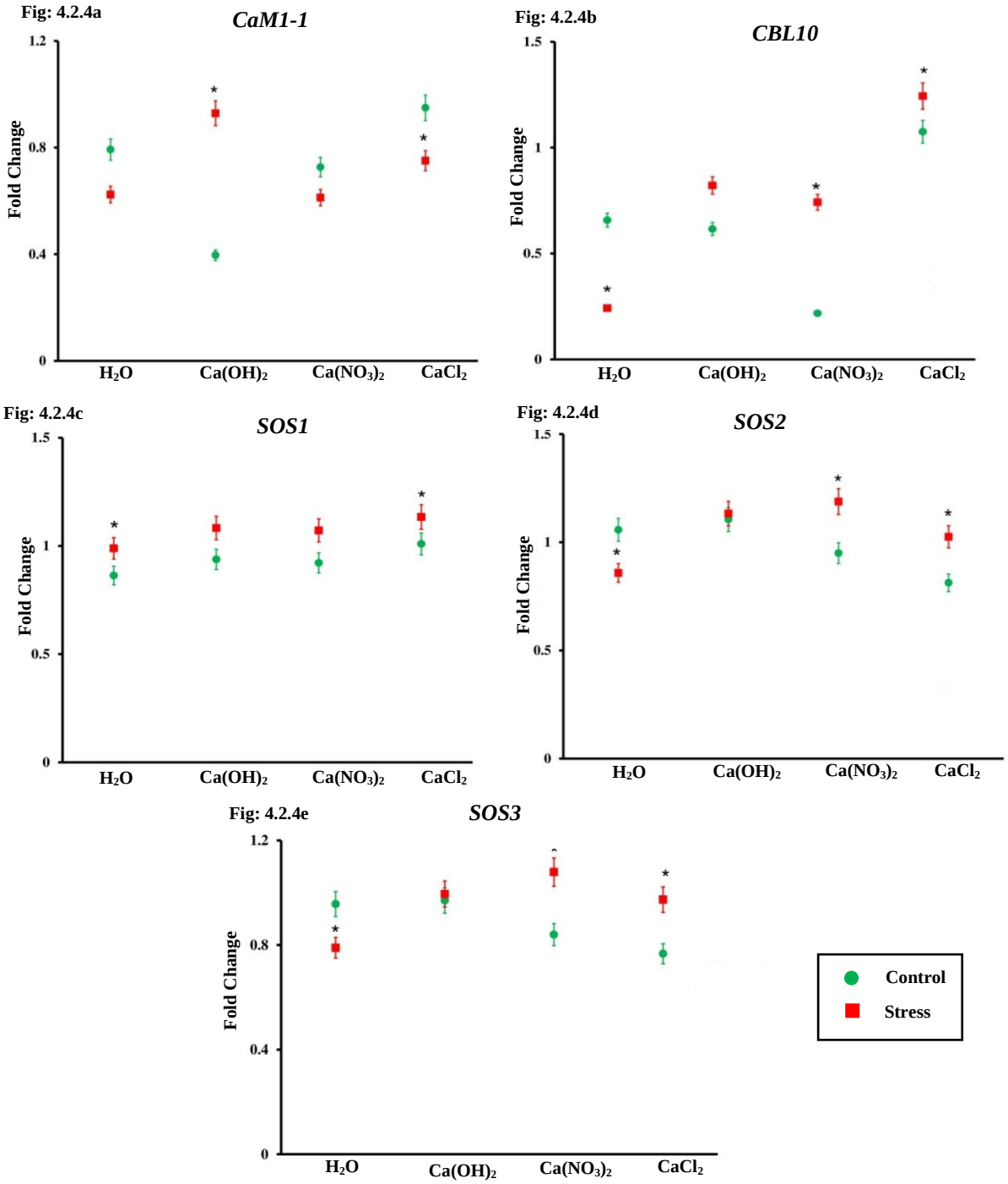


Fig. 4.2.4: Effect of seed priming with calcium compounds, viz., $Ca(OH)_2$ and $Ca(NO_3)_2$ (0.3 mM) and $CaCl_2$ (0.5 mM), for 24 h on expression level of genes involved in Ca signaling pathway, *CaM1-1* (**a**), *CBL10* (**b**), *SOS1* (**c**), *SOS2* (**d**), and *SOS3* (**e**), in seedlings under stressed condition (25 mg L^{-1} NaF) for 10 days; the untreated seedlings (with or without calcium pre-treatment of seeds) served as experimental control. Data are the mean value (n = 3) ± SE. Data with significant differences are labeled with '*' at $P \leq 0.05$, as compared with non-primed control set.

4.2.3.8. Calcium priming restored the content of organic acids in seedlings

Organic acids play a major role in energy production that enables the plants to adapt according to challenging environment. Fluoride stress reduces the content of pyruvate, citrate and malate by 1.5, 1.8 and 1.3 times, respectively in seedlings, as compared to that of control sets. However, on priming with 0.3 mM $Ca(OH)_2$, the level of the above mentioned organic acids was induced by 1.3, 1.6 and 1.1 fold, respectively in the stressed seedlings. Similarly, the level was enhanced by 1.2, 1.4 and 1.1 fold, respectively in $Ca(NO_3)_2$ primed stressed seedlings, as compared to that of water primed stressed seedlings (Fig 4.2.5). Reduced content of organic acids in fluoride stressed seedlings clearly demonstrated that fluoride ions significantly inhibited the energy productions in cells that increased the vulnerability of KH seedlings. Lower content of organic acids during salt stress in rice and barely seedlings was earlier reported by Das et al. (2019) and Widodo et al. (2009), respectively that also supported our observation. Priming of seeds with calcium compounds restored the level of organic acids which suggested increased flow of carbon to TCA cycle from glycolytic pathway, thus leading to higher formation of energy rich molecules such as NADH, $FADH_2$ and ATP which in turn supported the growth of the seedlings. Moreover, organic acids also presumably helped to maintain the osmotic balance of the cells which helped to combat the effects of fluoride induced oxidative damage in seedlings.

Fig: 4.2.5a

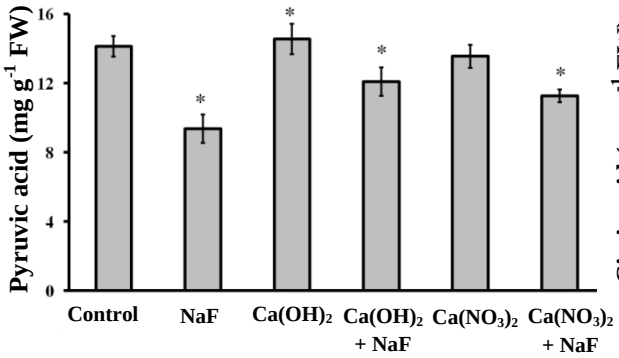


Fig: 4.2.5b

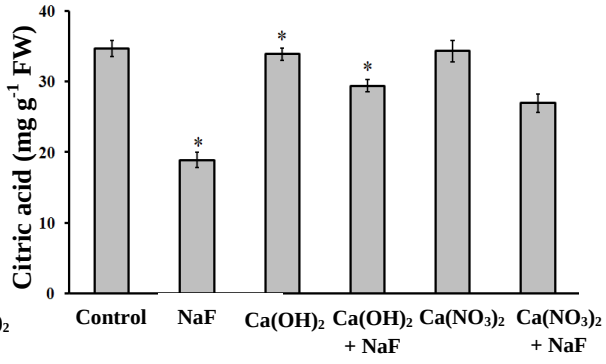


Fig: 4.2.5c

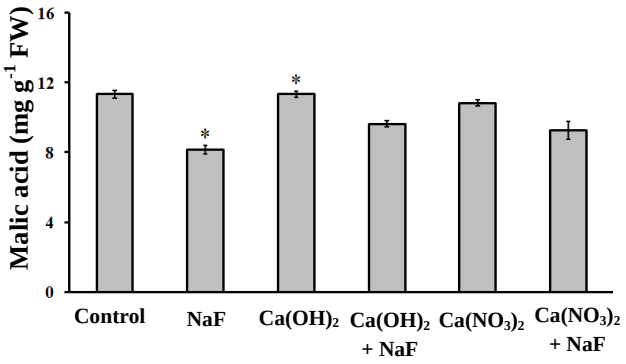


Fig. 4.2.5: Content of organic acids, viz., pyruvic acid (**a**), citric acid (**b**) and malic acid (**c**) during fluoride (25 mg L^{-1} NaF) stress for 10 days in the seedlings raised from seeds primed with either $Ca(OH)_2$ (0.3 mM) or $Ca(NO_3)_2$ (0.3 mM) for 24 h, as against water-primed seeds; the non-stressed seedlings were used as control. Data are the mean value of triplicate sets (n = 3) ± SE. Data with significant differences are labeled with '*' at $P \leq 0.05$, as compared to water-primed set.

4.2.3.9. Calcium regulated activity and expression of enzymes of TCA cycle

Fluoride toxicity drastically inhibited the activity of enzymes of TCA cycle, viz., PyDH (2.1 times), CS (1.7 times), IDH (1.5 times) and SDH (1.6 times) in water primed stressed seedlings (Table 4.2.7). Additionally, the gene expression pattern of the above mentioned enzymes was also down regulated in fluoride stressed seedlings (Fig 4.2.7). Upon priming of seedlings with $Ca(OH)_2$, the activity of the above mentioned enzymes was enhanced by 1.7, 1.4, 1.3 and 1.1 fold, respectively whereas priming with $Ca(NO_3)_2$ enhanced the same by 1.7, 1.3, 1.3 and 1.1 fold, respectively, as compared to that of water primed stressed seedlings (Table 4.2.7). Similarly, on priming with both the calcium compounds, the expression of *PyDH*, *CS* and *SDH* was also enhanced in fluoride stressed seedlings, whilst the expression of *IDH* remain unchanged on priming with $Ca(OH)_2$ but was enhanced in $Ca(NO_3)_2$ primed stressed seedlings (Fig 4.2.6). In contrast, the activity of fumarase and MDH was induced by 1.8 and 1.5 times, respectively in water primed fluoride stressed seedlings, whereas priming with $Ca(OH)_2$ reduced their activity by 1.5 and 1.2 fold, respectively. However, priming with $Ca(NO_3)_2$ showed negligible change in the activity of enzymes, as compared to that water primed stressed seedlings (Table 4.2.7). The gene expression of *MDH* coincided with enzyme activity in water primed and calcium primed stressed seedlings (Fig 4.2.6). PyDH is the

major enzyme that links glycolysis with TCA cycle. Inhibited activity and gene expression of PyDH leads to decreased supply of carbon to the TCA cycle that lowered the energy production in cells, resulting in higher cellular damage. Our results can be further supported by the work of Che-Othman et al. (2019) where they showed reduced PyDH activity in salt susceptible wheat variety. Priming of seeds with calcium compounds induced the expression and activity of PyDH that maintained the carbon supply and energy production in cells which in turn reduced the signs of fluoride induced damage. Decreased activity of CS can be linked with lower formation of citrate in fluoride stressed seedlings along with its lower gene expression. However on calcium priming, the level of citrate was enhanced which eventually led to higher CS activity, resulting in higher carbon condensation. Another major enzyme of TCA cycle that links carbon and nitrogen metabolism is IDH. Fluoride stress substantially lowered IDH activity and expression which signified higher mitochondrial damage during fluoride stress, that was rescued on priming with calcium. Similar to our observation, Bouthour et al. (2015) showed that higher activity of IDH contributed toward higher tolerance level of wheat seedlings exposed to salt stress. Reduced activity of SDH can be supported by the work of Banaei-Asl et al. (2016) where they reported that salt stress inhibited the activity of SDH in canola seedlings that hampered both TCA cycle and electron transport chain, leading to lower energy production in the cells. However, on calcium priming, the activity of SDH was restored that aided in higher energy production which was again utilized by the cells to overcome the fluoride toxicity. Additionally, the activity of the above mentioned enzymes was also severely retarded in salt stressed rice and *Vigna radiata* seedlings, supporting our observation (Das et al. 2019; Saha et al. 2012). On calcium priming, the activity of the mitochondrial respiratory enzymes was rescued in cucumber seedlings under hypoxia stress as reported by He et al. (2015) that coincided with our observation. Higher fumarase and MDH activity in fluoride stressed KH seedlings can be supported by the work of Chojak-Koźniewska et al. (2018) and Sil et al. (2018) where they showed induced fumarase activity in salt stressed cucumber seedlings and MDH activity in arsenic stressed wheat seedlings, respectively. MDH converts malate to oxaloacetate that leaks out from the cell membrane into the cytoplasm and acts as a marker of higher cell damage. Thus, higher MDH activity during fluoride stress indicates towards higher cellular damage. On priming of seeds with calcium, the activity of fumarase and MDH was reduced in seedlings which highlight the role of calcium as an effective stress ameliorating agent.

Table 4.2.7: Activity of respiratory enzymes, viz., PyDH, CS, IDH, SDH, fumarase and MDH during fluoride (25 mg L^{-1} NaF) stress for 10 days in the seedlings raised from seeds primed with either $Ca(OH)_2$ (0.3 mM) or $Ca(NO_3)_2$ (0.3 mM) for 24 h, as against water-primed seeds; the non-stressed seedlings were used as control. Data are the mean value of triplicate sets (n = 3) ± SE. Data with significant differences are labelled with '*' at $P \leq 0.05$ as compared to water-primed set.

Enzyme activity	Control	NaF	$Ca(OH)_2$		$Ca(NO_3)_2$	
			Control	NaF	Control	NaF
PyDH (µmol NADH min^{-1} mg^{-1} protein)	45.67 ± 2.03	21.19 ± 2.99*	47.74 ± 1.30	36.19 ± 2.94*	43.67 ± 1.75	36.84 ± 1.94*
CS (µmol TNB min^{-1} mg^{-1} protein)	74.77 ± 1.77	43.72 ± 1.66*	80.81 ± 1.58	59.70 ± 1.32*	76.99 ± 1.58	55.54 ± 1.16*
IDH (µmol NADH min^{-1} mg^{-1} protein)	10.90 ± 0.57	7.07 ± 0.64*	12.02 ± 0.58	9.45 ± 0.69	11.50 ± 0.53	9.51 ± 0.61
SDH (µmol INT min^{-1} mg^{-1} protein)	15.82 ± 1.11	10.07 ± 0.76*	13.87 ± 0.99*	11.10 ± 0.46	12.49 ± 0.70	10.98 ± 0.68
Fumarase (µmol fumarate min^{-1} mg^{-1} protein)	167.79 ± 12.25	309.18 ± 11.22*	154.88 ± 12.55	208.45 ± 13.71*	150.95 ± 10.62	296.14 ± 11.89
MDH (µmol NADH min^{-1} mg^{-1} protein)	0.16 ± 0.009	0.25 ± 0.009*	0.17 ± 0.003	0.21 ± 0.006*	0.16 ± 0.007	0.24 ± 0.004

Fig: 4.2.6a

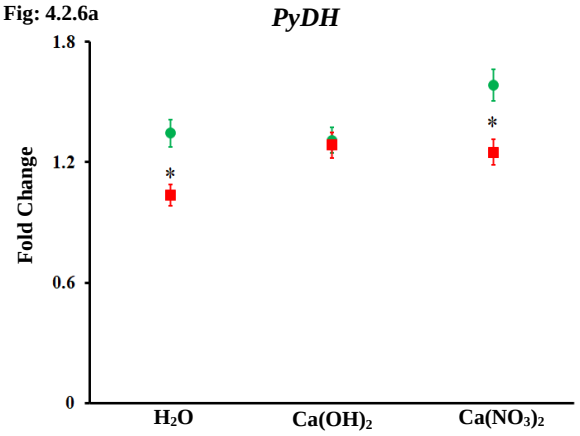
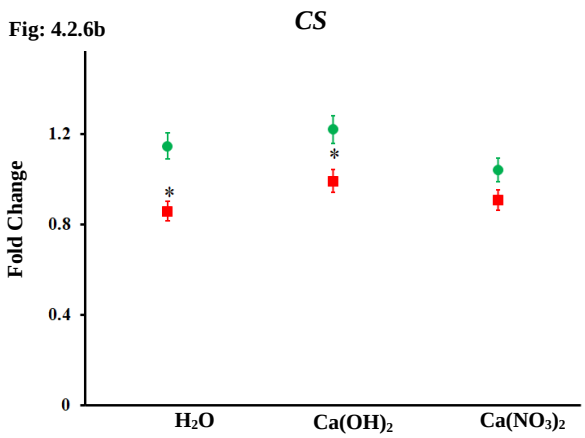


Fig: 4.2.6b



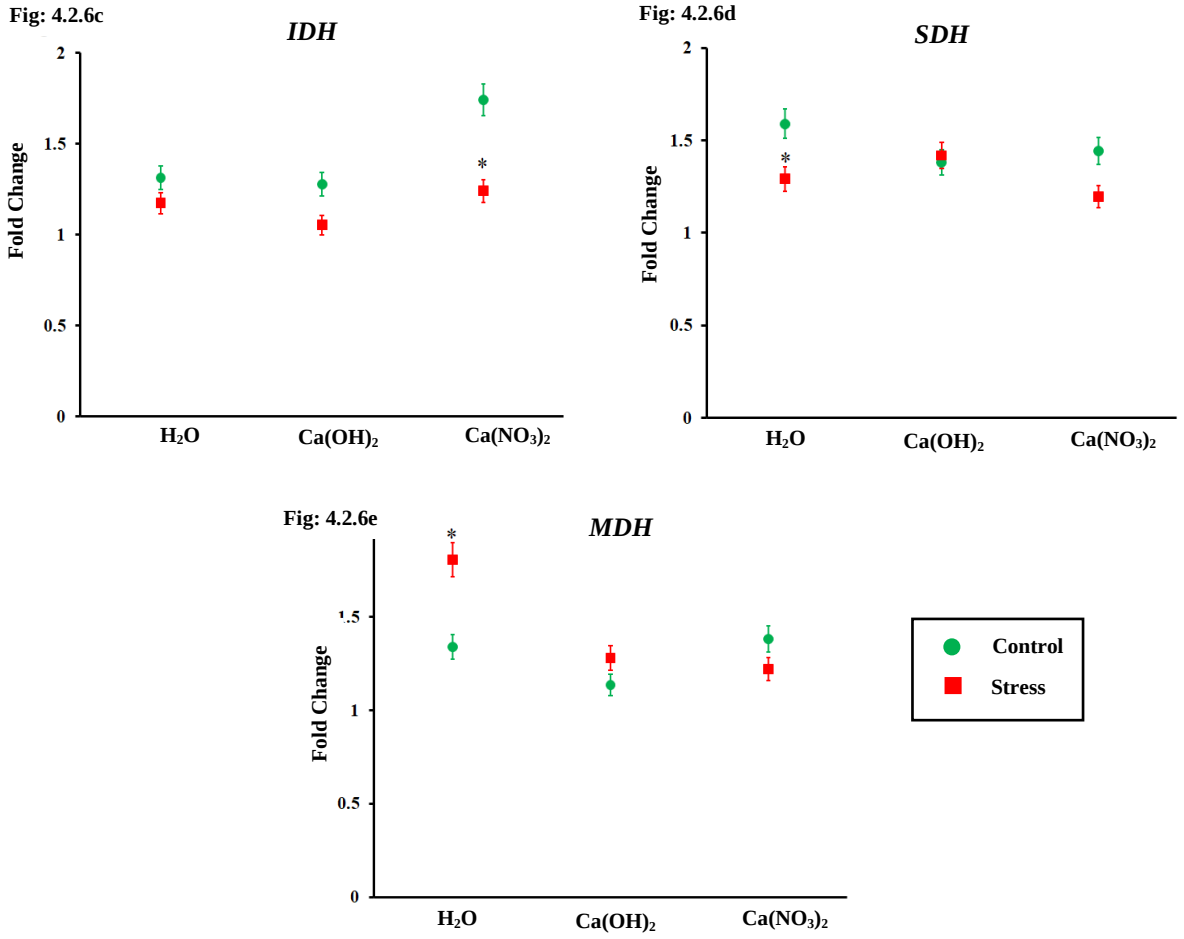


Fig. 4.2.6: Expression analysis of genes, viz., *PyDH* (**a**), *CS* (**b**), *IDH* (**c**), *SDH* (**d**) and *MDH* (**e**) during fluoride (25 mg L^{-1} NaF) stress for 10 days in the seedlings raised from seeds primed with either Ca(OH)2 (0.3 mM) or Ca(NO3)2 (0.3 mM) for 24 h, as against water-primed seeds; the non-stressed seedlings were used as control. Data are the mean value of the triplicate sets (n = 3) ± SE. Data with significant differences are labeled with '*' at $P \leq 0.05$ as compared to water-primed set.

4.2.3.10. Calcium regulated sucrose formation and activity and expression of concerned enzymes

2.3 fold reduction in the level of sucrose was noted in water primed fluoride stressed seedlings, whereas priming with $Ca(OH)_2$ and $Ca(NO_3)_2$ restored the level of the same by 1.8 and 1.4 fold, respectively as compared to that of water primed stressed seedlings (Table 4.2.8). Reduced content of sucrose in the fluoride stressed seedlings can be justified by the reduced activity of SPS by 1.5 fold. However, on priming, the activity of SPS was increased

by 1.4 and 1.1 times, respectively in fluoride stressed seedlings. Furthermore, the expression of *SPS* was also drastically reduced in fluoride stressed seedlings, whereas it was restored in calcium primed seedlings which coincided with the activity of SPS in seedlings (Fig 4.2.7). Additionally, the activity of INV was also enhanced by 2.1 times in water primed stressed seedlings that contributed towards lower sucrose content in the seedlings. Calcium priming inhibited the activity of INV by 1.5 and 1.2 times, respectively that led to higher preservation of endogenous sucrose pool of the seedlings. Likewise, the expression of INV was up regulated in the stressed seedlings, whereas priming with $Ca(OH)_2$ and $Ca(NO_3)_2$ down regulated the expression of *INV*. Fluoride stress lowered the activity of SS by 1.5 times in water primed seedlings, whilst the activity of same was enhanced by 1.2 fold on priming with either $Ca(OH)_2$ or $Ca(NO_3)_2$. Similarly, the expression of *SS* was reduced in fluoride stressed seedlings and was restored on priming with calcium compounds. Sucrose is an important osmoprotectant that scavenge ROS generated during environmental stresses and also balance the osmotic level of the cells. Reduced content of sucrose in stressed seedlings might be due to over utilization of sucrose pool by the seedlings, leading to its exhaustion. During abiotic stress, higher activity of INV and reduced activity of SPS lead to the lower formation of sucrose, as shown earlier by Choudhury et al. (2010) and Yang et al. (2019). Moreover, lower activity of SS might be due to conservation of sucrose pool by the seedlings for further utilization as an osmoprotectant. The sucrose level along with higher activity of SPS and SS and lower activity of INV in presence of calcium compounds, suggested lesser utilization of sucrose as an osmoprotectant, thus exhibiting the efficacy of calcium as an efficient ameliorating agent. Higher sucrose content on exogenous application of calcium was earlier noted in beetroot that efficiently minimized the extent of drought induced oxidative damage in seedlings as reported by Hosseini et al. (2019) that supports our observations.

Table 4.2.8: Sucrose and starch content and activity of enzymes, viz., SPS, SS, INV, PHO and α-amylase during fluoride (25 mg L^{-1} NaF) stress for 10 days in the seedlings raised from seeds primed with either $Ca(OH)_2$ (0.3 mM) or $Ca(NO_3)_2$ (0.3 mM) for 24 h, as against water-primed seeds; the non-stressed seedlings were used as control. Data are the mean value of triplicate sets (n = 3) ± SE. Data with significant differences are labeled with '*' at $P \leq 0.05$ as compared to water-primed set.

Parameters	Control	NaF	$Ca(OH)_2$		$Ca(NO_3)_2$	
			Control	NaF	Control	NaF
Sucrose content (mg g^{-1} FW)	15.41 ± 0.54	6.77 ± 0.36*	14.92 ± 0.50	12.33 ± 0.57*	16.00 ± 0.43	9.71 ± 0.43*
Starch content (mg g^{-1} FW)	148.01 ± 4.37	100.54 ± 3.50*	146.29 ± 3.89	120.95 ± 3.63*	143.77 ± 4.30	117.28 ± 4.19
SPS activity (µmol of sucrose formed min^{-1} mg^{-1} protein)	44.28 ± 1.48	29.10 ± 1.31*	47.47 ± 1.30	39.63 ± 1.52	47.37 ± 1.72	32.12 ± 1.56*
SS activity (µmol of sucrose hydrolyzed min^{-1} mg^{-1} protein)	39.98 ± 1.47	25.88 ± 1.44*	42.56 ± 2.25	31.49 ± 1.49	43.42 ± 1.54	29.89 ± 1.59*
INV activity (µmol of sucrose hydrolyzed min^{-1} mg^{-1} protein)	18.71 ± 1.48	39.61 ± 1.44*	19.44 ± 1.54	25.82 ± 1.87*	19.51 ± 1.51	32.38 ± 1.80*
PHO activity (µmol of Pi liberated min^{-1} mg^{-1} protein	45.30 ± 1.72	73.05 ± 1.75*	45.71 ± 2.15	60.94 ± 2.95*	51.65 ± 2.87	71.50 ± 2.19
α-amylase activity (µg of starch hydrolyzed min^{-1} mg^{-1} protein	18.71 ± 1.48	35.87 ± 1.53*	20.40 ± 1.63	22.80 ± 1.53*	19.86 ± 1.44	28.96 ± 1.49*

Fig: 4.2.7a

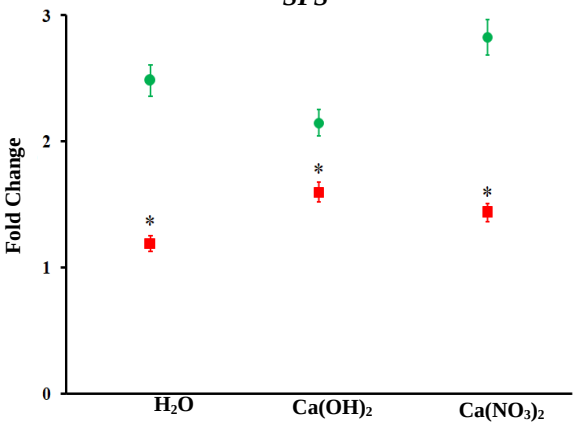
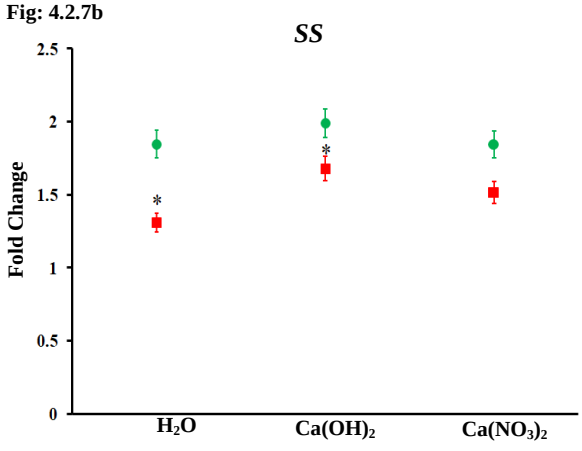


Fig: 4.2.7b



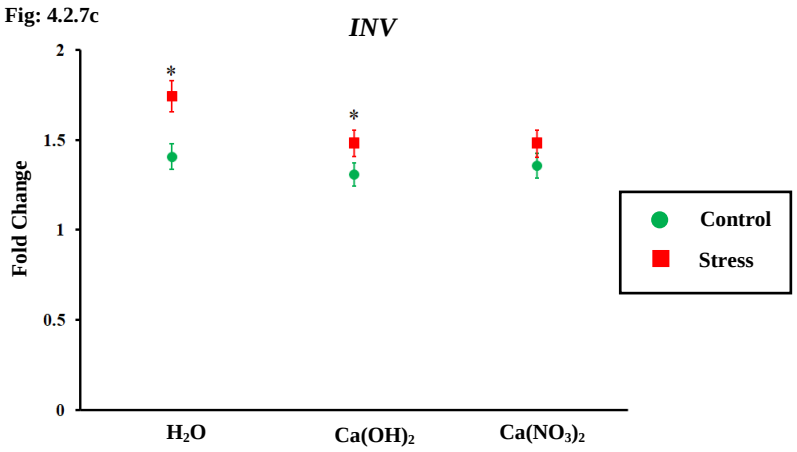


Fig. 4.2.7: Expression analysis of genes, viz., *SPS* (**a**), *SS* (**b**) and *INV* (**c**) during fluoride (25 mg L^{-1} NaF) stress for 10 days in the seedlings raised from seeds primed with either $Ca(OH)_2$ (0.3 mM) or $Ca(NO_3)_2$ (0.3 mM) for 24 h, as against water-primed seeds; the non-stressed seedlings were used as control. Data are the mean value of the triplicate sets (n = 3) ± SE. Data with significant differences are labeled with '*' at $P \leq 0.05$ as compared to water-primed set.

4.2.3.11. Calcium regulated starch formation and activity and expression of concerned enzymes

Fluoride stress reduced the endogenous starch content of the seedlings by 1.5 times which could be justified by the enhanced activity of PHO (1.6 times) and α-amylase (1.9 times) (Table 4.2.8). Moreover, the expression of both *PHO* and *α-amylase* genes was enhanced in water primed stressed seedlings (Fig. 4.2.8). On priming with $Ca(OH)_2$, the activity of PHO and α-amylase was reduced by 1.2 and 1.6 times, respectively which was reflected by higher endogenous level of starch (1.2 times). Similarly, priming with $Ca(NO_3)_2$ also enhanced the level of starch in the seedlings by 1.2 fold and reduced the activity of PHO and α-amylase by 1.1 and 1.2 times, respectively (Table 4.2.7). Reduced activity of both the above mentioned enzymes on calcium priming could be justified by down regulated gene expression in fluoride stressed seedlings (Fig 4.2.8). Moreover, lower starch content could be justified by the fact that seedlings are trying to overcome the fluoride stress by catabolizing the stored form of carbon, i.e., starch, to produce osmoregulatory molecules by inducing PHO and α-amylase activity. Yang et al. (2001) also reported lower starch content in rice seedlings on being exposed to drought stress that supports our observation. On priming of seeds with calcium

compounds, the activity of both the enzymes was reduced along with their gene expression. This proved the efficacy of calcium in abating the effects of fluoride induced damage in seedlings without using the endogenous starch pool that led to its lower catabolic breakdown.

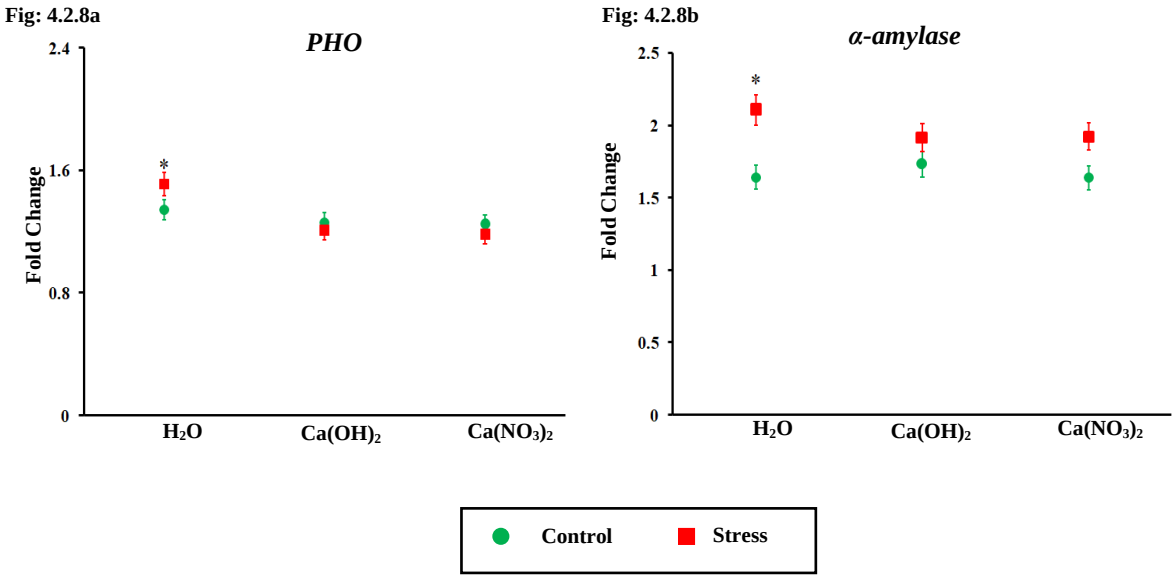
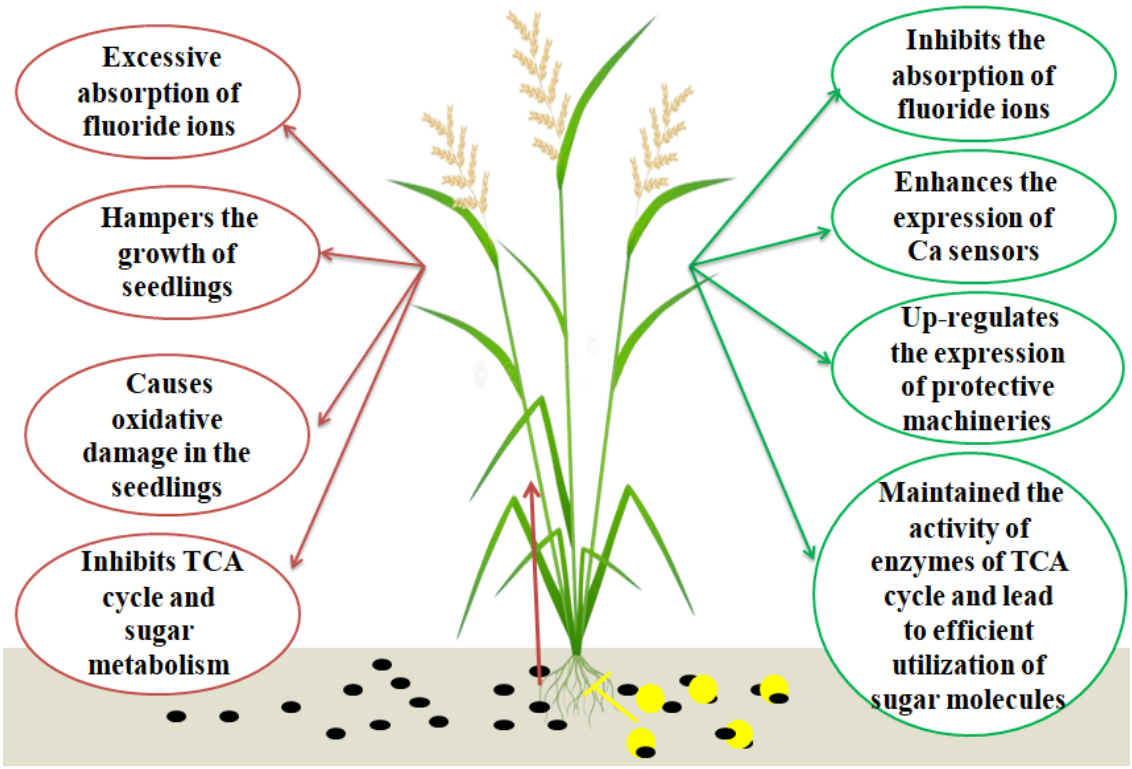


Fig. 4.2.8: Expression analysis of genes, viz., *PHO* (**a**) and *α-amylase* (**b**) during fluoride (25 mg L^{-1} NaF) stress for 10 days in the seedlings raised from seeds, primed with either $Ca(OH)_2$ (0.3 mM) or $Ca(NO_3)_2$ (0.3 mM) for 24 h, as against water-primed seeds; the non-stressed seedlings were used as control. Data are the mean value of the triplicate sets (n = 3) ± SE. Data with significant differences are labeled with '*' at $P \leq 0.05$ as compared to water-primed set.

4.2.4. Conclusion of the chapter

Overall, for this study, three different calcium compounds, i.e., $Ca(OH)_2$, $Ca(NO_3)_2$ and $CaCl_2$ were examined to abrogate the fluoride induced oxidative damage and inhibition of carbon metabolism (TCA cycle and sucrose and starch metabolism) in rice seedlings. From the data obtained, it was clearly visible that $Ca(OH)_2$ was the most effective in curtailing the signs of fluoride damage in seedlings, followed by $Ca(NO_3)_2$, whereas $CaCl_2$ was the least effective of all the three calcium compounds used. It was also apparent from our study that for $Ca(OH)_2$ and $Ca(NO_3)_2$, 0.3 mM concentration worked better, as compared with $CaCl_2$, where 0.5 mM appeared to be the better concentration to abrogate fluoride toxicity. Lower efficacy of $CaCl_2$ against fluoride stress, as compared with $Ca(OH)_2$ and $Ca(NO_3)_2$, whether at 0.3 mM or 0.5 mM is rather difficult to explain and possibly needs further investigation in the future. Thus, it could be concluded that pre-soaking of rice seeds with calcium compounds, prior to sowing, acts as a promising strategy to overcome the negative effects of fluoride induced oxidative damage and inhibited carbon metabolism in rice seedlings that can be adopted by the farmers as a strategy to deal with fluoride pollution in the agricultural fields (Fig. 4.2.9).”



"**Fig. 4.2.9:** Fluoride ions in the soil are taken up by the roots of the plants that induce the formation of ROS leading to oxidative damage. On priming of seeds with calcium compounds the level of fluoride deposited in the tissues was reduced that restored the normal metabolic cycle in the seedlings. Additionally, calcium application also regulates the level of protective metabolites that in turn mediates the tolerance capability of seedlings."

Chapter 4.3

Silicon modulates the fluoride tolerance level of susceptible rice cultivar

Data Published in this chapter has already been published in:
Plant Physiology and Biochemistry (2020) 154:758-769,
doi: 10.1016/j.plaphy.2020.06.023

"4.3.1 Overview of the chapter

In the previous chapter, the potency of calcium in nullifying the negative effects of fluoride stress in rice seedlings was established. This chapter elaborately elucidates the ameliorative effects of exogenous application of silicon in the seedlings of Khitish rice variety, subjected to fluoride stress. Fluoride stress (25 and 50 mg L^{-1} NaF) restricted the growth of seedlings by enhancing the extent of oxidative damages in the seedlings. Exogenous silicon application, irrespective of fluoride concentrations, restored the normal growth of the seedlings by restricting the uptake of fluoride ions from the surrounding media. Moreover, higher deposition of silicon in the tissues efficiently lowered the fluoride-mediated methylglyoxal formation in the tissues by inducing the activity of Gly I and Gly II. The activity of enzymatic antioxidants (APX, SOD, GPoX, GR and GPX) which was up regulated in the stressed seedlings was lowered in presence of silicon, whilst the fluoride induced inhibition in the activity of CAT was restored. Additionally, silicon application also reduced the level of non-enzymatic antioxidants such as anthocyanins, flavonoids, ascorbate, xanthophylls and reduced glutathione, and osmolytes such as proline, glycine betaine and amino acids along with the activity of P5CS and PAL which altogether signified the promising role of silicon in curtailing the negative effects of fluoride stress. Thus, overall it was established that oxidative damage incited due to fluoride stress was efficiently augmented on application of silicon that sustained the growth of the seedlings.

4.3.2. Introduction of the chapter

In recent times, silicon is identified as one of the most beneficial elements for plants that performs an array of functions, including tolerance to both biotic and abiotic stresses. Silicon is the second most abundant element present in both soil and earth's crust (Ranjan et al. 2021). Silicon is predominantly present as silica in soil which cannot be taken up by the plants. Silicon also exists as silicic acid and monosilic acid in soil that can be easily absorbed by the silicon transporters present in the plant roots (Vivancos et al. 2016). Once being absorbed, silicon is transported from xylem to xylem parenchyma cells and subsequently gets deposited in root endodermis, epidermal cells of leaf and inflorescence bracts (Mabagala et al. 2020). Silicon not only protects plants from stresses, but also plays a vital role in the regulation of various physiological processes such as plant development, seed germination, gene expression and regulation of enzymatic activities which altogether improves the adaptability of plants. Moreover, silicon-induced antioxidant defense system and its property to confer protection form oxidative damage has been reported in many plant species such as tomato, sorghum, wheat and spinach under drought stress (Cao et al. 2015; Galvez and Clark 1991; Kim et al. 2016; Gunes et al. 2007b). Many studies have also demonstrated the protective role of silicon in other abiotic stresses such as salinity, chilling, cadmium and aluminium toxicity and UV-B stress (Liang et al. 2007; Liu et al. 2009; Kollarova et al. 2019; Singh et al. 2011; Chen et al. 2016). In a recent study, Mishra et al. (2020) using EDXRF technique has shown higher accumulation of silicon in Pokkali rice variety over IR-64 rice cultivar which contributed to much needed salinity tolerance of the former in the seedling stage. Similarly, Garg and Bhandari (2016) and Tuna et al. (2008) showed that exogenous application of silicon reduced the concentration of Na^+ ions in salt stressed chickpeas and wheat seedlings, leading to higher K^+/Na^+ ratio. Silicon application has also been shown to reduce the metal toxicity either by reducing the apoplastic concentration of metals in cell walls or by apoplastic metal detoxification (Wu et al. 2019).

Any form of abiotic stress lead to direct or indirect production of ROS that causes oxidative damage in plants. However, silicon application has been proved to minimize the concentration of ROS in cells either by inhibiting its formation or by enhancing its detoxification (Kim et al. 2017). Yin et al. (2019) and Cao et al. (2015) reported that silicon application reduced the effects of oxidative damage caused due to salt and light stress in cucumber and tomato seedlings, respectively by enhancing the activity of catalase (CAT),

ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD). Similarly, Ma et al. (2016) reported that drought-induced lipid peroxidation and flavonoid and reduced glutathione content (GSH) level were reduced by the application of silicon in wheat plants. Thus, in past, a plethora of information regarding defensive mode of action of silicon against many abiotic stressors in plants have been documented.

The biological effects of silicon application during fluoride toxicity in rice seedlings have not been explored. Therefore, the main aim of this chapter was to showcase the beneficial role of silicon during fluoride stress in rice seedlings, since the efficacy of silicon in reducing the detrimental effects of other forms of abiotic stress is widely recognized. The physiological parameters, including fluoride-induced oxidative damage and defense machinery were analyzed in presence of silicon. The ameliorative role of silicon in fluoride stress was verified through determination of endogenous silicon content and the participation of enzymatic and non-enzymatic antioxidants, along with osmolytes and glyoxalase systems. Based on the earlier works, we hypothesized that silicon application could restrict the uptake of fluoride ions from the media and also regulate the formation and activity of protective metabolites and enzymatic antioxidants, so as to maintain the tolerance level of the seedlings.

4.3.3. Results and discussion

4.3.3.1. Physiological parameters

Initial standardization using three different concentrations of silicon, viz., 1, 2 and 4 mM Na_2SiO_3 showed that 2 mM Na_2SiO_3 was the most suitable for overall plant growth (Fig. 4.3.1a). Exogenous application of silicon restored the overall growth of the seedlings that was retarded in the presence of 25 and 50 mg L^{-1} NaF solution (Fig. 4.3.1b). NaF solution (25 and 50 mg L^{-1}) significantly reduced the germination of seeds by 1.4 and 1.9 fold, respectively as compared to that of control seeds, whereas the same was restored by 1.2 and 1.3 fold in silicon treated stressed seeds, respectively. Similarly, application of 25 mg L^{-1} NaF solution reduced the shoot and root length by 1.3 and 1.4 fold, respectively. Shoot and root length was further reduced by 1.6 and 1.7 fold, respectively in fluoride (50 mg L^{-1} NaF) stressed seedlings. Silicon restored the growth of shoot and root length by 1.2 and 1.3 fold, respectively in 25 mg L^{-1} NaF-treated seedlings and by 1.3 and 1.4 fold, respectively in 50 mg L^{-1} NaF-treated seedlings. Moreover, silicon application also restored the biomass of the seedlings by 1.3 fold which was reduced by 1.5 and 1.7 fold, respectively in 25 and 50 mg L^{-1} NaF-treated seedlings, respectively (Table 4.3.1). Similar to our observations in the earlier chapters, fluoride application detrimentally affected the physiological parameters of 10 day-old KH cultivar. On silicon supplementation, these parameters were restored to nearly their control value. The data can be supported by the earlier work of Haghighi et al. (2012) where they reported higher germination rate in silicon-treated salt-stressed tomato seedlings. Similarly, higher biomass and root and shoot length of the seedlings on silicon treatment in the present work are in accordance with work of Sattar et al. (2013) in wheat seedlings.

Fig. 4.3.1a

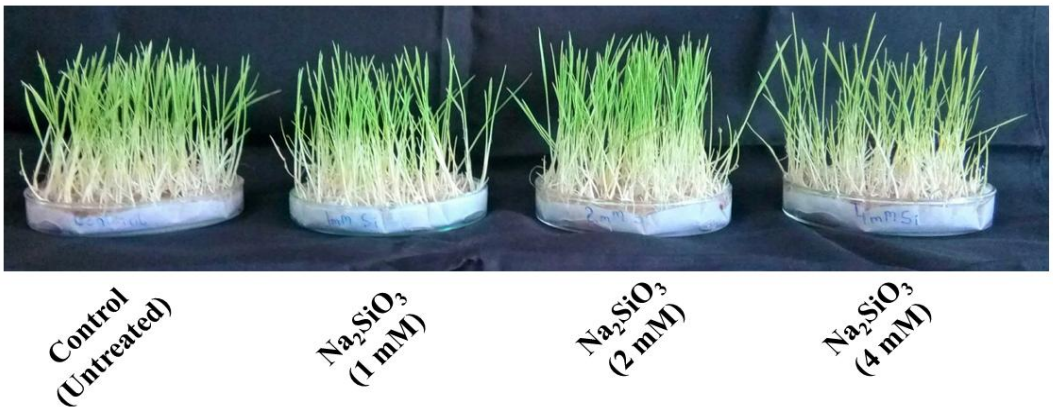


Fig. 4.3.1b

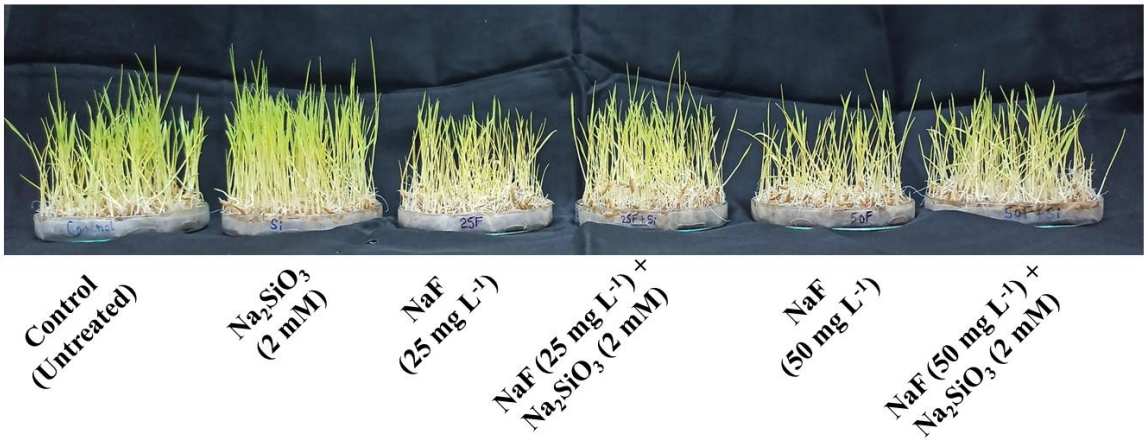


Fig. 4.3.1: Standardization of silicon concentration for optimum growth of rice seedlings **(a)** and the effect of application of 2 mM Na_2SiO_3 on growth performance of seedlings in presence or absence of 25 and 50 mg L^{-1} NaF **(b)** monitored for 10 days; untreated (non-stressed) seedlings served as experimental control.

Table 4.3.1: Percentage of seed germination, total biomass of seedlings, and shoot and root length of seedlings, grown either in presence of 25 or 50 mg L^{-1} NaF in absence or presence of 2 mM Na_2SiO_3. Data represented are the mean value (n = 3) ± SE and '*' represents data with significant differences at $P \leq 0.05$ against control set.

Parameters	Control	Na_2SiO_3 (2 mM)	NaF (25 mg L^{-1})	NaF (25 mg L^{-1}) + Na_2SiO_3 (2 mM)	NaF (50 mg L^{-1})	NaF (50 mg L^{-1}) + Na_2SiO_3 (2 mM)
Percentage germination (%)	93.62 ± 1.27	96.57 ± 2.07	69.14 ± 0.98*	82.14 ± 0.47	49.17 ± 0.57*	65.17 ± 0.84
Seedling biomass (mg)	154.00 ± 1.97	160.00 ± 2.98	105.47 ± 0.49*	132.78 ± 1.02	91.02 ± 0.43*	120.87 ± 1.49
Shoot length (cm)	9.20 ± 0.24	10.90 ± 0.45	7.32 ± 0.43*	8.95 ± 0.40	5.81 ± 0.49*	7.54 ± 0.59
Root length (cm)	4.27 ± 0.14	4.67 ± 0.27	3.12 ± 0.34*	3.98 ± 0.13	2.54 ± 0.37*	3.54 ± 0.50

4.3.3.2. Fluoride and silicon content of the seedlings

The fluoride content of the seedlings was drastically enhanced by 17.9 and 36.8 fold in 25 and 50 mg L^{-1} NaF treated seedlings, respectively, as compared to that of control seedlings. Application of 2 mM Na_2SiO_3 enhanced the content of silicon in seedlings by 12.1 times. Higher accumulation of silicon inhibited the uptake of fluoride ions in seedlings as noted from reduced endogenous content of fluoride ions by 1.9 and 2.9 folds in 25 and 50 mg L^{-1} NaF treated seedlings, respectively, as compared to that of their respective stressed seedlings grown in absence of silicon (Fig 4.3.2). Based on these observations, it appeared that silicon application inhibited the uptake of fluoride ions via roots which could be justified by the previous work of Emamverdian et al. (2018) where they showed that silicon deposition in roots acts as a physical barrier that inhibit the absorption and translocation of heavy metals from the soil. Earlier, Adrees et al. (2015) also reported that silicon stimulates the production of root exudates that can chelate metals in soil, thereby reducing their uptake via roots. Such results also coincide with our observation of lower fluoride deposition in seedlings on application of silicon.

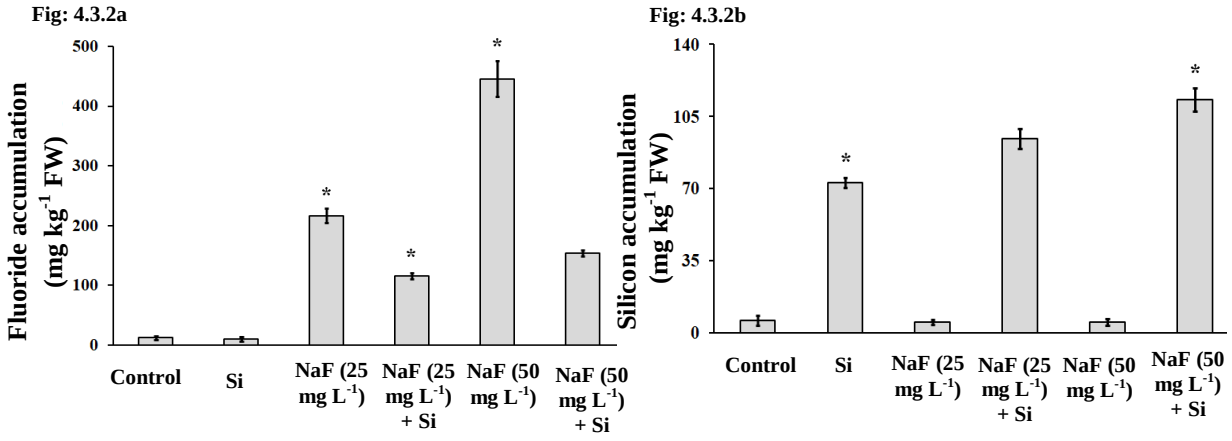


Fig. 4.3.2: Accumulation of endogenous fluoride (**a**) and silicon (**b**) in rice seedlings grown in presence of 25 or 50 mg L^{-1} NaF, either in absence or presence of 2 mM Na_2SiO_3. Data represented are the mean value (n = 3) ± SE and '*' represent data with significant differences at $P \leq 0.05$ against control set.

4.3.3.3. Extent of oxidative damage in seedlings

On application of NaF solution, the level of fluoride ions in the tissues was enhanced that up regulated the formation of H_2O_2 by 1.4 and 1.8 fold in 25 and 50 mg L^{-1} NaF applied seedlings, respectively. Fluoride-induced oxidative stress also hampered the integrity of cell membrane, enhancing the electrolyte leakage from the cells by 2.4 and 3.2 fold, respectively, along with the formation of cytotoxic metabolites such as MDA by 1.8 fold and 2.6 fold in 25 and 50 mg L^{-1} NaF applied seedlings, respectively. Additionally, higher oxidative stress also induced the activity of LOX and protease by 1.7 and 2.0 fold in 25 mg L^{-1} NaF stressed seedlings, respectively and by 2.2 and 2.4 fold in 50 mg L^{-1} NaF stressed seedlings, respectively. Higher protease activity led to 3.4 fold and 4.3 fold higher protein carbonylation in 25 and 50 mg L^{-1} NaF stressed seedlings, respectively. Similarly, higher accumulation of fluoride ions also reduced the chlorophyll content by 1.8 and 2.3 fold, respectively in the stressed seedlings (Table 4.3.2). Gill and Tuteja (2010) reported that fluoride accumulation imposes oxidative stress on the cells and triggered the formation of ROS that led to lipid peroxidation along with higher formation of cytotoxic metabolites like MDA that coincides with our observation. Additionally, Moller et al. (2007) reported that protein carbonylation is an important consequence of stress mediated oxidative damage to cellular proteins and is regarded as an important marker of oxidative stress. Protein carbonylation inhibits the normal functions of proteins and enhances their proteolytic cleavage by distorting their stable structure.

Exogenous application of silicon inhibited the uptake of fluoride ions by the roots that almost restored the normal physiological growth of the seedlings and alleviated the extent of oxidative damage in the seedlings. In presence of silicon, the level of H_2O_2 was reduced by 1.4 and 1.5 fold in 25 and 50 mg L^{-1} NaF stressed seedlings, respectively, as compared to that of their respective stressed counterparts. Lower accumulation of ROS also restored the integrity of cell membrane that ultimately led to 1.6 fold lower electrolyte leakage in case of both 25 and 50 mg L^{-1} NaF-stressed seedlings. Similarly, the level of MDA and protein carbonylation in the seedlings was lowered by 1.6 and 1.5 fold in 25 mg L^{-1} NaF stressed seedlings, respectively and by 1.8 and 1.6 fold in 50 mg L^{-1} NaF stressed seedlings, respectively, in presence of silicon. Fluoride-mediated enhanced activity of LOX and protease was also down regulated by 1.4 and 1.7 fold, respectively, in 25 mg L^{-1} NaF-stressed seedlings and by 1.5 and 1.5 fold, respectively, in 50 mg L^{-1} NaF-stressed seedlings

in presence of silicon. Similarly, silicon application also stabilized chlorophyll synthesis which was enhanced by 1.3 and 1.4 fold in 25 and 50 mg L^{-1} NaF stressed seedlings, respectively (Table 4.3.2). Based on such data, it could be concluded that silicon accumulation in seedlings acted as a physical barrier that inhibited the uptake of fluoride ions from the surrounding media. Furthermore, lesser accumulation of fluoride ions also lowered the formation of ROS, minimized the extent of lipid peroxidation that was reflected in lower MDA level, and down regulated LOX and protease activity, leading to lower protein carbonylation and chlorophyll loss in the seedlings. Similar results were also reported by Khan et al. (2019) where they showed that exogenous application of silicon inhibited the absorption of Na^+ ions via roots during pH stress in tomato seedlings, restoring the normal growth of the seedlings. Srivastava et al. (2014) also observed lowered protein carbonylation in cadmium-stressed rice seedlings during exogenous application of silicon that appeared to be advantageous for the rice plants. Thus, based on these observations, it could be stated that silicon application reduced the effects of fluoride toxicity, either by reducing the concentration of fluoride in cells wall or by its efficient detoxification by depositing fluoride ions in metabolically inactive tissues.

Table 4.3.2: Chlorophyll content, electrolyte leakage, MDA, H_2O_2, protein carbonylation, LOX activity and protease activity of rice seedlings grown in presence of either 25 or 50 mg L^{-1} NaF in absence or presence of 2 mM Na_2SiO_3. Data represented here are the mean value (n = 3) ± SE and '*' represents data with significant differences at $P \leq 0.05$ against control set.

Parameters	Control	Na_2SiO_3 (2 mM)	NaF (25 mg L^{-1})	NaF (25 mg L^{-1}) + Na_2SiO_3 (2 mM)	NaF (50 mg L^{-1})	NaF (50 mg L^{-1}) + Na_2SiO_3 (2 mM)
Chlorophyll (µg g^{-1} FW)	95.47 ± 4.35	100.79 ± 4.13	53.70 ± 1.69*	70.74 ± 3.77	41.63 ± 1.13*	60.16 ± 3.08*
Electrolyte leakage (%)	13.71 ± 1.61	12.40 ± 1.60	33.64 ± 0.91*	21.54 ± 1.69*	43.49 ± 0.73*	26.92 ± 1.29
MDA (µM g^{-1} FW)	1.48 ± 0.14	1.25 ± 0.15	2.68 ± 0.20*	1.62 ± 0.21	3.89 ± 0.13*	2.21 ± 0.16*
H_2O_2 (µM g^{-1} FW	0.27 ± 0.01	0.24 ± 0.03	0.38 ± 0.02*	0.28 ± 0.01	0.44 ± 0.02	0.30 ± 0.04

Protein carbonylation (mol carbonyl mol^{-1} BSA)	0.69 ± 0.07	0.58 ± 0.06	2.37 ± 0.31*	1.51 ± 0.05	2.94 ± 0.12*	1.75 ± 0.02*
LOX activity (U mg^{-1} leaf protein)	5.96 ± 0.46	4.91 ± 0.48	10.20 ± 1.02*	7.27 ± 0.51*	13.01 ± 0.50*	8.40 ± 0.89*
Protease (µg of protein cleaved min^{-1} mg^{-1} leaf protein)	2.17 ± 0.15	2.03 ± 0.24	4.30 ± 0.20*	2.57 ± 0.21	5.28 ± 0.25*	3.41 ± 0.10*

4.3.3.4. Glyoxalase cycle in seedlings

Along with other cytotoxic metabolites, fluoride stress also enhanced the formation of methylglyoxal by 1.3 and 1.4 fold in 25 and 50 mg L^{-1} NaF-treated seedlings, respectively. The activity of glyoxalase (Gly) I and Gly II was also enhanced by 1.5 and 1.2 times, respectively, in 25 mg L^{-1} NaF-stressed seedlings and by 1.6 and 1.9 fold, respectively, in 50 mg L^{-1} NaF-stressed seedlings (Fig. 4.3.3). On exogenous application of silicon, the content of methylglyoxal in the seedlings was reduced by 1.2 and 1.3 fold, respectively, which could be attributed to 1.2 and 1.5 fold rise in the activity of Gly I and Gly II in 25 mg L^{-1} NaF-stressed seedlings, respectively and 1.7 and 1.5 fold rise in the activity of Gly I and Gly II in 50 mg L^{-1} NaF-stressed seedlings, respectively (Fig. 4.3.3). Methylglyoxal is an α-oxoaldehyde and is one of the major cytotoxic metabolites whose level was enhanced in fluoride-stressed seedlings in a concentration-dependent manner. Accumulation of methylglyoxal above threshold level can readily modify nucleic acids, proteins and phospholipids that inevitably distort their structure and hamper their activity. Exogenous silicon application lowered the formation of methylglyoxal in the seedlings which can be supported by the earlier work of Hasanuzzaman et al. (2019) where they reported the beneficial role of silicon application in methylglyoxal reduction in nickel-stressed rice seedlings. They further reported that silicon application aggravated the activity of Gly I and Gly II that helped in detoxification of methylglyoxal which is in accordance with the present observation.

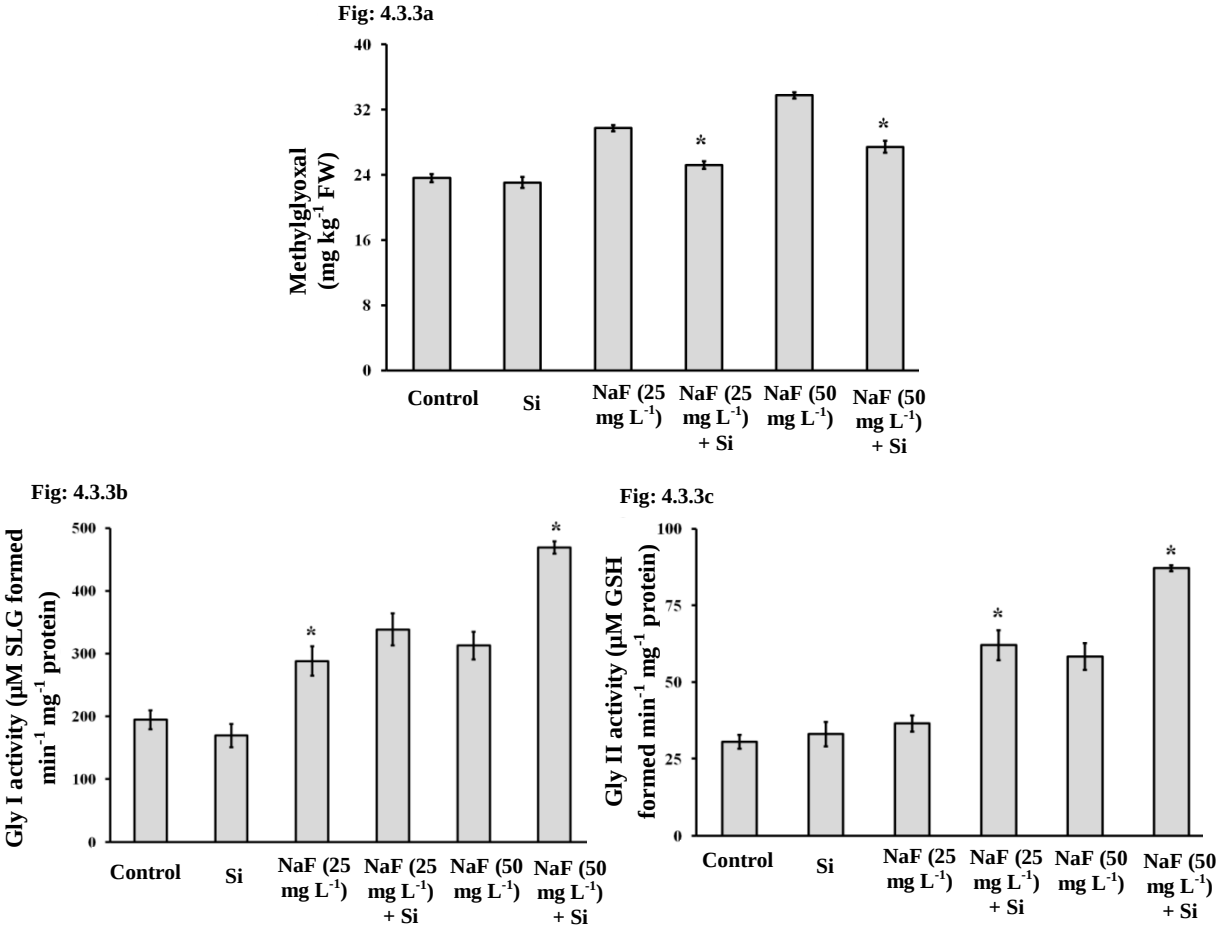


Fig. 4.3.3: Level of methylglyoxal (**a**) and activity of Gly I (**b**) and Gly II (**c**) enzymes in rice seedlings, grown in presence of 25 or 50 mg L^{-1} NaF, either in absence or presence of 2 mM Na_2SiO_3. Data represented are the mean value (n = 3) ± SE and '*' represent data with significant differences at $P \leq 0.05$ against control set.

4.3.3.5. Level of osmolytes and activity of P5CS in the seedlings

The level of the major osmolytes like proline was enhanced by 2.1 and 2.7 fold in 25 and 50 mg L^{-1} NaF-stressed seedlings, respectively which could be linked with the higher activity (1.8 and 2.5 fold, respectively) of Δ^1-pyrroline-5-carboxylate synthetase (P5CS) in the stressed seedlings. However, application of silicon reduced the extent of fluoride-induced damage in the seedlings, restoring the level of proline by 1.2 fold for both the treatments along with 1.1 and 1.4 fold lower activity of P5CS in 25 and 50 mg L^{-1} NaF-stressed seedlings, respectively. Similar pattern was also noted in case of amino acids and glycine betaine where fluoride application enhanced the level of osmolytes by 1.8 and 1.7 fold, respectively in 25 mg L^{-1} NaF stressed-seedlings and by 2.4 and 2.2 fold, respectively in 50

mg L^{-1} NaF-stressed seedlings. In presence of silicon, the concentration of amino acids and glycine betaine was lowered in seedlings, treated either with 25 mg L^{-1} NaF (1.3 and 1.2 fold, respectively) or with 50 mg L^{-1} NaF (1.2 and 1.1 fold, respectively) (Fig. 4.3.4). Osmolytes are low molecular weight compounds that scavenge ROS and protect the cellular membrane from oxidative damages (Roychoudhury et al. 2011). During fluoride stress, higher formation of osmolytes could be justified by the previous work of Banerjee and Roychoudhury (2019a). Based on the data obtained, the examined osmolytes aided in maintaining the osmotic balance, detoxified the ROS and also stabilized the cellular membrane of stressed seedlings. Application of silicon reduced the requirement of these protective metabolites in the seedlings, thus leading to their lower synthesis. Moreover, the reduced activity of P5CS in presence of silicon lies in concurrence with the lower level of proline in fluoride stressed seedlings. The results of these observations can be supported by the previous work of Gong et al. (2005) in wheat and Mauad et al. (2016) in rice where they reported lower formation of osmolytes along with P5CS activity in water stressed seedlings on exogenous application of silicon.

Fig: 4.3.4a

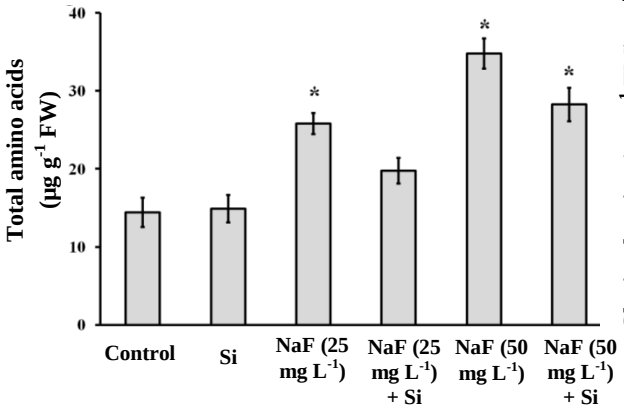


Fig: 4.3.4b

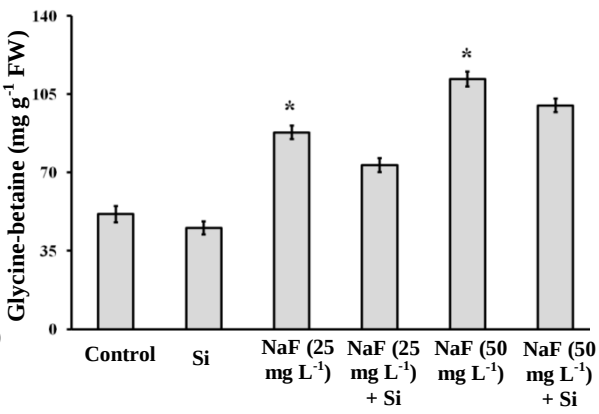


Fig: 4.3.4c

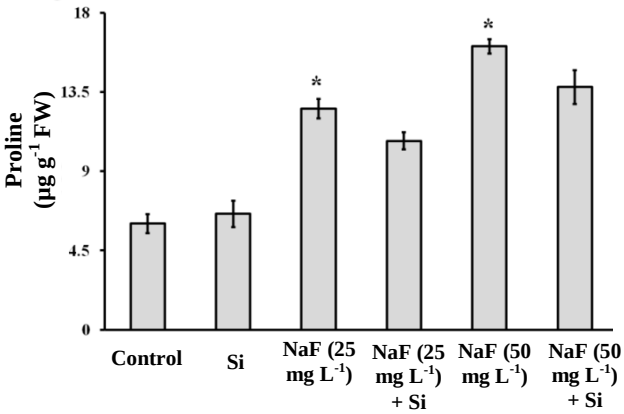


Fig: 4.3.4d

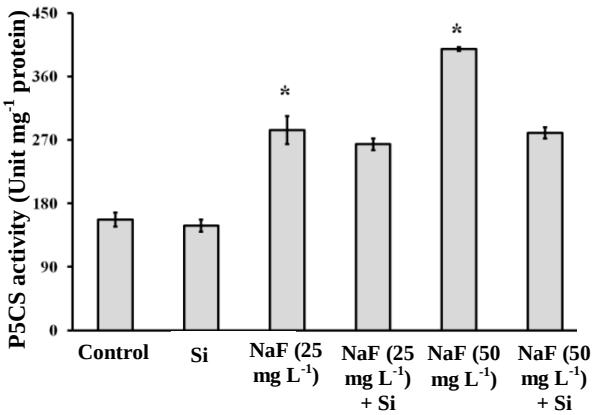


Fig. 4.3.4: Level of total amino acids (**a**), glycine-betaine (**b**), proline (**c**) and activity of P5CS (**d**) in rice seedlings grown in presence of 25 or 50 mg L^{-1} NaF, either in absence or presence of 2 mM Na_2SiO_3. Data represented are the mean value (n = 3) ± SE and '*' represent data with significant differences at $P \leq 0.05$ against control set.

4.3.3.6. Activity of enzymatic antioxidants in the seedlings

Enzymatic antioxidants work coordinately to detoxify ROS formed during abiotic stresses. Silicon application efficiently reduced the activity of SOD by 1.2 and 1.6 fold in 25 and 50 mg L^{-1} NaF-applied seedlings, respectively; the activity was enhanced by 1.3 and 1.8 fold, respectively, in the stressed seedlings. In contrast, NaF application inhibited the CAT activity by 2.2 and 3.3 fold in 25 and 50 mg L^{-1} NaF treated seedlings, respectively, whereas in presence of silicon, the activity of CAT was restored by 1.4 and 2.2 fold, respectively in the above mentioned sets. The activity of both the enzymatic antioxidants, i.e., APX and GPoX was enhanced by 1.4 fold in 25 mg L^{-1} NaF treated seedlings, whereas the same was enhanced by 1.6 and 1.8 fold, respectively in 50 mg L^{-1} NaF treated seedlings. Silicon exhibited the intrinsic potential of protecting the seedlings, as reflected in 1.2 fold reduction in the GPoX activity in both 25 and 50 mg L^{-1} NaF-treated seedlings and 1.1 fold reduction in the APX activity in both 25 and 50 mg L-1 NaF-treated seedlings. The activity of GR and GPX was up regulated by 1.8 and 1.6 fold, respectively in 25 mg L^{-1} NaF-treated seedlings and by 2.5 and 2.3 fold, respectively in 50 mg L^{-1} NaF-treated seedlings. Silicon application lowered the extent of ROS accumulation in seedlings that respectively led to 1.3 and 1.1 fold lower GR and GPX activity in 25 mg L^{-1} NaF-treated seedlings, whilst the same were reduced by 1.2 and 1.3 fold, respectively in 50 mg L^{-1} NaF-treated seedlings (Table 4.3.3).

In fluoride-stressed seedlings, the seedlings managed to detoxify the superoxide radicals generated in the cells into less toxic H_2O_2 molecule via up regulated activity of SOD. Moreover, the H_2O_2 formed in the seedlings was further detoxified by the activity of enzymatic antioxidants, viz., APX, GPoX, GR and GPX. However, fluoride stress inhibited the activity of CAT which could be supported by the earlier work of Kumar et al. (2009) where they reported that accumulation of fluoride ions above threshold level could inhibit the activity of CAT by replacing the hydroxyl group present in the active site of the enzyme. Enhanced activity of SOD, APX and GPoX as observed here is also in concurrence with the previous work of Banerjee and Roychoudhury (2019a). Thus, increment in the activity of the

antioxidative enzymes clearly indicates their active participation in the detoxification ROS. In presence of silicon, the activity of all the enzymatic antioxidants (except CAT whose activity was restored in seedlings) was lowered which proved the efficacy of silicon in minimizing ROS accumulation due to lesser deposition of fluoride ions in the tissues. Moreover, the current observations coincide with the earlier work of Maksimovic et al. (2012) where lower GPoX activity was noted in cucumber seedlings on exogenous application of silicon during Mg stress. Similar observation was also reported by Gunes et al. (2008) who observed lower SOD and APX activity and higher CAT activity in drought-stressed sunflower seedlings in presence of silicon.

Table 4.3.3: Activity of major enzymatic antioxidants like CAT, GPOX, APX, SOD, GPX and GR in rice seedlings, grown in presence of either 25 or 50 mg L^{-1} NaF in absence or presence of 2 mM Na_2SiO_3. Data represented are the mean value (n = 3) ± SE and '*' represents data with significant differences at $P \leq 0.05$ against control set.

Parameters	Control	Na_2SiO_3 (2 mM)	NaF (25 mg L^{-1})	NaF (25 mg L^{-1}) + Na_2SiO_3 (2 mM)	NaF (50 mg L^{-1})	NaF (50 mg L^{-1}) + Na_2SiO_3 (2 mM)
CAT activity (µM H_2O_2 used min^{-1} mg^{-1} protein)	14.06 ± 1.29	12.30 ± 1.23	6.40 ± 0.05*	8.96 ± 0.93*	4.27 ± 0.64*	9.22 ± 0.29
SOD activity (Unit mg^{-1} protein)	2.37 ± 0.13	2.48 ± 0.19	3.13 ± 0.01	2.55 ± 0.21	4.38 ± 0.22*	2.67 ± 0.21
GPOX activity (µM tetraguaiacol formed min^{-1} mg^{-1} protein)	18.07 ± 1.15	16.86 ± 1.99	25.53 ± 0.69*	22.04 ± 0.85	31.95 ± 1.89*	25.96 ± 2.52
APX activity (µM ascorbate oxidised min^{-1} mg^{-1} protein)	160.50 ± 2.55	160.14 ± 14.43	224.21 ± 8.35*	209.87 ± 4.97	254.84 ± 7.42	224.11 ± 2.30
GPX activity (µM of NADPH used min^{-1} mg^{-1} protein)	47.97 ± 3.44	54.56 ± 0.92	74.89 ± 1.51*	65.98 ± 0.42	108.64 ± 5.20*	83.36 ± 0.15

GR activity (µM of NADPH used min^{-1} mg^{-1} protein)	23.33 ± 2.78	23.81 ± 3.83	42.43 ± 4.02*	33.64 ± 3.01	57.33 ± 4.31*	48.83 ± 2.33*

4.3.3.7. Level of non-enzymatic antioxidants and PAL activity in the seedlings

Fluoride application enhanced the level of anthocyanins by 1.8 and 2.4 fold in 25 and 50 mg L^{-1} NaF-treated seedlings, whereas the application of silicon reduced the same by 1.2 and 1.3 fold, respectively in the above mentioned stressed sets. Similarly, the level of both ascorbate and GSH was elevated by 1.5 fold in seedlings treated with 25 mg L^{-1} NaF solution and by 1.8 and 2.0 fold, respectively in seedlings treated with 50 mg L^{-1} NaF solution, whereas application of silicon lowered the content of both the metabolites by 1.2 and 1.1 fold, respectively in 25 mg L^{-1} NaF-treated seedlings and by 1.2 and 1.6 fold in 50 mg L^{-1} NaF-treated seedlings. The level of xanthophylls was enhanced by 1.6 and 1.8 fold, respectively, in 25 and 50 mg L^{-1} NaF-treated seedlings, whilst silicon application lowered the same by 1.3 and 1.2 fold, respectively in the above mentioned stressed seedlings. In contrast, the level of carotenoids and phenolics was reduced by 1.4 and 1.6 times, respectively in 25 mg L^{-1} NaF-stressed seedlings and by 1.6 and 1.9 fold, respectively, in 50 mg L^{-1} NaF-stressed seedlings. Exogenous application of silicon restored the content of carotenoids and phenolics by 1.1 and 1.5 fold, respectively in 25 mg L^{-1} NaF-treated seedlings and by 1.3 and 1.5 fold, respectively in 50 mg L^{-1} NaF-treated seedlings. The level of flavonoids was up regulated by 1.4 and 1.6 fold, respectively in 25 and 50 mg L^{-1} NaF-treated seedlings which can be linked with the higher activity of PAL (1.5 and 1.6 fold, respectively). On application of silicon, the extent of flavonoid accumulation in seedlings was reduced by 1.1 and 1.2 fold, respectively in stressed seedlings which might be due to reduced activity of PAL by 1.4 and 1.1 fold, respectively in 25 and 50 mg L^{-1} NaF-treated seedlings (Table 4.3.4).

The level of the major non-enzymatic antioxidants, viz., anthocyanins, ascorbate, GSH, xanthophylls and flavonoids was induced in fluoride-stressed seedlings which proved the efficacy of these antioxidants as important redox homeostasis managers. Moreover, the present observation lies in congruence with the earlier work of Banerjee et al. (2019a). Roychoudhury and Basu (2012) also observed that ascorbate and GSH are the key metabolites for ascorbate-glutathione cycle under harsh conditions to detoxify the toxic free

radicals. Reduced level of phenolics and carotenoids could be justified by the fact that seedlings were actively utilizing these metabolites to overcome the damaging effects of

fluoride-induced oxidative damages. Moreover, the enhanced level of flavonoids can be attributed to the higher activity of PAL, the key rate-limiting enzyme of phenylpropanoid pathway. In presence of silicon, the level of the above mentioned protective metabolites (except carotenoids and phenolics) along with the activity of PAL was reduced that again showcase the efficacy of silicon in abrogating the negative symptoms of fluoride toxicity. Earlier studies conducted by Ma et al. (2016) and Metwally et al. (2018) also reported that exogenous application of silicon reduced the level of ROS in drought-stressed wheat plants and boron-stressed canola plants, respectively that led to the lower formation of ascorbate, GSH and flavonoids in the stressed seedlings. The trend was bit different in case of phenolics and carotenoids where silicon application enhanced the content of both the metabolites in the stressed seedlings in the present study. This data can be supported by the earlier work of Pontigo et al. (2017) and Al-Huqail et al. (2019), respectively.

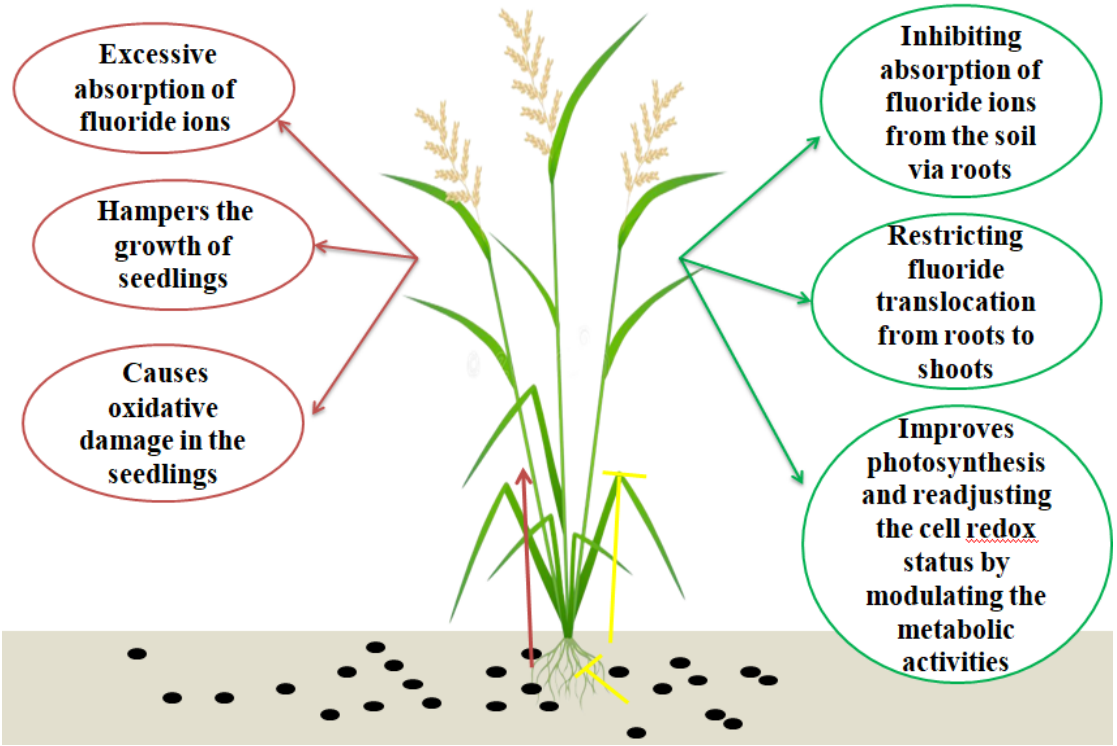
Table 4.4.4: Level of major non-enzymatic antioxidants like anthocyanins, total phenolics, carotenoids, xanthophylls, flavonoids, ascorbate and GSH and activity of PAL in rice seedlings, grown in presence of either 25 or 50 mg L^{-1} NaF, in absence or presence of 2 mM Na_2SiO_3. Data represented here are the mean value (n = 3) ± SE and '*' represents data with significant differences at $P \leq 0.05$ against control set.

Parameters	Control	Na_2SiO_3 (2 mM)	NaF (25 mg L^{-1})	NaF (25 mg L^{-1}) + Na_2SiO_3 (2 mM)	NaF (50 mg L^{-1})	NaF (50 mg L^{-1}) + Na_2SiO_3 (2 mM)
Anthocyanins (µM g^{-1} FW)	24.31 ± 1.56	26.89 ± 2.08	43.35 ± 1.49*	35.33 ± 2.08	58.43 ± 1.93*	43.35 ± 1.49
Total phenolics (µg g^{-1} FW)	91.72 ± 3.35	95.97 ± 4.06	56.90 ± 4.37*	83.81 ± 3.15	49.49 ± 1.42*	72.58 ± 3.15*
Carotenoids (µM g^{-1} FW)	16.04 ± 0.61	15.77 ± 0.30	11.75 ± 0.37*	13.42 ± 0.37	9.96 ± 0.49*	13.20 ± 0.62
Xanthophylls (µM g^{-1} FW)	4.64 ± 0.16	4.46 ± 0.11	7.26 ± 0.11*	5.42 ± 0.26	8.22 ± 0.30*	6.64 ± 0.20

Flavonoids (µg g^{-1} FW)	113.20 ± 3.44	116.53 ± 4.53	161.79 ± 3.62*	144.61 ± 8.34*	183.46 ± 4.89*	150.64 ± 3.44
Ascorbate (µg g^{-1} FW)	12.06 ± 0.54	12.61 ± 0.93	17.65 ± 0.67*	14.27 ± 0.83	21.20 ± 0.99*	17.11 ± 0.93
GSH (mM g^{-1} FW)	29.88 ± 1.16	32.58 ± 1.66	46.17 ± 1.41*	40.52 ± 1.08*	60.23 ± 2.49 *	37.05 ± 0.99
PAL activity (µM trans-cinnamic acid formed min^{-1} mg^{-1} protein)	2.00 ± 0.17	1.86 ± 0.11	3.07 ± 0.04*	2.24 ± 0.09	3.10 ± 0.18	2.80 ± 0.15

4.3.4. Conclusion of the chapter

Overall, this chapter clearly demonstrated the efficacy of silicon in curtailing the negative effects of fluoride-induced oxidative damages. Exogenous application of silicon elevated the endogenous content of silicon in the tissues that acted as a physical barrier to check the absorption of fluoride ions via roots. Furthermore, based on previous studies, it could also be assumed that higher endogenous level of silicon detoxified the fluoride ions by depositing them into metabolically inactive tissues. Exogenous application of NaF solution led to higher deposition of fluoride ions in the tissues that restricted the growth of the seedlings along with induced formation of ROS (H_2O_2) leading to higher peroxidation of lipid membrane (reflected by higher electrolyte leakage) and formation of cytotoxic species such as MDA and methylglyoxal. Higher activity of protease and LOX was also noted in the stressed seedlings, leading to higher protein carbonylation in a concentration dependent manner. As a result of exogenous application of silicon, there was enhancement in the endogenous silicon level that efficiently checked the formation of ROS, thereby mitigating fluoride toxicity, together with stabilizing the chlorophyll molecule. Moreover, lower formation of ROS in presence of silicon also reduced the burden on protective metabolites such as osmolytes (proline, amino acids and glycine betaine) and non-enzymatic antioxidants (anthocyanins, xanthophylls, ascorbate, GSH and flavonoids) that eventually lowered their accumulation in the seedlings. Lower level of proline and flavonoids in presence of silicon could be further linked with the down regulated activity of P5CS and PAL, respectively in the stressed seedlings. Similarly, the activity of the enzymatic antioxidants such as SOD, APX, GPoX, GR and GPX was also down regulated in silicon-treated, stressed seedlings that further highlighted the efficacy of silicon in restraining the formation of ROS in stressed seedlings and thus reducing the burden of the enzymatic antioxidants. Thus, based on these observations, deployments of silicon rich fertilizer in fluoride contaminated soil is recommended for the farmers that will improve the growth of rice plants by refurbishing the cellular homeostasis (Fig. 4.3.5).”



"Fig. 4.3.5: Exogenous application of silicon elevated the endogenous content of silicon in the tissues that acts as a physical barrier to check the absorption of fluoride ions via roots and also maintains growth and development of seedlings."

Chapter 4.4

Fluoride tolerance is independent or is negatively regulated by ABA level in rice seedlings

Data Published in this chapter has already been published in:
Protoplasma (2022) 259:1331-1350,
doi:10.1007/s00709-022-01740-7

"4.4.1. Overview of the chapter

Plants perceive and respond adaptively to abiotic stress mainly using phytohormones. ABA is one such phytohormone whose role has been widely reported in plants surviving under challenging environment. However, the role of ABA during fluoride stress still remains unexplored in rice. This chapter focuses upon investigating whether ABA promotes fluoride tolerance in rice seedlings, similar to triggering salt tolerance in rice. Suppressed ABA biosynthesis and down regulated expression of ABA inducible genes, viz., *TRAB1*, *Osem* and *Rab16A* triggered NaCl induced ROS formation such as H_2O_2 that led to oxidative damage (electrolyte leakage, formation of MDA and methylglyoxal and chlorophyll degradation) and growth inhibition in Matla seedlings. Reduced ABA accumulation led to higher formation of gibberellic acid and melatonin in 50 mg L^{-1} NaF-stressed Matla and Nonabokra that promoted fluoride tolerance of the seedlings. Higher content of ABA in fluoride-stressed Jarava seedlings up regulated the expression of chloride channels that caused higher deposition of fluoride ions in tissues. Thus, accumulation of fluoride ions led to severe oxidative damage in spite of higher production of ABA-associated osmolytes like polyamines, proline and glycine betaine in Jarava seedlings. Increased accumulation of compatible solutes in presence of high endogenous ABA promoted salt tolerance in Jarava; the same was insufficient to ameliorate fluoride-induced injuries in this cultivar. Treatment with Na_2WO_4 (ABA biosynthetic inhibitor) reversed the effects of fluoride damage in Jarava seedlings, whilst further application of ABA resulted in reversion back to fluoride susceptible seedlings. Thus based on these observations, we hypothesized that unlike salt stress, fluoride tolerance is negatively regulated by ABA, but promoted by ABA antagonist phytohormones like melatonin and gibberellic acid.

4.4.2. Introduction of the chapter

Abscisic acid (ABA) is a well know phytohormone and its role in curtailing the damaging effects of abiotic stress is well established along with its pivotal role in controlling growth and development of the plants under normal conditions. It also plays a major influential role in various physiological processes in plants such as stomatal opening, seed dormancy and development, protein and lipid synthesis, flowering, grain filling and plant-microbe interaction (Govind et al. 2011; Sreenivasulu et al. 2010). β-carotenoids serve as the precursor of ABA in plants. Initially, β-carotenoids are converted into 9′-cis-neoxanthin and 9′-cis-violaxanthin which is again converted into ABA by the enzymatic action of 9-cis-epoxycarotenoid dioxygenase (NCED; the rate limiting enzyme of ABA biosynthetic pathway). Another major enzyme that regulates the level of ABA in cells is ABA-8-oxidase 1 (ABA8ox1) which degrades ABA into phaseic acid (Roychoudhury and Banerjee 2017).

On being exposed to abiotic stress, plants efficiently up regulates the endogenous ABA pool either by enhancing its formation or by reducing its degradation that in turn ensures tolerance against salinity and drought stress (Chen et al. 2020). Basu and Roychoudhury (2014) reported that ABA-dependent signaling pathway led to the up regulation of osmotic stress responsive genes, viz., *TRAB1*, *dehydration-responsive element binding 2A* (*DREB2A*), *Oryza sativa embryonic abundant* (*Osem*) and *Responsive to ABA 16A* (*Rab16A*) that countered the effects of salinity-induced oxidative stress in rice plants. Yu et al. (2016) demonstrated that *Osem* acts as a positive regulator of drought tolerance and its over-expression in rice plants resulted in the up regulated expression of *late embryogenesis abundant* (*LEA*) genes like *Rab16A*, *Rab16C* and other related genes, involved in maintaining systemic integrity against unfavorable environmental conditions. Similar to that of ABA-dependent genes, ABA-independent genes such as *DREB2A* and *DREB2B* also contributes in maintaining the tolerance level of plants against dehydration stress as earlier reported by Roychoudhury et al. (2013).

ABA also maintains the level of protective metabolites such as polyamines (putrescine, spermidine and spermine), glycine betaine and proline along with other phytohormones such as melatonin and gibberellic acid. Polyamines are low molecular weight compounds carrying two or more amino groups. Arginine and ornithine are converted into putrescine by the catalytic action of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC),

respectively. Putrescine is again converted into spermidine and spermine by the sequential addition of aminopropyl group to the putrescine backbone by the action of spermidine synthase (SPDS) and spermine synthase (SPMS). Polyamine oxidase (PAO) and diamine oxidase (DAO) catalyze the degradation of polyamines in plants. Pal et al. (2018) demonstrated that over-expression of *ADC, SAMDC* or *SPMS* up regulated the expression of *NCED* that triggered the formation of ABA in plants. Additionally, the protective roles of proline and glycine betaine against fluoride stress have been highlighted in the previous chapter. Planchet et al. (2014) and Zhang et al. (2012) reported a positive feedback regulation between ABA and proline and glycine betaine, respectively. ABA application induces the formation of proline in water deficient *Medicago truncatula* which might be due to its lower degradation or its higher formation (Planchet et al. 2014). Along with ABA, melatonin and gibberellic acid are the two major phytohormones that act antagonistically to ABA. Melatonin reduced the level of ABA in drought-stressed apple seedlings and salt stressed cucumber seedlings by up and down regulating the expression of ABA catabolic and anabolic genes, respectively (Li et al. 2015; Zhang et al. 2014). Gomez-Cadenas et al. (2001) also demonstrated that gibberellic acid acts antagonistically to ABA in order to regulate internodal growth and germination in plants.

Tungsten (W) is a rare transition element that has high resemblance with molybdenum (Mo). Due to its high physico-chemical similarity with Mo, W is largely used as an inhibitor of enzymes containing Mo as a cofactors. Adamakis et al. (2012) reported that Mo is present as a cofactor in the active site of abscisic aldehyde oxidase (AAO) that catalyzes the formation of ABA from abscisic aldehyde. Jiang et al. (2004) reported that exogenous application of Mo upregulated the expression of *AAO* that eventually triggered the formation of ABA, whilst application of W above threshold level lowered ABA content in barley seedlings.

Although the protective role of ABA against various abiotic stresses like drought and salinity in several crops is well documented (Roychoudhury et al. 2013; Shevyakova et al. 2013; Zhang et al. 2012), the precise function and importance of ABA in fluoride tolerance in plants still remain to be extensively investigated. In this study, three rice genotypes, viz., Nonabokra (NB), Matla (MT), and Jarava (JV) were selected. These varieties are well characterized for their salt-tolerant trait and also high endogenous ABA level. The role of ABA in rice cultivars under salt stress is extensively studied earlier. All observations on ABA response during fluoride stress were compared against salt stress as reference. The parameters

related to physiological damages such as chlorophyll loss, electrolyte leakage, H_2O_2, MDA, and MG formation in the tissues were analyzed, along with monitoring the ABA level and endogenous Na^+ and fluoride ion accumulation within the tissues. The level of ABA-regulated metabolites like polyamines, glycine betaine and proline and that of ABA-antagonistic growth regulators like melatonin and gibberellic acid along with the expression profiling of associated genes were adequately monitored. To further justify the findings, the fluoride-stressed cultivars with exogenous ABA were grown in presence or absence of W so as to demonstrate the overall physiological effects in order to arrive at a clear cut conclusion on the role of ABA in mediating fluoride stress response in rice.

4.4.3. Results and discussion

4.4.3.1. Growth and ABA content and expression of ABA metabolic genes

Under non-stressed condition, the highest ABA level was recorded in NB seedlings as compared to that of MT and JV. During salt stress, the level of ABA was enhanced by 1.1 and 1.2 times in NB and JV seedlings, respectively, whereas nominal fall in the level of ABA was noted in salt-stressed MT seedlings (Fig. 4.4.1a). Rise in ABA level in NB and JV seedlings supported their growth during salt stress, whilst fall in ABA content led to higher salt-induced oxidative damage in MT seedlings (Fig. 4.4.2). Moreover, higher ABA content in NB and JV seedlings can be supported by the up and down regulated expression of *NCED3* and *ABA8ox1*, respectively in both the cultivars. In case of MT seedlings, no change in expression of *NCED3* and reduced expression of *ABA8ox1* indicated toward efficient utilization of ABA in stress amelioration (Fig 4.4.1b, c). Higher tolerance level of NB and JV could be linked with their ABA content that can be supported by the earlier work of Paul and Roychoudhury (2019) where they showed that up and down regulated expression of *NCED3* and *ABA8ox1*, respectively are required for maintaining higher ABA content which in turn regulates the tolerance capability of salt-stressed rice seedlings. In contrast to NB and JV, the level of ABA was reduced in MT seedlings that somewhat led to oxidative damage in the seedlings.

As compared to that of salt stress, higher ABA level (1.5 times) failed to maintain the growth of JV seedlings during fluoride stress, whereas luxuriant growth was noted in case of NB and MT seedlings, in spite of lower ABA content that was reduced by 1.3 and 1.1 fold in seedlings, respectively, as compared to that of control seedlings (Fig 4.4.1a). According to Reyes and Chua (2007), drought stress induced the formation of ABA in *Arabidopsis* seedlings that led to higher formation of miRNA159 in ABA insensitive 3 (ABI3) dependent manner. Transcription factors, viz., MYB101 and MYB33 are the prime targets of miRNA159 and are positive regulators of ABA responses. Thus, higher ABA formation cleaves these transcription factors that arrest the growth of seedlings. Higher level of ABA in fluoride stressed JV seedlings could be justified by the up regulated expression of *NCED3*, whilst higher expression of *ABA8ox1* reduced the level of ABA in fluoride-stressed NB and MT seedlings (Fig 4.4.1 b, c). Based on such observations, it could be inferred that higher expression of *NCED3* and *ABA8ox1* led to higher ABA formation followed by its subsequent

breakdown into phaseic acid that regulated the fluoride tolerance capability of NB seedlings. In contrast, the expression of *NCED3* was comparable to that of NB in JV seedlings; however, the expression of *ABA8ox1* was significantly lower in JV. This led to higher accumulation of ABA in JV seedlings that curbed the tolerance level of seedlings. Thus, higher ABA level failed to confer tolerance against fluoride stress as it does against salt stress.

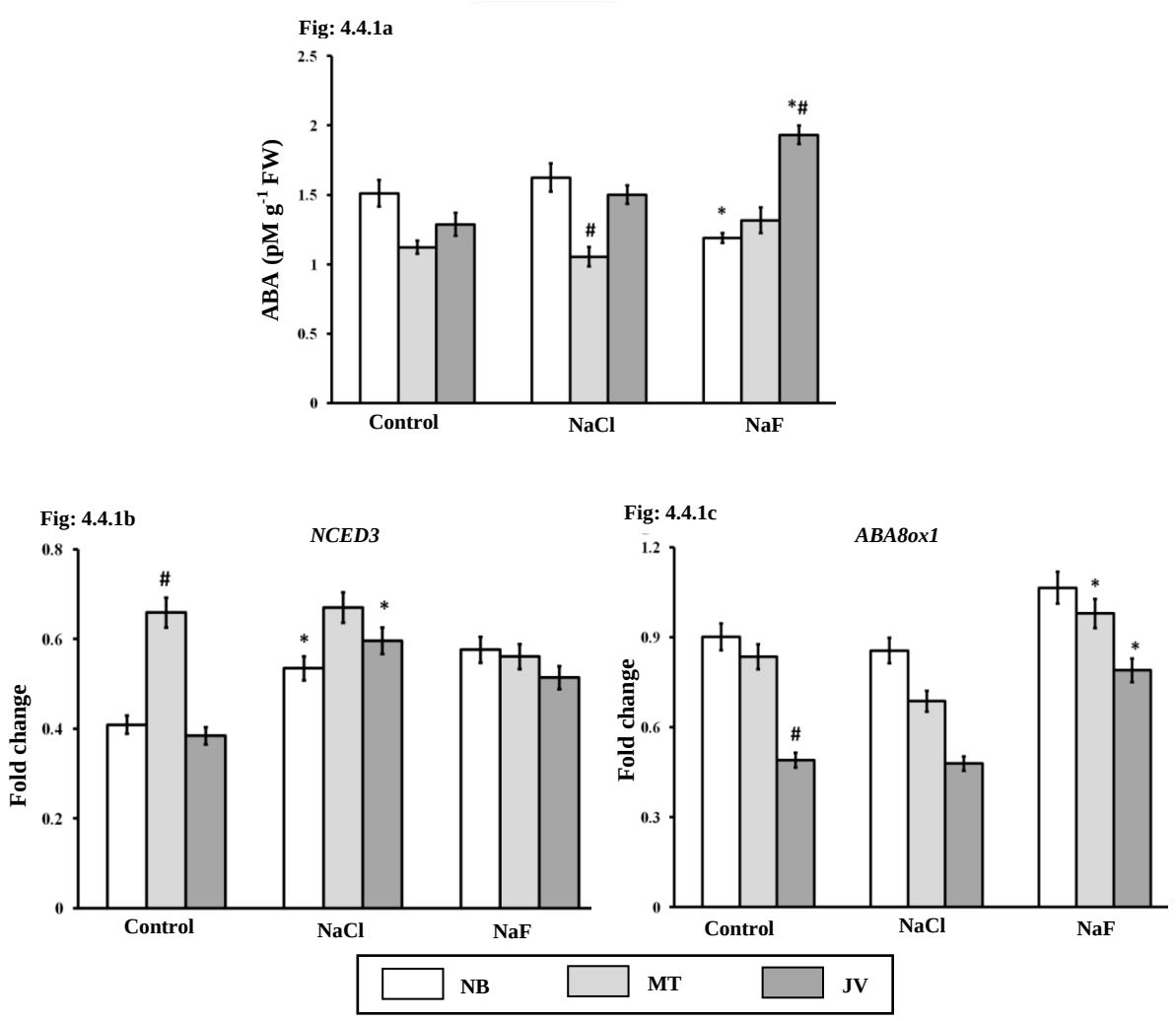


Fig. 4.4.1: ABA level (**a**), and expression of *NCED3* (**b**) and *ABA8ox1* (**c**) in NB, MT and JV cultivars in presence of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Data represented are the mean value (n = 3) ± standard error (SE); '*' and '#' represent data with significant differences between treatment and cultivars, respectively, at $P \leq 0.05$ against control set.

Fig. 4.4.2

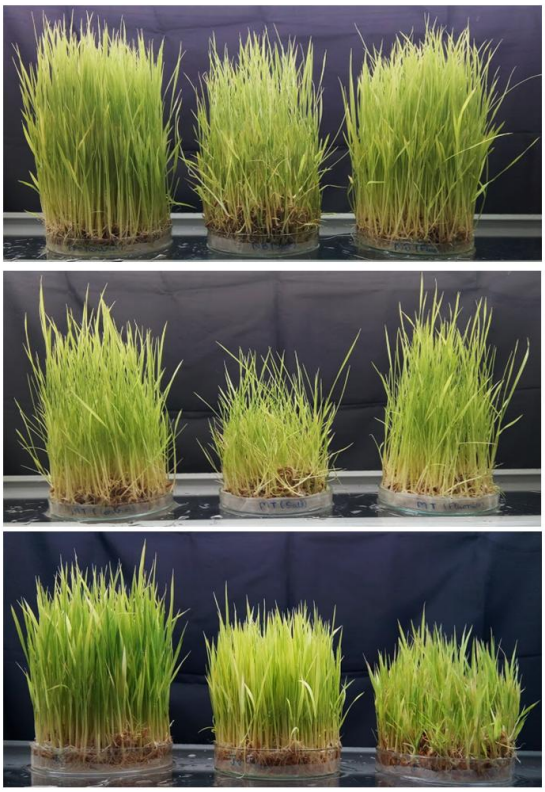


Fig. 4.4.2: Effect of exogenous application of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF solution for 20 days on overall growth performance of NB, MT and JV seedlings. Untreated (maintained with water only) seedlings served as experimental control.

4.4.3.2. Expression pattern of ABA dependent and ABA independent genes

In salt stressed NB and JV seedlings, the expression of ABA dependent genes such as *TRAB1*, *Osem* and *Rab16A* was upregulated that could be linked with higher ABA content of the seedlings. This rise in the expression of stress responsive genes promoted the growth of the seedlings. On the contrary, in MT seedlings, the expression of the above mentioned genes was reduced or remained comparable to that of control seedlings that hampered the growth of salt stressed seedlings (Fig 4.4.3a-c). Lower expression of ABA dependent genes in MT seedlings could be linked with lower ABA level in seedlings. Paul and Roychoudhury (2019) demonstrated that salt stress (250 mM NaCl) induced the level of ABA that up regulated the expression of the above mentioned genes in the tolerant rice cultivar, whereas no significant change was noted in case of the sensitive variety that coincided with our observation. Interestingly, the expression of *DREB2A* (ABA independent gene) was upregulated in NB

and MT and down regulated in JV seedlings (Fig 4.4.3d). Thus, higher ABA level in NB and JV regulated the expression of ABA dependent genes that positively modulated the tolerance level of the stressed seedlings unlike in MT where lower ABA level reduced the expression of ABA dependent genes that made it susceptible toward salinity.

Upon treatment with NaF solution (50 mg L^{-1}), the expression of ABA dependent (*TRAB1*, *Osem* and *Rab16A*) and independent genes (*DREB2A*) was downregulated in NB seedlings which could be linked with their lower ABA content, as compared to that of water treated seedlings. In contrast, the expression of ABA dependent and ABA independent genes was up regulated in fluoride-stressed MT seedlings which might be due to nominal rise in the level of ABA in seedlings, as compared to control seedlings. In case of fluoride stressed JV seedlings, the expression of ABA dependent genes was up regulated and ABA independent gene was down regulated that can be supported by their higher endogenous ABA level, as compared to that control seedlings (Fig 4.4.3 a-d). Thus, it can be presumed that fluoride tolerance is not governed by ABA dependent genes, since it was clearly noticeable in JV that higher expression of ABA dependent genes (due to higher endogenous ABA pool) failed to restore the normal growth of the seedlings on exogenous application of NaF solution, unlike in salt stress where higher ABA content up regulated the expression of the above mentioned stress responsive genes that abrogated the signs of fluoride-induced damages in seedlings.

Fig: 4.4.3a

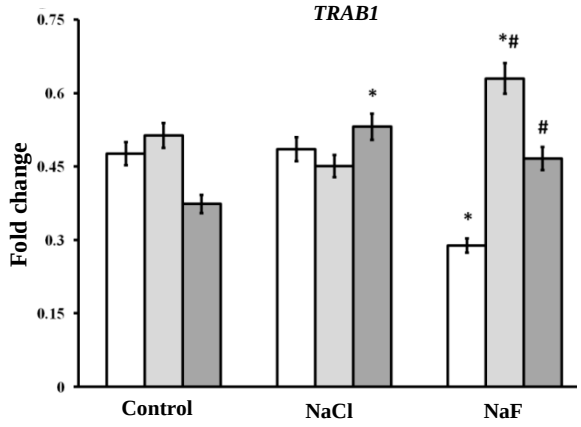
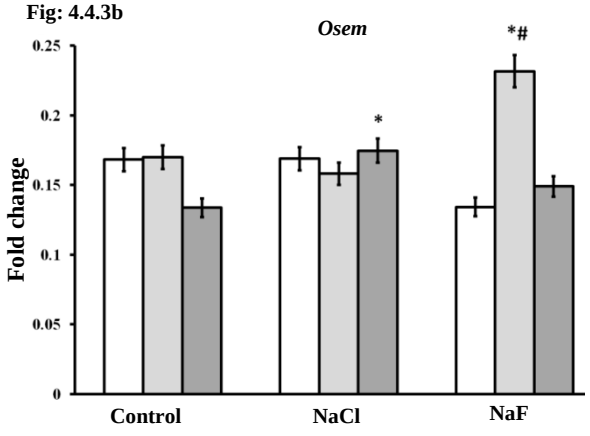


Fig: 4.4.3b



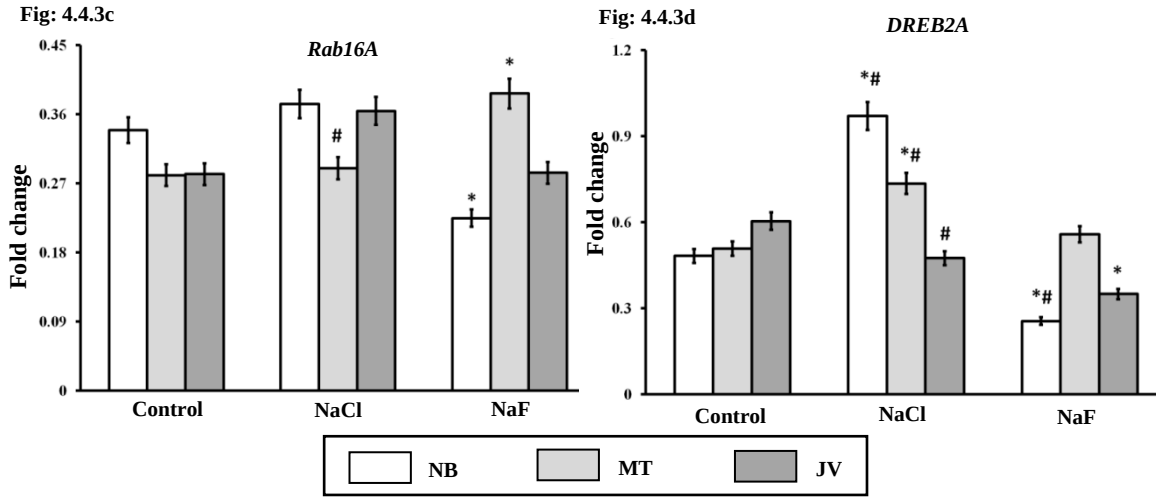


Fig. 4.4.3: Gene expression pattern of *TRAB1* (**a**), *Osem* (**b**), *Rab16A* (**c**), and *DREB2A* (**d**) in NB, MT, and JV cultivars in presence of 12 g L^{-1} or 50 mg L^{-1} NaF. Data represented are the mean value (n = 3) ± SE; '*' and '#' represent data with significant differences between treatment and cultivars, respectively at $P \leq 0.05$ against control set.

4.4.3.3. Na and fluoride deposition in seedlings

During salt stress, the highest Na^+ ion accumulation was noted in salt stressed MT seedling which was 1.2 and 1.3 fold higher, as compared to that of salt stressed NB and JV seedlings, respectively (Fig 4.4.4a). Additionally, the level of fluoride ions in all the three salt stressed cultivars was comparable to that of their respective control seedlings where no uptake of fluoride ions during salt stress was noted (Fig 4.4.4b). Our observation can be supported by the work of Saeedipour (2011) where they showed higher Na^+ ion accumulation in salt sensitive variety, as compared to that of tolerant cultivar. They further showed that on exogenous application of fluoridone (ABA inhibitor), the level of ABA was reduced in seedlings that significantly raised the endogenous accumulation of Na^+ ion accumulation in both the seedlings.

On exogenous application of NaF, the level of fluoride ions was drastically enhanced in JV seedlings (1.3 fold higher as compared to that of both fluoride stressed NB and MT seedlings) (Fig 4.4.4b). Thus, it can be evidently stated that higher ABA level induces the accumulation of fluoride ions in JV seedlings. Furthermore, no significant rise in the level of Na^+ ions was noted in fluoride stressed cultivars which suggested that damaging effects manifested on application of 50 mg L^{-1} NaF solution was due to accumulation of fluoride ions and not Na^+ ions (Fig 4.4.4b).

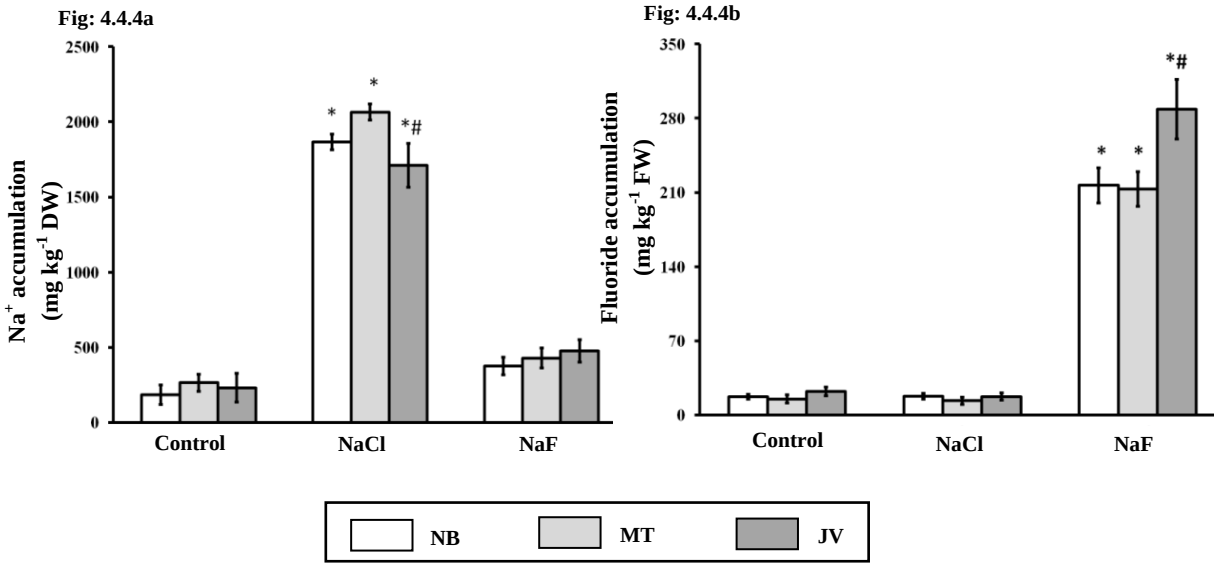


Fig. 4.4.4: Na^+ (**a**) and F^- (**b**) accumulation in NB, MT and JV cultivars in presence of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Data represented are the mean value (n = 3) ± SE; '*' and '#' represent data with significant differences between treatment and cultivars, respectively at $P \leq 0.05$ against control set.

4.4.3.4. Expression of chloride channels, viz., CLC1 and CLC2 in seedlings

Chloride channels have been reported to regulate the uptake of chloride ions via roots during salt stress in rice (Nakamura et al. 2006). In addition, Um et al. (2018) reported that higher ABA level maintains elevated or steady state of chloride channels in salt stressed rice seedlings. In fluoride stressed JV seedlings, there was no significant change in the expression of chloride channels which can be linked with higher ABA level. Constant expression of chloride channels led to unregulated uptake of fluoride ions via roots that elevated the symptoms of fluoride induced oxidative damage in JV seedlings. Such observation coincided with the earlier work of Banerjee and Roychoudhury (2019a) where they showed that chloride channels passively mediates the translocation of fluoride ions in rice seedlings due to similarity between the structures of chloride ions and fluoride ions (Fig 4.4.5). Lower content of ABA in NB seedlings down regulated the expression of both the chloride channels, thereby restricting the deposition of fluoride ions in tissue biomass. Similarly, the expression of *CLC1* was also reduced in fluoride stressed MT seedlings which might be due to reduced ABA level in the seedlings (Fig 4.4.5a). Banerjee et al. (2019a) showed that fluoride stress induces the expression of chloride channels, i.e., *CLC1* and *CLC2* in fluoride sensitive rice

cultivar (IR 64) that led to severe physiological damage to seedlings which lies in concurrence with our observation.

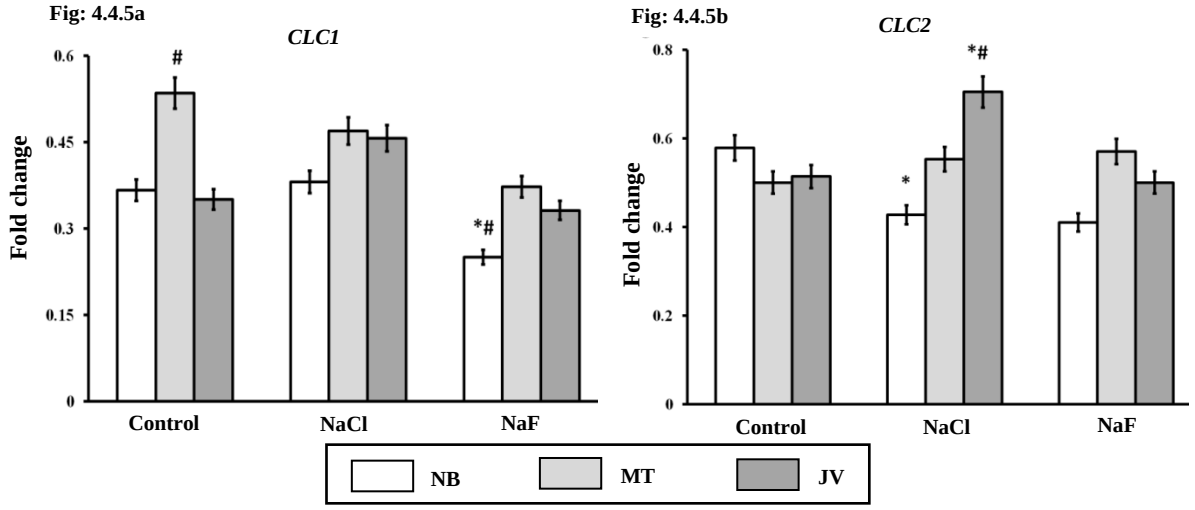


Fig. 4.4.5: Gene expression pattern of *CLC1* (**a**) and *CLC2* (**b**) in NB, MT and JV cultivars in presence of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Data represented are the mean value (n = 3) ± SE; '*' and '#' represent data with significant differences between treatment and cultivars, respectively at $P \leq 0.05$ against control set.

4.4.3.5. Extent of salt and fluoride induced oxidative damage in the cultivars

The germination percentage of salt stressed MT seeds was reduced by 1.2 fold, whilst no significant change was noted in case of NB and JV seedlings which can be linked with higher ABA content and accounting for their higher tolerance nature. Similar results was also reported by Saeedipour (2011) where they demonstrated that higher ABA level maintained the growth and development of salt stressed rice cultivars. During fluoride stress, the germination of NB and JV was reduced by 1.3 and 1.1 times, whereas no significant change was noted in case of MT seeds. Lower germination rate of fluoride stressed NB and JV seeds could be linked with their higher initial ABA. Such data lies in concurrence with the previous observations that showed higher fluoride induced damage in presence of elevated ABA content.

On treatment with 12 g L^{-1} NaCl solution, the level of H_2O_2 was induced by 1.5 times in MT seedlings that hampered the integrity of lipid membrane, thus leading to 2.2 fold higher electrolyte leakage. Furthermore, higher ROS accumulation also induced the formation of cytotoxic metabolites such as MDA and methylglyoxal by 1.7 and 1.6 times and reduced the chlorophyll level by 1.2 times. In case of NB and JV, no such prominent symptom of salt

stress was observed as compared to water treated seedlings (Table 4.4.1). Exposure to salt stress triggered the formation of ROS, leading to higher electrolyte leakage, MDA and methylglyoxal formation and chlorophyll loss which could be justified by lower ABA content of seedlings. However, higher ABA content of NB and JV seedlings rescued the seedlings from salt induced oxidative damage that lies in concurrence with the previous work of Roychoudhury et al. (2008) where they showed that application of 200 mM NaCl solution led to higher Na^+ deposition and associated oxidative injuries in salt sensitive rice cultivars, viz., Gobindobhog and M-1-48, as compared to salt tolerant variety, i.e., NB. Shevyakova et al. (2013) further reported that application of 1 µM ABA protected ice plant seedlings from 100 and 300 mM NaCl induced oxidative damage.

During fluoride stress, the growth and physiology of JV seedlings was highly compromised, whilst no major effect was noted in case of NB and MT. Fluoride stress led to 2.2 times higher H_2O_2 formation that caused 2.5 fold higher electrolyte leakage. Additionally, accumulation of ROS caused lipid membrane peroxidation producing 2.1 and 1.9 times higher cytotoxic metabolites like MDA and methylglyoxal, respectively. Fluoride stress also induced chlorophyll degradation in the seedlings by 2.0 times (Table 4.4.1). Similar effects were also reported by Banerjee and Roychoudhury (2019a) where they showed that application of NaF resulted in unrestricted uptake of fluoride ions in IR64 (fluoride sensitive cultivar) that caused higher oxidative damage in seedlings as compared to that of fluoride tolerant variety (Gobindobhog). Thus, overall it was observed that higher ABA content of JV seedlings failed to protect the seedlings from fluoride induced oxidative damage, ultimately leading to higher membrane perturbation, electrolyte leakage, chlorophyll loss and formation of cytotoxic metabolites. Banerjee and Roychoudhury (2019b) also demonstrated that higher ABA content of rice seedlings is not associated with fluoride tolerance.

Table 4.4.1: Germination percentage, chlorophyll, H_2O_2, electrolyte leakage, MDA and MG content of NB, MT and JV cultivars in presence of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Data represented are the mean value (n = 3) ± SE; '*' and '#' represent data with significance differences between treatment and cultivars respectively at $P \leq 0.05$ against control set.

Treatment	Cultivars	Germination percentage (%)	Chlorophyll (µg g^{-1} FW)	H_2O_2 (µM g^{-1} FW)	Electrolyte leakage (%)	MDA (µM g^{-1} FW)	MG (mg g^{-1} FW)
Control	NB	98.23 ± 1.98	106.54 ± 5.51	0.27 ± 0.05	13.76 ± 0.66	2.32 ± 0.18	8.35 ± 0.52
	MT	97.54 ± 2.04	101.54 ± 4.88	0.28 ± 0.04	13.78 ± 0.70	2.45 ± 0.15	8.63 ± 0.50
	JV	98.01 ± 2.20	106.69 ± 2.02	0.26 ± 0.06	14.51 ± 0.39	2.22 ± 0.18	8.11 ± 0.34
12 g L^{-1} NaCl	NB	95.47 ± 1.58	98.82 ± 1.18	0.28 ± 0.04	17.38 ± 0.84	2.73 ± 0.21	9.72 ± 0.34
	MT	78.95 ± 1.49$^{*\#}$	83.49 ± 6.69*	0.41 ± 0.08$^{*\#}$	29.8 ± 1.69*	4.17 ± 0.19$^{*\#}$	13.54 ± 0.78*
	JV	90.41 ± 1.74	99.84 ± 1.34	0.28 ± 0.06	17.85 ± 0.84	2.74 ± 0.25	9.60 ± 0.45
50 mg L^{-1} NaF	NB	76.24 ± 1.21$^{*\#}$	93.39 ± 5.21	0.28 ± 0.04	16.38 ± 0.21	2.82 ± 0.22	9.29 ± 0.42
	MT	92.12 ± 2.54	87.69 ± 4.60	0.31 ± 0.06	17.34 ± 0.64	2.99 ± 0.45	9.83 ± 0.57
	JV	89.54 ± 1.97	52.79 ± 6.25$^{*\#}$	0.58 ± 0.05$^{*\#}$	35.94 ± 0.56$^{*\#}$	4.59 ± 0.24$^{*\#}$	15.74 ± 0.89$^{*\#}$

4.4.3.6. Level of polyamines and expression of associated genes

Salt stress induced the level of putrescine by 1.6, 1.4 and 1.7 times in NB, MT and JV, respectively. Similarly, the level of spermidine and spermine was also elevated by 2.2 and 1.5 times, respectively in NB, 1.1 and 1.6 times, respectively in MT and 1.7 and 1.5 times, respectively, in JV during salt stress (Table 4.4.2). Higher formation of putrescine in all the three cultivars can be justified by the up regulated expression of *ODC* and *ADC* that are involved in the formation of putrescine from ornithine and arginine, respectively (Fig 4.4.6a, b). Additionally, down regulated expression of *DAO* (involved in putrescine catabolism) also supports the higher endogenous putrescine level of the seedlings (Fig 4.4.6c). Moreover, higher endogenous putrescine pool was further utilized by *SPDS* and *SPMS* during the formation of spermidine and spermine. Down regulated expression of *PAO* also inhibited the oxidation of spermine to putrescine (Fig 4.4.6d). Higher ABA level was responsible for elevation in the expression of *SAMDC* (the rate limiting enzyme of polyamine biosynthesis) in all the three genotypes (Fig 4.4.6e). The expression of SPDS and SPMS was enhanced in the salt stressed NB and JV that resulted in higher spermidine and spermine formation in these seedlings, as compared to that of salt stressed MT seedlings (Fig 4.4.6 f, g). Higher

level of polyamines in NB and JV seedlings could be directly linked with their higher endogenous ABA content as shown earlier by Pal et al. (2018). Significant rise in the level of polyamines in NB and JV seedlings efficiently curtailed the effects of fluoride induced oxidative damage in the seedlings that positively regulated the tolerance capability of seedlings. However, in case of MT seedlings, induced polyamine level was insufficient to scavenge the ROS formed that restrained the growth of seedlings. Do et al. (2014) screened 21 rice varieties and observed that salt stress induced the level of polyamines in the tolerant ones as compared to that of susceptible cultivars that coincided with our observation.

On application of NaF solution, the level of putrescine was enhanced by 1.3, 1.8 and 2.3 times in NB, MT and JV seedlings, respectively (Table 4.4.2). The highest level of putrescine in JV seedlings could be linked with their highest ABA level during fluoride stress. Additionally, ABA dependent up regulated expression of *ODC* and *ADC* also contributed to enhance the level of putrescine in JV cultivars (Fig 4.4.6a, b). Moreover, down regulated expression of *DAO* and *PAO* also justified higher endogenous content of putrescine in fluoride stressed JV seedlings (Fig 4.4.6 c, d). Ndayiragije and Lutts (2006) linked higher putrescine level of the stressed seedlings with their susceptible nature that lies in concurrence with the present observation. The level of spermidine and spermine was elevated by 1.4 and 1.1 times, respectively in NB, 1.5 and 1.5 times, respectively in MT and 2.5 and 1.5 times, respectively in JV seedlings during fluoride stress. Again, extended accumulation of ABA in seedlings led to the highest formation of spermidine and spermine in JV seedlings; however, this rise in the level of polyamines was insufficient to abrogate the symptoms of fluoride induced oxidative damage in seedlings. Based on these observations, JV seedlings were unable to efficiently utilize the endogenous polyamine pool to scavenge the ROS generated in response to fluoride stress that significantly hampered their growth and aggravated cellular damage. In contrast, the endogenous polyamines were efficiently employed by NB and MT seedlings during NaF treatment, restoring the growth of seedlings and ameliorating the damaging effects of fluoride toxicity.

Table 4.4.2: Putrescine, spermidine and spermine content of NB, MT and JV cultivars in presence of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Data represented here are the mean value (n = 3) ± SE; '*' and '#' represent data with significance differences between treatment and cultivars respectively at $P \leq 0.05$ against control set.

Treatment	Cultivars	Putrescine (nM g^{-1} FW)	Spermidine (nM g^{-1} FW)	Spermine (nM g^{-1} FW)
Control	NB	3.12 ± 0.57	1.12 ± 0.09	2.09 ± 0.15
	MT	2.74 ± 0.49	1.97 ± 0.05	1.89 ± 0.21
	JV	2.98 ± 0.39	1.41 ± 0.03	1.97 ± 0.08
12 g L^{-1} NaCl	NB	4.98 ± 0.16*	2.51 ± 0.09*	3.19 ± 0.13*
	MT	3.97 ± 0.59	2.13 ± 0.19	2.99 ± 0.04*
	JV	5.12 ± 0.37*#	2.41 ± 0.21*	2.87 ± 0.09
50 mg L^{-1} NaF	NB	4.12 ± 0.09	1.54 ± 0.19	2.24 ± 0.81
	MT	4.97 ± 0.23	2.97 ± 0.11	2.74 ± 0.15*
	JV	6.79 ± 0.57#	3.57 ± 0.17#	3.04 ± 0.29*#

Fig: 4.4.6a

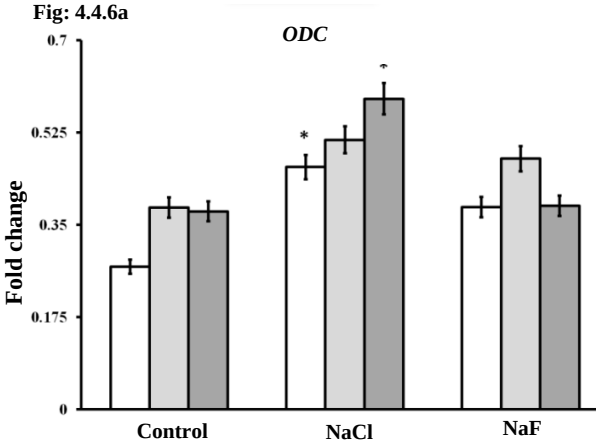


Fig: 4.4.6b

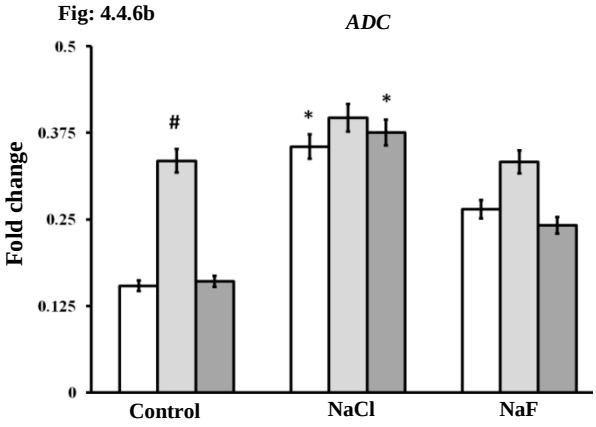


Fig: 4.4.6c

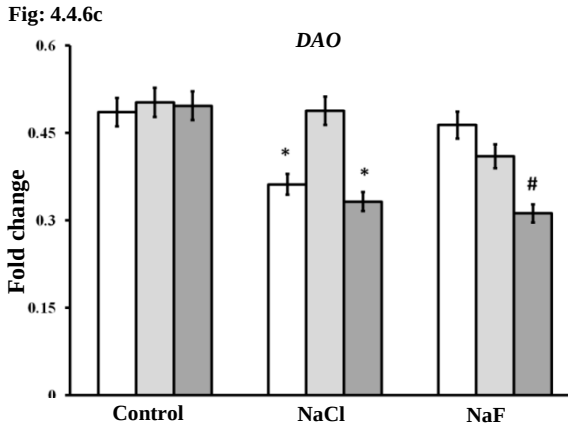
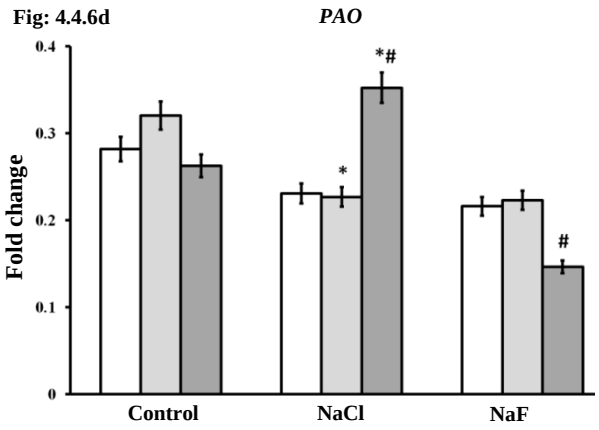


Fig: 4.4.6d



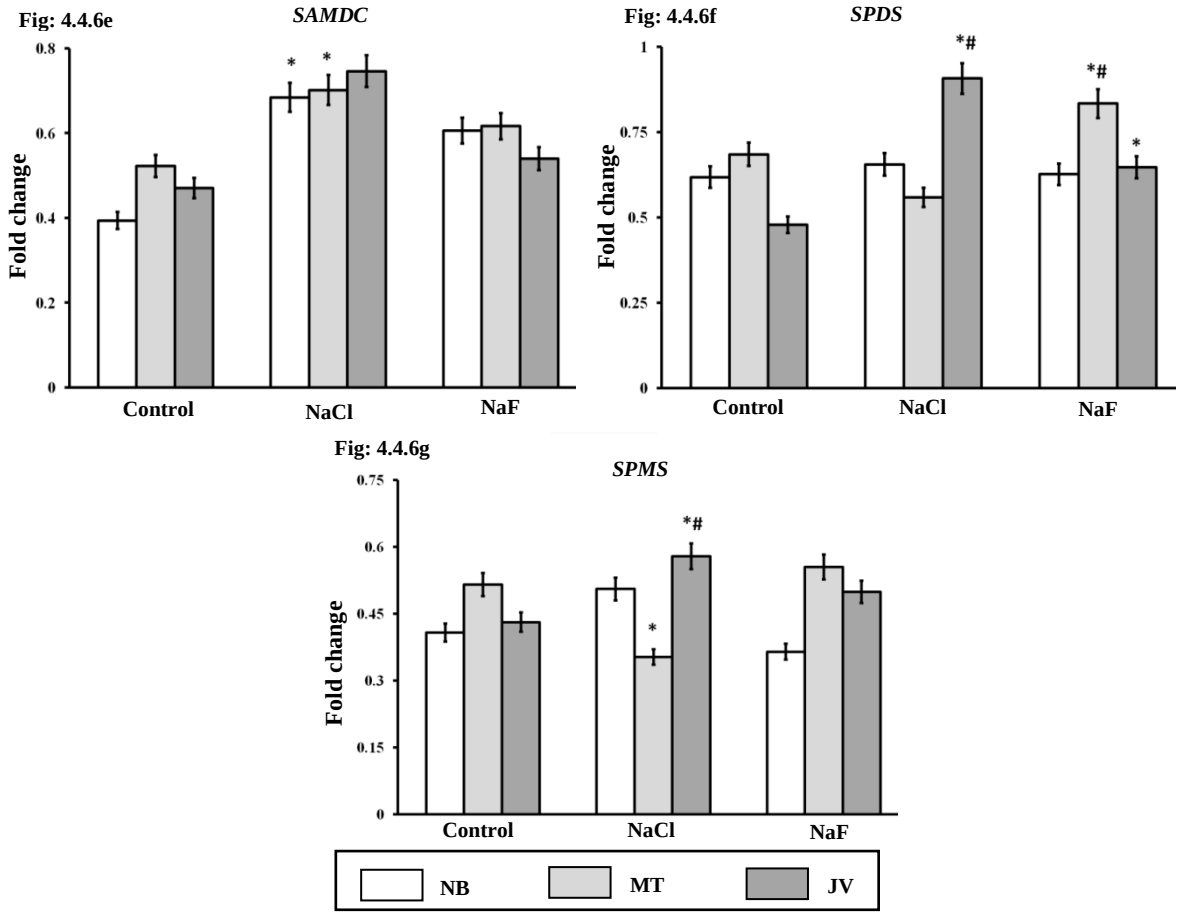


Fig. 4.4.6: Gene expression pattern of *ODC* (**a**), *ADC* (**b**), *DAO* (**c**), *PAO* (**d**), *SAMDC* (**e**), *SPDS* (**f**), and *SPMS* (**g**) in NB, MT and JV cultivars in presence of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Data represented are the mean value (n = 3) ± SE; '*' and '#' represent data with significant differences between treatment and cultivars, respectively at $P \leq 0.05$ against control set.

4.4.3.7. Glycine betaine and proline content in seedlings along with *PDH* expression

The level of glycine betaine was induced by 1.6, 1.8 and 1.9 times in salt stressed NB, MT and JV seedlings, respectively, whereas the level of proline was induced by 1.8, 3.5 and 1.3 fold, respectively in the above mentioned stressed varieties (Table 4.4.3). Significant rise in the level of proline in salt stressed MT seedlings can be supported by the down regulated expression of *PDH* that conserve the endogenous proline content of seedlings (Fig 4.4.7a). Contrastingly, the expression of PDH was induced in both NB and JV seedlings which could be justified by the fact that seedlings efficiently channelized the endogenous proline pool to other protective metabolites for effective stress mitigation. Highest level of proline in MT seedlings could be supported by the earlier work of Roychoudhury et al. (2008) where they

reported significant rise in the level of proline in salt sensitive rice variety, as compared to that of tolerant one.

Similar to that of salt stress, fluoride stress also induced the level of glycine betaine by 1.7, 1.5 and 2.5 times, respectively in NB, MT and JV seedlings (Table 4.4.3). Amongst all the cultivars, the highest glycine betaine level was noted in JV seedlings that coincided with its highest ABA level. Nawaz and Wang (2020) reported that exogenous application of ABA stimulated the formation of gylcine betaine in drought stressed *Axonopus compressus*, highlighting a direct correlation between glycine betaine and ABA content of seedlings. The level of proline was also induced by 1.5, 2.8 and 2.7 times in the above mentioned fluoride stressed rice cultivars, respectively (Table 4.4.3). The higher level of proline in JV seedlings could be linked with their higher ABA content. Additionally, higher proline level in MT could be linked with significantly reduced expression of *PDH* in fluoride stressed seedlings (Fig 4.4.7a). Similar to that of polyamines, the level of glycine betaine and proline was also enhanced in fluoride stressed JV seedlings, but interestingly, the rise in the level of these protective metabolites was still insufficient to curtail the negative impact of fluoride toxicity. Such antagonist relation was attributed primarily to prominent negative role by ABA in fluoride adaptation. Unlike JV seedlings, lower polyamines, glycine betaine and proline level in fluoride stressed NB and MT seedlings as compared to that of JV seedlings were highly efficient to maintain the growth and development of the seedlings which might be due to suppressed or unaltered endogenous ABA level in fluoride treated seedlings, as compared to that of control seedlings.

Table 4.4.3: Proline, glycine betaine, melatonin and gibberellic acid content of NB, MT and JV cultivars, in presence of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Data represented here are the mean value (n = 3) ± SE; '*' and '#' represent data with significance differences between treatment and cultivars respectively at $P \leq 0.05$ against control set.

Treatment	Cultivars	Proline (µg g^{-1} FW)	Glycine betaine (mg g^{-1} FW)	Melatonin (µg g^{-1} FW)	Gibberellic acid (µg g^{-1} FW)
Control	NB	15.92 ± 1.17	50.96 ± 2.43	164.32 ± 4.15	12.15 ± 0.89
	MT	11.65 ± 1.38	52.47 ± 2.12	184.24 ± 3.34	13.54 ± 1.21
	JV	16.47 ± 1.24	41.72 ± 2.96	169.67 ± 3.74	11.91 ± 1.14

12 g L^{-1} NaCl	NB	29.10 ± 0.83*	82.68 ± 1.97*	132.19 ± 3.66	9.87 ± 1.54
	MT	40.96 ± 1.38$^{*\#}$	97.05 ± 2.19*	160.23 ± 3.41	8.74 ± 0.98*
	JV	21.12 ± 0.97*	77.95 ± 2.58*	143.96 ± 2.60*	10.90 ± 1.97
50 mg L^{-1} NaF	NB	23.76 ± 1.17	84.48 ± 2.79	247.42 ± 2.93*	15.43 ± 0.54
	MT	32.63 ± 1.22	80.96 ± 3.80	196.10 ± 3.98	13.82 ± 0.81
	JV	44.03 ± 0.83$^{*\#}$	102.54 ± 3.49$^{*\#}$	107.87 ± 2.41$^{*\#}$	7.97 ± 1.41$^{*\#}$

4.4.3.8. Gibberellic acid and melatonin level in seedlings and *TDC* expression

Salt stress reduced the level of gibberellic acid and melatonin by 1.3 and 1.2 times, respectively in NB, 1.5 and 1.1 times, respectively in MT and 1.1 and 1.2 times, respectively in JV seedlings (Table 4.4.3). Lower gibberellic acid and melatonin content in the seedlings can be supported by the previous work of Gomez-Cadenas et al. (2001) and Zhang et al. (2014), where they reported that higher ABA level suppressed the synthesis of gibberellic acid and melatonin, respectively in seedlings. On the contrary, in spite of lower *TDC* expression, the level of melatonin in MT seedlings was the highest in all the salt stressed cultivars which might be due to its lower ABA content (Fig 4.4.7b). In case of NB and JV seedlings, the expression of *TDC* was up regulated, whilst melatonin level was reduced that could probably be due to its higher utilization in stress amelioration as earlier demonstrated by Liu et al. (2020) where they showed the efficiency of melatonin in generating salt tolerant seedlings.

During fluoride stress, the level of gibberellic acid and melatonin was enhanced by 1.3 and 1.5 times in NB seedling, but was lowered by 1.5 and 1.6 times in JV seedlings, respectively, whereas no such significant change was noted in MT seedlings (Table 4.4.3). Lower level of melatonin in JV seedlings could be directly linked with their higher ABA level. The present observation lies in line with the previous work of Banerjee and Roychoudhury (2019b) where they demonstrated that exogenous application of melatonin (20 µM) inhibited the uptake of fluoride ions and stimulated the level of endogenous melatonin and gibberellic acid in fluoride stressed seedlings, thereby maintaining the growth of seedlings by regulating the

level of protective metabolites and enzymes. Thus, lower level of gibberellic acid and melatonin in fluoride stressed JV seedlings clearly showcase its inability to adapt against fluoride toxicity. Hence, based on these observations, it could be stated that fluoride tolerance is negatively regulated or is independent of ABA signaling pathway and is more prominently controlled by melatonin and gibberellic acid.

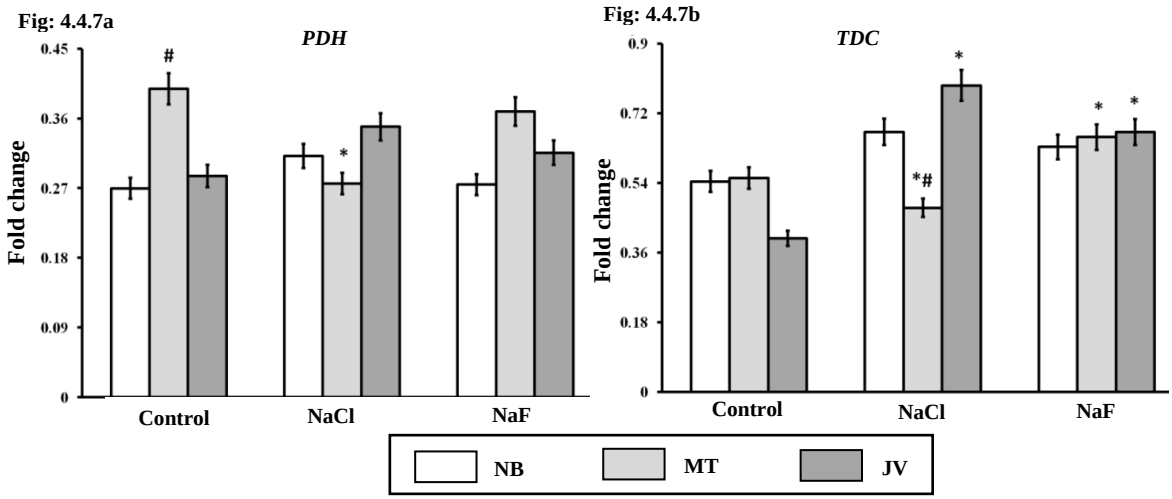


Fig. 4.4.7: Gene expression pattern of *PDH* (**a**) and *TDC* (**b**) in NB, MT and JV cultivars in presence of 12 g L^{-1} NaCl or 50 mg L^{-1} NaF. Data represented are the mean value (n = 3) ± SE; '*' and '#' represent data with significant differences between treatment and cultivars, respectively at $P \leq 0.05$ against control set.

4.4.3.9. Application of Na_2WO_4 firmly establish the negative role of ABA in fluoride tolerance

To further corroborate the role of ABA during fluoride stress, a specific inhibitor of ABA biosynthetic pathway, viz., sodium tungstate (Na_2WO_4) was applied to rice seedlings. Adamakis et al. (2012) stated that the presence of tungstate above threshold level replaces the Mo ions present in the active site of AAO that catalyzes the conversion of ABA aldehyde to ABA. For this work, three dosage of Na_2WO_4, i.e., 1, 2 and 4 mM were used. Prolonged application of 2 mM Na_2WO_4 could significantly suppress the level of ABA in all the three cultivars (Table 4.4.4, 4.4.5 and 4.4.6). The current observation was in line with the work of Jiang et al. (2004) where they showed that application of Na_2WO_4 repressed the level of ABA in barley seedlings by inhibiting the AAO activity. Interestingly, on application of Na_2WO_4 solution, the growth of fluoride stressed JV seedlings was restored and was comparable to that of control seedlings. However, on combined application of ABA (30 mg L^{-1}) and NaF,

the symptoms of damage were again reverted back even in presence of Na_2WO_4 (Fig 4.4.8a). Moreover, treatment of fluoride stressed NB and MT seedlings with Na_2WO_4 solution showed no such appreciable change in the growth of the seedlings which could be linked with their tolerant nature. Treatment of fluoride stressed NB and MT seedlings with ABA compromised the growth of seedlings, either in presence or absence of Na_2WO_4 (Fig 4.4.8b, 4.4.8c). These observations clearly highlight the negative role of ABA in controlling fluoride tolerance in these rice seedlings.

To further validate the present observations, major deleterious parameters linked with fluoride toxicity were monitored in presence or absence of ABA and Na_2WO_4. All the stress related parameters such as chlorophyll loss, electrolyte leakage, fluoride accumulation, H_2O_2, MDA and methylglyoxal formation were the highest in fluoride stressed JV seedlings, as compared to that of NB and MT which could be associated with its highest ABA level in all the three cultivars upon application of ABA exogenously (Table 4.4.4). However, application of Na_2WO_4 restored the chlorophyll (1.5 times) level of the stressed JV seedlings along with appreciably reducing the effects of fluoride induced damage, whilst exogenous application of ABA again restored all the symptoms of fluoride induced damage in JV seedlings even in presence of Na_2WO_4 close to that of only fluoride treated seedlings. Jiang and Zhang (2002) demonstrated that desiccation stress promoted ABA formation in maize seedlings that ultimately triggered oxidative damage in seedlings due to excess accumulation of ROS that supports the current observation. Based on these observations, it could be stated that Na_2WO_4 application significantly lowered the endogenous ABA content (2.0 times) in fluoride stressed JV seedlings that restricted the uptake and deposition of fluoride ions in seedlings along with restoration of the membrane integrity and chlorophyll level by reducing the formation of ROS and cytotoxic metabolites (MDA and methylglyoxal).

NB and MT exhibited fluoride tolerance due to reduced endogenous ABA pool; however, exogenous application of ABA elevated their endogenous ABA content that aggravated the symptoms of fluoride toxicity due to unrestricted uptake of fluoride ions. In presence of Na_2WO_4, the level of ABA was reduced by 1.5 times in both the fluoride stressed seedlings that countered the signs of fluoride toxicity. Furthermore, application of ABA along with NaF and Na_2WO_4 solution triggered ABA accumulation in NB and MT seedlings by 3.1 and 2.4 times, respectively that drastically reduced the chlorophyll content and also enhanced ROS formation, leading to higher membrane damage, as compared to that of seedlings treated

with NaF and Na_2WO_4 solution (Table 4.4.5, 4.4.6). These observations clearly highlight the negative role of ABA in regulating fluoride tolerance in NB, MT and JV seedlings. Thus, ABA formation during fluoride stress aggravated the level of oxidative damage, resulting in the generation of fluoride sensitive phenotype. Additionally, tolerance against fluoride stress was tightly mediated by melatonin and gibberellic acid, unlike salt stress where tolerance nature is significantly governed by ABA. Moreover, it was noted that treatment of seedlings with ABA inhibitor reduced their endogenous ABA pool that restored the normal growth of seedlings. However, on treatment with ABA again, the signs of oxidative damage reappeared. Thus, it could be concluded that fluoride tolerance is negatively regulated or is independent of endogenous ABA level in the examined rice varieties.

Table 4.4.4: Chlorophyll, H_2O_2, electrolyte leakage, MDA, methylglyoxal, fluoride and ABA content in JV seedlings, in absence or presence of 2 mM Na_2WO_4 and exposed to 50 mg L^{-1} NaF solution alone or in conjunction with 30 mg L^{-1} ABA. Data represented are the mean value (n = 3) ± SE; '*' represent data with significance differences between treatment at $P \leq 0.05$ against control set.

	In absence of 2 mM Na_2WO_4				**In presence of 2 mM Na_2WO_4**			
Parameters	Control	50 mg L^{-1} NaF	30 mg L^{-1} ABA	50 mg L^{-1} NaF + 30 mg L^{-1} ABA	Control	50 mg L^{-1} NaF	30 mg L^{-1} ABA	50 mg L^{-1} NaF + 30 mg L^{-1} ABA
Chlorophyll (µg g^{-1} FW)	104.17 ± 1.54	52.64 ± 5.03*	107.02 ± 2.28	41.06 ± 1.94	99.15 ± 1.88	79.60 ± 2.33	94.06 ± 5.54	67.65 ± 6.06*
H_2O_2 (µM g^{-1} FW)	0.27 ± 0.03	0.52 ± 0.09*	0.24 ± 0.07	0.72 ± 0.03*	0.26 ± 0.05	0.38 ± 0.04	0.26 ± 0.07	0.56 ± 0.06
Electrolyte leakage (%)	13.66 ± 0.60	33.44 ± 1.55*	10.86 ± 0.88	38.85 ± 0.50	13.78 ± 0.33	30.65 ± 1.75	14.71 ± 0.98	34.39 ± 1.80
MDA (µM g^{-1} FW)	2.45 ± 0.18	4.50 ± 0.29*	1.77 ± 0.22	7.79 ± 0.25*	2.55 ± 0.15	3.24 ± 0.87	2.90 ± 0.32	6.00 ± 0.54*
Methylglyoxal (mg g^{-1} FW)	8.48 ± 0.34	16.36 ± 0.39*	7.46 ± 0.52	25.18 ± 0.42*	8.91 ± 0.79	14.69 ± 0.60	8.69 ± 0.31	17.07 ± 0.45
Fluoride (mg kg^{-1} FW)	24.65 ± 7.07	300.42 ± 16.97*	16.50 ± 1.27	327.72 ± 11.45	15.88 ± 2.26	287.53 ± 30.68	23.72 ± 7.77	279.81 ± 27.71

ABA (pM g^{-1} FW)	1.26 ± 0.09	1.82 ± 0.06	3.81 ± 0.47*	4.28 ± 0.37	0.77 ± 0.14	0.90 ± 0.02	2.45 ± 0.17*	1.69 ± 0.41

Table 4.4.5: Chlorophyll, H_2O_2, electrolyte leakage, MDA, methylglyoxal, fluoride and ABA content in NB seedlings, in absence or presence of 2 mM Na_2WO_4 and exposed to 50 mg L^{-1} NaF solution alone or in conjunction with 30 mg L^{-1} ABA. Data represented are the mean value (n = 3) ± SE; '*' represent data with significance differences between treatment at P ≤ 0.05 against control set.

	In absence of 2 mM Na_2WO_4				**In presence of 2 mM Na_2WO_4**			
Parameters	Control	50 mg L^{-1} NaF	30 mg L^{-1} ABA	50 mg L^{-1} NaF + 30 mg L^{-1} ABA	Control	50 mg L^{-1} NaF	30 mg L^{-1} ABA	50 mg L^{-1} NaF + 30 mg L^{-1} ABA
Chlorophyll (µg g^{-1} FW)	103.34 ± 2.26	96.55 ± 6.75	102.44 ± 1.82	59.97 ± 3.07*	98.08 ± 1.69	104.32 ± 2.25	91.05 ± 0.54	71.04 ± 1.36
H_2O_2 (µM g^{-1} FW)	0.27 ± 0.04	0.29 ± 0.06	0.23 ± 0.01*	0.40 ± 0.05*	0.26 ± 0.03	0.28 ± 0.09	0.33 ± 0.06	0.36 ± 0.05
Electrolyte leakage (%)	14.61 ± 0.87	16.88 ± 0.48	13.22 ± 0.34	38.53 ± 0.67*	15.98 ± 0.03	13.30 ± 0.40	15.90 ± 0.89	31.50 ± 0.71*
MDA (µM g^{-1} FW)	2.32 ± 0.18	2.82 ± 0.22	2.18 ± 0.45	6.20 ±0.07*	2.39 ± 0.22	2.50 ± 0.29	2.46 ± 0.30	4.81 ± 0.28*
Methylglyoxal (mg g^{-1} FW)	8.54 ± 0.26	9.47 ± 0.68*	7.46 ± 0.52	13.48 ± 0.65*	9.81 ± 0.31	8.22 ± 0.44	8.02 ± 0.73	11.60 ± 0.52*
Fluoride (mg kg^{-1} FW)	14.60 ± 1.69	216.62 ± 16.68*	26.78 ± 5.79	263.40 ± 21.77	15.15 ± 5.23	216.74 ± 3.81	17.18 ± 3.25	214.90 ± 18.52
ABA (pM g^{-1} FW)	1.52 ± 0.89	1.22 ± 0.54	3.02 ± 0.24*	3.26 ± 0.97*	0.70 ± 0.07*	0.8 ± 0.05	1.85 ± 0.09	2.44 ± 0.14*

Table 4.4.6: Chlorophyll, H_2O_2, electrolyte leakage, MDA, methylglyoxal, fluoride and ABA content in MT seedlings, in absence or presence of 2 mM Na_2WO_4 and exposed to 50 mg L^{-1} NaF solution alone or in conjunction with 30 mg L^{-1} ABA. Data represented are the mean value (n = 3) ± SE; '*' represent data with significance differences between treatment at $P \leq 0.05$ against control set.

	In absence of 2 mM Na_2WO_4				In presence of 2 mM Na_2WO_4			
Parameters	Control	50 mg L^{-1} NaF	30 mg L^{-1} ABA	50 mg L^{-1} NaF + 30 mg L^{-1} ABA	Control	50 mg L^{-1} NaF	30 mg L^{-1} ABA	50 mg L^{-1} NaF + 30 mg L^{-1} ABA
Chlorophyll (µg g^{-1} FW)	101.54 ± 4.88	95.27 ± 6.11	110.01 ± 1.70	64.77 ± 3.51*	98.87 ± 1.24	98.08 ± 1.46	102.45 ± 1.17	79.96 ± 1.13
H_2O_2 (µM g^{-1} FW)	0.26 ± 0.03	0.34 ± 0.07*	0.25 ± 0.02	0.47 ± 0.04*	0.28 ± 0.06	0.30 ± 0.05	0.26 ± 0.07	0.28 ± 0.03
Electrolyte leakage (%)	13.28 ± 1.40	17.84 ± 1.35	11.02 ± 0.60	32.33 ± 1.52*	14.36 ± 1.19	13.91 ± 0.53	13.75 ± 1.71	26.32 ± 1.53*
MDA (µM g^{-1} FW)	2.35 ± 0.15	2.91 ± 0.13	2.00 ± 0.12	5.67 ± 0.30*	2.74 ± 0.19	2.53 ± 0.27	2.31 ± 0.22	4.10 ± 0.30*
Methylglyoxal (mg g^{-1} FW)	8.82 ± 0.23	9.45 ± 0.87	7.68 ± 0.31	12.77 ± 0.60*	9.19 ± 0.39	7.85 ± 0.55	8.26 ± 0.39	10.61 ± 0.71
Fluoride (mg kg^{-1} FW)	16.75 ± 1.55	213.12 ± 16.54*	21.42 ± 4.80	224.23 ± 5.93	21.91 ± 3.25	241.49 ± 23.47*	21.29 ± 2.26	221.09 ± 24.04
ABA (pM g^{-1} FW)	1.14 ± 0.57	1.81 ± 0.08	4.18 ± 0.97*	3.11 ± 0.23*	0.58 ± 0.04*	1.19 ± 0.97	2.50 ± 0.41	2.80 ± 0.57

Fig. 4.4.8a

In absence of 2 mM Na_2WO_4

In presence of 2 mM Na_2WO_4

Control | 50 mg L^{-1} NaF | 30 mg L^{-1} ABA | 50 mg L^{-1} NaF + 30 mg L^{-1} ABA

Fig. 4.4.8b

In absence of 2 mM Na_2WO_4

In presence of 2 mM Na_2WO_4

Control | 50 mg L^{-1} NaF | 30 mg L^{-1} ABA | 50 mg L^{-1} NaF + 30 mg L^{-1} ABA

Fig. 4.4.8c

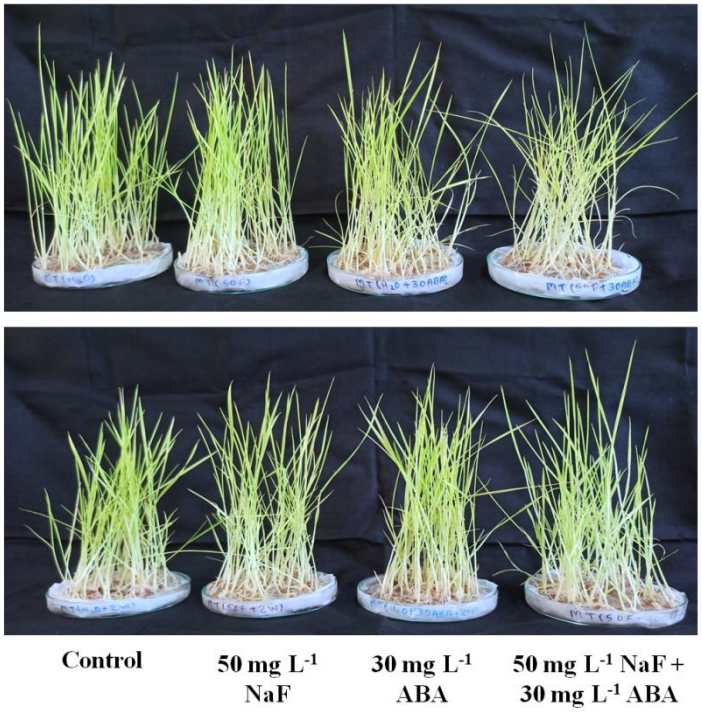
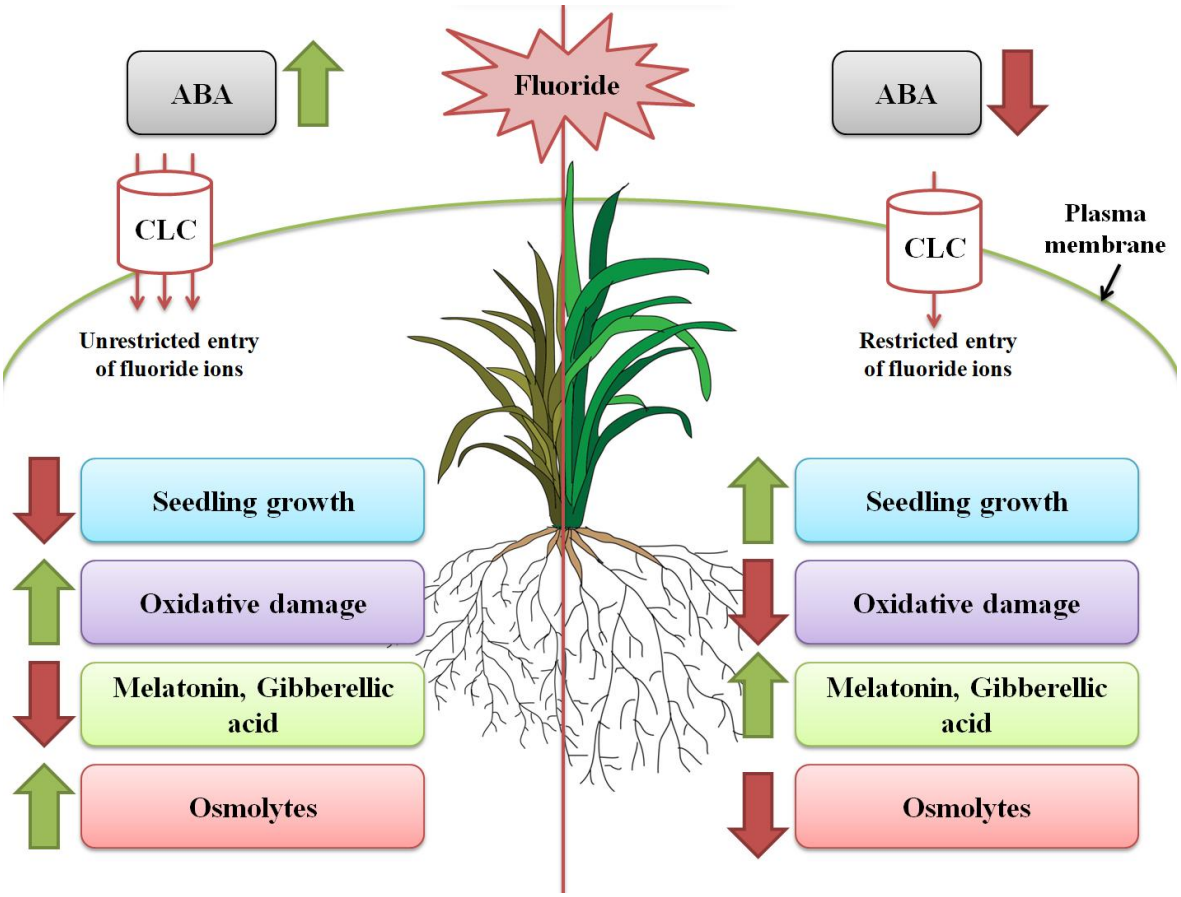


Fig. 4.4.8: Effect of exogenous application of 50 mg L^{-1} NaF alone or in conjunction with 30 mg L^{-1} ABA, either in absence or presence of 2 mM Na_2WO_4 solution for 20 days on growth performance in JV (**a**), NB (**b**) and MT (**c**) seedlings; untreated (maintained with water only) seedlings served as the experimental control.

4.4.4. Conclusion of the chapter

The role of ABA is widely documented against abiotic stresses like salinity, drought and heat stress; however, in case of fluoride stress, no such elaborate work has been done. For the present work, we selected three rice cultivars having high endogenous ABA level and exposed them to salt and fluoride stress separately, followed by estimation of damage incurred in the seedlings. While higher endogenous ABA level governed the tolerance nature of JV seedlings against salt stress, the same was unable to restore the growth and development of JV seedlings against fluoride stress in spite of higher formation of protective metabolites such as polyamines, proline and glycine betaine. Interestingly, reduced ABA level resulted in salt sensitivity in MT seedlings, but the same efficiently curtailed the symptoms of fluoride induced damage in both NB and MT seedlings. Moreover, lower ABA content of NB and MT during fluoride stress induced the level of two other phytohormones, viz., gibberellic acid and melatonin that stimulated the growth of seedlings. This data established the importance of these two phytohormones in fluoride stress tolerance and also highlighted that fluoride tolerance in rice seedlings is either ABA independent or is unregulated by ABA. On further investigation, it was observed that exogenous application of ABA resulted in higher oxidative damage, whereas application of ABA biosynthetic inhibitor (Na_2WO_4) checked the endogenous ABA accumulation in all the three rice cultivars that rescued the inhibition of seedling growth. These observations provide a strong support to establish the fact that fluoride tolerance is not governed by ABA level or ABA dependent signaling pathway, unlike majority of the other abiotic stress where ABA acts as a major player in dictating the tolerant trait (Fig. 4.4.9)."



"**Fig. 4.4.9:** Model depicting that fluoride tolerance is independent or is negatively regulated by endogenous ABA pool of the rice seedlings"

Chapter 5

Conclusion of Thesis

Due to rapid industrialization and gradual rise in human population, xenobiotic discharge in the environment has risen enormously that has significantly raised the concern of environmentalist. Fluoride is one such potent xenobiotic whose unregulated discharge in the ground and surface water has significantly affected the farmers utilizing such contaminated field and water resource for agriculture. Over exploitation of such contaminated water bodies has enhanced the deposition of fluoride in the agricultural field. The present study exclusively demonstrated the hazardous effects of fluoride toxicity in two *indica* rice cultivars, viz., Khitish and MTU1010 exposed to NaF (25 mg L^{-1}) solution for 10 and 20 days. Unregulated uptake of fluoride ions via chloride channels and their accumulation in seedlings (10 and 20 day-old Khitish and MTU1010, respectively) led to higher accumulation of superoxide anion and H_2O_2 that incited chlorophyll loss and electrolyte leakage along with higher formation of ammonium ions, MDA and methylglyoxal. Moreover, fluoride stress also aggravated the activity of LOX and NOX that further induced oxidative damages. However, in case of 20 day-old Khitish and 10 day-old MTU1010 seedlings, plant growth was maintained with lesser oxidative damages due to up regulated expression of H^+-ATPase and FEX along with the elevated level of nitrogen (nitrate, nitrite and total nitrogen) and sulfur (cysteine and H_2S) containing compounds. Higher accumulation of nitrogen was due to higher activity and expression of NR and NiR. Similarly, sulfur assimilation was catalyzed by the higher activity of ATP-S and OAS-TL. The activity of the enzymatic antioxidants and level of non-enzymatic antioxidants were also enhanced that strengthened the antioxidative potential of the seedlings. Thus, it can be concluded that differential metabolism of nitrogen and sulfur assimilatory pathway along with enzymatic antioxidant activity and level of non-enzymatic antioxidants were largely responsible for differential pattern of adaptive strategies in the two rice cultivars (Khitish and MTU1010), the former exhibiting resilience, while the latter showing greater susceptibility against long term stress.

In order to promote fluoride tolerance, exogenous application of protective agents such as calcium and silicon were pursued. These chemicals are eco-friendly, cheap and easily accessible to the farmers. On exogenous application of calcium and silicon, the symptoms of fluoride-induced damage were significantly lowered due to reduced bioaccumulation of fluoride ions in the tissues that restored the normal growth of the susceptible seedlings. Furthermore, application of the above mentioned protective chemicals also proved to be highly effective in regulating the formation of internal protective metabolites like osmolytes and non-enzymatic antioxidants and activity of enzymatic antioxidants that in turn

maintained the normal osmotic balance of the cells and also scavenged the ROS formed due to excess accumulation of fluoride ions. Priming of seeds with calcium compounds also rescued the normal functioning of the TCA cycle and also restored the endogenous pool of starch and sucrose that provided sufficient energy to the seedlings to overcome fluoride toxicity. Thus, the data obtained highlighted the promising role of calcium and silicon in curtailing the negative effects of fluoride toxicity by inhibiting its uptake and modulating the formation of protective metabolites.

The universal stress phytohormone ABA is known to mediate protective signaling responses against various abiotic stresses like drought and salinity and is considered to enhance stress tolerance. However, contrasting response was noted in case of three rice cultivars having high endogenous ABA level, upon being treated with NaF solution. Higher ABA content protected the JV seedlings from salt stress, whilst the same was insufficient to maintain the growth of the seedlings under fluoride stress, in spite of higher formation of osmolytes and polyamines. In contrast, lower ABA level caused higher salt-induced damage in MT seedlings, but the same was significantly effective in regulating the tolerance level of fluoride-stressed MT seedlings. Moreover, the level of the other two major phytohormones, viz., melatonin and gibberellic acid was antagonistically related to ABA that was effective in mitigating the effects of fluoride-induced damages in NB and MT seedlings, suggesting that fluoride tolerance in rice seedlings is controlled by melatonin and gibberellic acid-dependent pathways, but is independent of ABA, unlike other abiotic stresses where ABA plays a pivotal role. This was further verified by the exogenous application of ABA that triggered the effects of fluoride toxicity, whereas on application of ABA biosynthetic inhibitor (Na_2WO_4), the endogenous ABA content of the seedlings was reduced that restored the normal growth of the seedlings.

The inferences drawn from the overall Thesis work can be summarized as following:

1) Unregulated uptake of fluoride ions led to higher production of ROS that significantly affected the growth of rice cultivars.
2) Differential formation of protective metabolites and sulfur and nitrogen metabolism regulated differentially the fluoride tolerance level of the rice varieties investigated.
3) Priming of seed with calcium compounds, viz., $Ca(OH)_2$, $Ca(NO_3)_2$ and $CaCl_2$ inhibited the accumulation of fluoride ions and also enhanced the calcium-mediated signaling pathways and carbon metabolism, enhancing their adaptability.

4) Treatment of seedlings with silicon led to higher bioaccumulation of silicon in the tissues that acted as a protective barrier by inhibiting the uptake of fluoride ions, thereby reducing the effects of fluoride-mediated damages.
5) Fluoride tolerance was found to be independent or not regulated by ABA level, but was rather controlled primarily by other two major phytohormones, viz., melatonin and gibberellic acid, which usually act antagonistically to ABA.

References

Abbas T, Balal RM, Shahid MA, Pervez MA, Ayyub CM, Aqueel MA, Javaid MM (2015) Silicon-induced alleviation of NaCl toxicity in okra (*Abelmoschus esculentus*) is associated with enhanced photosynthesis, osmoprotectants and antioxidant metabolism. Acta Physiologiae Plantarum 37: 1–15.

Abdalla MM (2011) Impact of diatomite nutrition on two *Trifolium alexandrinum* cultivars differing in salinity tolerance. International Journal of Plant Physiology and Biochemistry 3: 233–246.

Abdelrahman M, El-Sayed M, Jogaiah S, Burritt DJ, Tran L-S P (2017) The "STAY-GREEN" trait and phytohormone signaling networks in plants under heat stress. Plant Cell Reports 36: 1009–1025.

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Shinozaki KY (2003) *Arabidopsi*s AtM.Y.C2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signalling. The Plant Cell 15: 63–78.

Abid M, Ali S, Qi LK, Zahoor R, Tian ZW, Jiang D, Snider JL, Dai TB (2018) Physiological and biochemical changes during drought and recovery periods at tillering and jointing stages in wheat (*Triticum aestivum* L.). Scientific Reports 8: 15.

Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. The Plant Cell 20: 2117–2129.

Adamakis I-D, Panteris E, Eleftheriou E (2012) Tungsten toxicity in plants. Plants 1: 82–99.

Adrees M, Ali S, Rizwan M, Zia-Ur-Rehman M, Ibrahim M, Abbas F, Farid M, Qayyum MF, Irshad MK (2015) Mechanisms of silicon-mediated alleviation of heavy metal toxicity in plants: A review. Ecotoxicology and Environmental Safety 119: 186-197.

Afzal I, Butt A, Rehman H, Basra SMA, Afzal A (2012) Alleviation of salt stress in fine aromatic rice by seed priming. Australian Journal of Crop Science 6: 1401–1407.

Agarwal R, Chauhan SS (2014) Bioaccumulation of sodium fluoride toxicity in *Triticum aestivum* var. RAJ. 3077. International Journal of Food, Agriculture & Veterinary Sciences 4: 98–101.

Agarwal S, Regon P, Rehman M, Tanti B, Panda SK (2021) Genome-wide analysis of fluoride exporter genes in plants. 3 Biotech 11: 124.

Ahmad P, Jaleel CA, Salem MA, Nabi G, Sharma S (2010) Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. Critical Reviews in Biotechnology 30: 161–175.

Akihiro T, Koike S, Tani R, Tominaga T, Watanabe S, Iijima Y, Aoki K, Shibata D, Ashihara H, Matsukura C, Akama K, Fujimura T, Ezura H (2008) Biochemical mechanism on GABA accumulation during fruit development in tomato. Plant Cell and Physiology 49: 1378–1389.

Al-aghabary K, Zhu Z, Shi Q (2005) Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidative enzyme activities in tomato plants under salt stress. Journal of Plant Nutrition 27: 2101–2115.

Al-Huqail AA, Alqarawi AA, Hashem A, Ahmad Malik J, Abd-Allah EF (2019) Silicon supplementation modulates antioxidant system and osmolyte accumulation to balance salt stress in *Acacia gerrardii* Benth. Saudi Journal of Biological Science 26: 1856-1864.

Ali F, Qanmber G, Li F, Wang Z (2022) Updated role of ABA in seed maturation, dormancy, and germination. Journal of Advanced Research 35: 199–214.

Alonso R, Elvira S, Castillo FJ, Gimeno BS (2001) Interactive effects of ozone and drought stress on pigments and activities of antioxidative enzymes in *Pinus halepensis*. Plant Cell and Environment 24: 905–916.

Al-Taweel K, Iwaki T, Yabuta Y, Shigeoka S, Murata N, Wadano A (2007) A bacterial transgene for catalase protects translation of d1 protein during exposure of salt-stressed tobacco leaves to strong light. Plant Physiology 145: 258–265.

Amirossadat Z, Ghehsareh AM, Mojiri A (2012) Impact of silicon on decreasing of salinity stress in greenhouse cucumber *(Cucumis sativus* L.) in soilless culture. Journal of Biological and Environmental Sciences 6: 171174.

Anazawa K (2006) Fluorine and coexisting volatiles in the geosphere: The Role in Japanese volcanic rocks. In Tressaud A (ed.), Fluorine and the environment: Atmospheric chemistry, emissions, & lithosphere. Amsterdam, Elsevier, pp. 187–224.

Ang HH, Lee KL (2005) Analysis of mercury in Malaysian herbal preparations. Journal of Medicine and Biomedical Research 4: 31–36.

Anjum NA, Sharma P, Gill SS, Hasanuzzaman M, Khan EA, Kachhap K, Mohamed AA, Thangavel P, Devi GD, Vasudhevan P, Sofo A, Khan NA, Misra AN, Lukatkin AS, Singh HP, Pereira E, Tuteja N (2016) Catalase and ascorbate peroxidase-representative H_2O_2-detoxifying heme enzymes in plants. Environmental Science and Pollution Research International 23: 19002–19029.

Anshumali B (2014) Fluoride in agricultural soil: A review on its sources and toxicity to plants. Global Sustainability Transitions: Impacts and Innovations 3: 29–37.

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55: 373–99.

Araya O, Wittwer F, Villa A (1993) Evolution of fluoride concentration in cattle and grass following a volcanic eruption. Veterinary and Human Toxicology 35: 437–440.

Arlappa N, Qureshi A, Srinivas R (2013) Fluorosis in India: an overview. International Journal of Research and Development of Health 1: 97–102.

Arnon DI (1949) Copper enzymes in isolated chloroplasts polyphenol oxidase in *Beta vulgaris*. Plant Physiology 24: 1–15.

Asad M, Zakari S, Zhao Q, Zhou L, Ye Y, Cheng F (2019) Abiotic stresses intervene with ABA signaling to induce destructive metabolic pathways leading to death: premature leaf senescence in plants. International Journal of Molecular Sciences 20: 256–279.

Asthir B, Tak Y (2017) Fluoride-induced changes in carbon and nitrogen metabolism in two contrasting cultivars of *Triticum aestivum* L. Fluoride 50: 334-342.

Attaelmanan AG (2012) Reliability of a new X-ray analytical microscope in archaeological research. Journal of Archaeological Science 39: 2552–2558.

Awasthi YC, Beutler E, Srivastava SK (1975) Purification and properties of human erythrocyte glutathione peroxidase. Journal of Biological Chemistry 250: 5144–5149.

Azevedo-Neto AD, Prisco JT, Gomes-Filho E (2009) Changes in soluble amino-N, soluble proteins and free amino acids in leaves and roots of salt-stressed maize genotypes. Journal of Plant Interaction 4: 137–144.

Back K, Tan DX, Reiter RJ (2016) Melatonin biosynthesis in plants: Multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. Journal of Pineal Research 61: 426–437.

Banaei-Asl F, Farajzadeh D, Bandehagh A, Komatsu S (2016) Comprehensive proteomic analysis of canola leaf inoculated with a plant growth-promoting bacterium, *Pseudomonas fluorescens*, under salt stress. Biochim Biophys Acta (BBA) 1864: 1222–1236.

Banerjee A, Roychoudhury A (2019a) Differential regulation of defence pathways in aromatic and non-aromatic indica rice cultivars towards fluoride toxicity. Plant Cell Reports 38: 1217–1233.

Banerjee A, Roychoudhury A (2019b) Melatonin application reduces fluoride uptake and toxicity in rice seedlings by altering abscisic acid, gibberellin, auxin and antioxidant homeostasis. Plant Physiology and Biochemistry 145: 164–173.

Banerjee A, Roychoudhury A (2021) Functional and molecular characterization of fluoride exporter (FEX) from rice and its constitutive overexpression in *Nicotiana benthamiana* to promote fluoride tolerance. Plant Cell Reports 40: 1751–1772.

Banerjee A, Roychoudhury A, Ghosh P (2019a) Differential fluoride uptake induces variable physiological damage in a non-aromatic and an aromatic indica rice cultivar. Plant Physiology and Biochemistry 142: 143–150.

Banerjee A, Samanta S, Roychoudhury A (2019b) Spermine ameliorates prolonged fluoride toxicity in soil-grown rice seedlings by activating the antioxidant machinery and glyoxalase system. Ecotoxicology and Environmental Safety 189: 109737.

Banerjee A, Singh A, Roychoudhury A (2019c) Spermidine application reduces fluoride uptake and ameliorates physiological injuries in a susceptible rice cultivar by activating diverse regulators of the defense machinery. Environmental Science and Pollution Research 26: 36598–36614.

Banerjee A, Singh A, Sudarshan M, Roychoudhury A (2021) Silicon nanoparticle-pulsing mitigates fluoride stress in rice by fine-tuning the ionomic and metabolomic balance and refining agronomic traits. Chemosphere 262: 127826.

Basu S, Roychoudhury A (2014) Expression profiling of abiotic stress-inducible genes in response to multiple stresses in rice (*Oryza sativa* L.) varieties with contrasting level of stress tolerance. BioMed Research International 2014: 706890.

Basu S, Roychoudhury A, Sanyal S, Sengupta DN (2012) Carbohydrate content and antioxidative potential of the seed of three edible indica rice (*Oryza sativa* L.) cultivars. Indian Journal of Biochemistry and Biophysics 49: 115–123.

Baunthiyal M, Ranghar S (2014) Physiological and biochemical responses of plants under fluoride stress: An overview. Fluoride 47: 287–293.

Baunthiyal M, Sharma V (2014) Response of fluoride stress on plasma membrane H^+-ATPase and vacuolar H^+-ATPase activity in semi-arid plants. Indian Journal of Plant Physiology 19: 210-214.

Belanger RR, Benhamou N,Menzies JG (2003) Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (*Blumeria graminis* f. sp *tritici*). Phytopathology 93: 402–412.

Bellomo S, Aiuppa A, D'Alessandro W, Parello F (2007) Environmental impact of magmatic fluorine emission in the Mt. Etna area. Journal of Volcanology and Geothermal Research 165: 87–101.

Benderradji L, Saibi W, Brini F (2021) Role of ABA in overcoming environmental stress: Sensing, signaling and crosstalk. Annals of Agricultural & Crop Sciences 6: 1070.

Bhardwaj S, Sharma D, Singh S, Ramamurthy PC, Verma T, Pujari M, Singh J, Kapoor D, Prasad R (2023) Physiological and molecular insights into the role of silicon in improving plant performance under abiotic stresses. Plant Soil 486: 25–43.

Bhargava D, Bhardwaj N (2010) Effect of sodium fluoride on seed germination and seedling growth of *Triticum aestivum* var. Raj. 4083. Journal of Phytology 2: 41–43.

Bharti VK, Giri A, Kumar K (2017) Fluoride sources, toxicity and its amelioration: a review. Annals of Environmental Science and Toxicology 2: 21–32.

Bhattacharya P, Samal AC (2018) Fluoride contamination in groundwater, soil and cultivated foodstuffs of India and its associated healthrisks: a review. Research Journal of Recent Science 7: 36–47.

BIS (2012) Drinking Water-Specification B.S.10500; Bureau of Indian Standard: New Delhi, India, 2012. Available online: http://cgwb.gov. in/Documents/WQ-standards.pdf

Bolat I, Kaya C, Almaca A, Timucin S (2006) Calcium sulfate improves salinity tolerance in rootstocks of plum. Journal of Plant Nutrition 29: 553–564.

Bonifacio A, Martins MO, Ribeiro CW, Fontenele AV, Carvalho F, Margis-Pinheiro M, Silveira JAG (2011) Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress. Plant Cell and Environment 34: 1705–1722.

Borkowska B, Szczerba J (1991) Influence of different carbon sources on invertase activity and growth of sour cherry (*Prunus cerasus* L.) shoot cultures. Journal of Experimntal Botany 42: 911–915.

Bouthour D, Kalai T, Chaffei HC, Gouia H, Corpas FJ (2015) Differential response of NADP-dehydrogenases and carbon metabolism in leaves and roots of two durum wheat (*Triticum durum* Desf.) cultivars (Karim and Azizi) with different sensitivities to salt stress. Journal of Plant Physiology 179: 56–63.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Camargo JA (2003) Fluoride toxicity to aquatic organisms: A review. Chemosphere 50: 251–264.

Camargo JA, Alonso Á (2006) Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environment International 32: 831–849.

Canovas FM, Canton FR, Gallardo F, Garcia-Gutierrez A, de Vincente A (1991) Accumulation of glutamine synthetase during early development of maritime pine (*Pinus pinaster*) seedlings. Planta 185: 372–378.

Cao BL, Ma Q, Zhao Q, Wang L, Xu K (2015) Effects of silicon on absorbed light allocation, antioxidant enzymes and ultrastructure of chloroplasts in tomato leaves under simulated drought stress. Scientia Horticulturae 194: 53–62.

Cape JN, Fowler D, Davison A (2003) Ecological effects of sulfur dioxide, fluorides, and minor air pollutants: Recent trends and research needs. Environment International 29: 201–211.

Cataldo DA, Haroon M, Schrader LE, Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Communications in Soil Science and Plant Analysis 6: 71–80.

Caverzan A, Passaia G, Rosa SB, Ribeiro CW, Lazzarotto F, Margis-Pinheiro M (2012) Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. Genetics and Molecular Biology 35: 1011–1019.

Chakrabarti S, Patra PK (2013) Effect of fluoride on superoxide dismutase activity in four common crop plants. Fluoride 46: 59–62.

Chakrabarti S, Patra PK (2015) Biochemical and antioxidant responses of paddy (Oryza sativa L.) to fluoride stress. Fluoride 48: 56–61.

Chakraborti D, Rahman MM, Chatterjee A, Das D, Das B, Nayak B, Pal A, Chowdhury UK, Ahmed S, Biswas BK, Sengupta MK, Lodh D, Samanta G, Chakraborty S, Roy MM, Dutta RN, Saha KC, Mukherjee SC, Pati S, Kar PB (2016) Fate of over 480 million inhabitants living in arsenic and fluoride endemic Indian districts: Magnitude, health, socio-economic effects and mitigation approaches. Journal of Trace Elements in Medicine and Biology 38: 33–45.

Chakraborty S, Singh A, Roychoudhury A (2022) Extensive cross-talk among stress-regulated protective metabolites, biogenic-amines and phytohormone-signalling, co-ordinated by dopamine-mediated seed-priming, governs tolerance against fluoride stress in rice. Plant Cell Reports 41: 2261-2278.

Chalanika De Silva HC, Asaeda T (2017) Effects of heat stress on growth, photosynthetic pigments, oxidative damage and competitive capacity of three submerged macrophytes. Journal of Plant Interactions 12: 228–236.

Chandrawanshi CK, Patel KS (1999) Fluoride deposition in central India. Environmental Monitoring and Assessment 55: 251–265.

Cha-Um S, Charoenpanich A, Roytrakul S, Kirdmanee C (2009) Sugar accumulation, photosynthesis and growth of two indica rice varieties in response to salt stress. Acta Physiologiae Plantarum 31: 477–486.

Chen D, Wang S, Yin L, Deng X (2018) How does silicon mediate plant water uptake and loss under water deficiency? Frontiers in Plant Science 9: 281.

Chen D, Yin L, Deng X, Wang S (2014) Silicon increases salt tolerance by influencing the two-phase growth response to salinity in wheat (*Triticum aestivum* L.) Acta Physiologiae Plantarum 36: 2531–2535.

Chen FL, Cullimore JV (1988) Two isoenzymes of NADH-dependent glutamate synthase in root nodules of *Phaseolus vulgare* L. purification, properties and activity changes during nodule development. Plant Physiology 88: 1411–1417.

Chen J, Zhang M, Eneji AE, Li J (2016) Influence of exogenous silicon on UV-B radiation-induced cyclobutane pyrimidine dimmers in soybean leaves and its alleviation mechanism. Journal of Plant Physiology 196-197: 20-27.

Chen K, Li G, Bressan RA, Song C, Zhu J, Zhao Y (2020) Abscisic acid dynamics, signaling and functions in plants. Journal of Integrative Plant Biology 62: 25–54.

Chen KM, Gong HJ, Chen GC, Wang SM, Zhang CL (2004) Gradual drought under field conditions influences the glutathione metabolism, redox balance and energy supply in spring wheat. Journal of Plant Growth Regulation 23: 20–28.

Chen THH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Current Opinion in Plant Biology 5: 250–257.

Cheng XT, Wang SX, Gao JG (2002) Analysis of MBP,NSE,F−content and CHE activity in brain tissue of rats with chronic fluorosis. Chinese Journal of Endemiology 2: 357–358.

Cheong YH, Kim KN, Pandey GK, Gupta R, Grant JJ, Luan S (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in Arabidopsis. Plant Cell 15: 1833–1845.

Che-Othman MH, Jacoby RP, Millar AH, Taylor NL (2019) Wheat mitochondrial respiration shifts from the TCA cycle to the GABA shunt under salt stress. New Phytologist 225: 1166–1180.

Chiba FY, Garbin CAS, Sumida DH (2012) Effect of fluoride intake on carbohydrate metabolism, glucose tolerance, and insulin signaling, Fluoride 45: 236–241.

Chinnusamy V, Jagendorf A, Zhu JK (2005) Understanding and improving salt tolerance in plants. Crop Sciences 45: 437–448.

Chiu RS, Nahal H, Provart NJ, Gazzarrini S (2012) The role of the Arabidopsis FUSCA3 transcription factor during inhibition of seed germination at high temperature. BMC Plant Biology 12: 15.

Chojak-Koźniewska J, Kuźniak E, Linkiewicz A, Sowa S (2018) Primary carbon metabolism-related changes in cucumber exposed to single and sequential treatments with salt stress and bacterial infection. Plant Physiology and Biochemistry 123: 160–169.

Choubisa SL (2001) Endemic fluorosis in southern Rajasthan, India, Fluoride 34: 61–70.

Choubisa SL (2017) A brief and critical review on hydrofluorosis in diverse species of domestic animals in India. Environmental Geochemistry and Health 2017: 1–16.

Choubisa SL, Choubisa DK, Joshi SC, Choubisa L (1997) Fluorosis in some tribal villages of Dungarpur district of Rajasthan, India. Fluoride 30: 223–228.

Choubisa SL, Choubisa L, Choubisa DK (2001) Endemic fluorosis in Rajasthan. Indian Journal of Environmental Health 43: 177–189.

Choubisa SL, Modasiya V, Bahura CK, Sheikh Z (2012) Toxicity of fluoride in cattle of the Indian Thar Desert, Rajasthan, India. Fluoride 45: 371–376.

Choubisa SL, Sompura K (1996) Dental fluorosis in tribal villages of Dungarpur district (Rajasthan). Pollution Research 15: 45–47.

Choudhury B, Mitra S, Biswas AK (2010) Regulation of sugar metabolism in rice (*Oryza sativa* L.) seedlings under arsenate toxicity and its improvement by phosphate. Physiology and Molecular Biology of Plants 16: 59–68.

Chouhan S, Flora SJ (2010) Arsenic and F: two major ground water pollutants. Indian Journal of Experimental Biology 48: 666-78.

Chrispeels MJ, Varner JE (1967) Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley and aleurone layers. Plant Physiology 42: 398–406.

Churchill HV, Rowley RJ, Martin LN (1948) Fluorine content of certain vegetation in a Western Pennsylvania area. Analytical Chemistry 20: 69–71.

Coelho SM, Taylor AR, Ryan KR, Sousa-Pinto I, Brown MT, Brownlee C (2002) Spatiotemporal pattering of reactive oxygen production and Ca^{2+} wave propagation in *Fucus Rhizoid* cells. The Plant Cell 14: 2369–2381.

Corpas FJ (2019) Hydrogen sulfide: A new warrior against abiotic stress. Trends in Plant Science 24: 983-988.

Cuevas JC, López-Cobollo R, Alcázar R, Zarza X, Koncz C, Altabella T, Salinas J, Tiburcio AF, Ferrando A (2008) Putrescine is involved in *Arabidopsis* freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. Plant Physiology 148: 1094–1105.

Das P, Manna I, Sil P, Bandyopadhyay M, Biswas AK (2019) Exogenous silicon alters organic acid production and enzymatic activity of TCA cycle in two NaCl stressed indica rice cultivars. Plant Physiology and Biochemistry 136: 76–91.

Datta JK, Maitra A, Mondal NK (2012) Studies on the fluoride toxicity on germination and seedlings growth of gram seed (*Cicer arietinum* L. cv. Anuradha). Journal of Stress Physiology & Biochemistry 8: 194–202.

Davidian JC, Kopriva S (2010) Regulation of sulfate uptake and assimilation—the same or not the same? Molecular Plant 3: 314-325.

Davies BH (1965) Chemistry and biochemistry of plant pigments. Goodwin, London.

Dawson AP (1997) Calcium signalling: How do IP3 receptor work? Current Biology 7: R544–R547.

de Carvalho MHC (2008) Drought stress and reactive oxygen species production, scavenging and signaling. Plant Signaling and Behavior 3: 156–165.

Debska K, Bogatek R, Gniazdowska A (2012) Protein carbonylation and its role in physiological processes in plants. Postepy biochemii 58: 34-43.

Delauney A, Hu C, Kishor P, Verma D (1993) Cloning of ornithine delta-aminotransferase cDNA from *Vigna aconitifolia* by trans-complementation in *Escherichia coli* and regulation of proline biosynthesis. Journal of Biological Chemistry 268: 18673–18678.

Dey U, Mondal NK, Das K, Datta JK (2012a) Dual effects of fluoride and calcium on the uptake of fluoride, growth physiology, pigmentation, and biochemistry of Bengal gram seedlings (Cicer arietinum L.). Fluoride 45: 389–393.

Dey RK, Swain SK, Mishra S, Sharma P, Patnaik T, Singh VK, Dehury BN, Jha U, Patel RK (2012b) Hydrogeochemical processes controlling the high fluoride concentration in groundwater: a case study at the Boden block area, Orissa, India. Environmental Monitoring and Assessment 184: 3279–3291.

Dey S, Goswami S, Ghosh UC (2004) Hydrous ferric oxide (HFO)—A scavenger for fluoride from contaminated water. Water, Air, and Soil Pollution 158: 311–323.

Dharmaratne RW (2015) Fluoride in drinking water and diet: the causative factor of chronic kidney diseases in the north Central Province of Sri Lanka. Environmental Health and Preventive Medicine 20: 237–242.

Do PT, Drechsel O, Heyer AG, Hincha DK, Zuther E (2014) Changes in free polyamine levels, expression of polyamine biosynthesis genes, and performance of rice cultivars under salt stress: a comparison with responses to drought. Frontiers in Plant Science 5: 182.

Dos Reis SP, Lima AM, De Souza CRB (2012) Recent molecular advances on downstream plant responses to abiotic stress. International Journal of Molecular Sciences 13: 8628–8647.

Dou HO, Xv KP, Meng QW, Li G, Yang XH (2015) Potato plants ectopically expressing Arabidopsis thaliana CBF3 exhibit enhanced tolerance to high-temperature stress. Plant Cell and Environment 38: 61–72.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colororimetric method for determination of sugars and related substances. Analytical Chemistry 28: 350–356.

Dumanović J, Nepovimova E, Natić M, Kuča K and Jaćević V (2021) The significance of reactive oxygen species and antioxidant defense system in plants: A concise overview. Frontier in Plant Science 11: 552969,

Edmunds WM, Smedley PL (2005) Fluoride in natural waters. In Selinus O (ed) Essentials of medical geology. Dordrecht, Springer, pp. 311–336.

El Sabagh A, Sorour S, Omar AE, Ragab A, Islam MS, Barutçular C, Ueda A, Saneoka H (2015a) Alleviation of adverse effects of salt stress on soybean (*Glycine max* L.) by using osmoprotectants and organic nutrients. International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering 9: 1014–1018.

El Sabagh A, Sorour S, Omar A, Islam M, Ueda A, Saneoka H, Barutçular C (2015b) Soybean (*Glycine max* L.) growth enhancement under water stress conditions. International Conference on Chemical, Agricultural and Biological Sciences Istanbul, Turkey 2015: 144–148.

El-Shora HE, Ali AS (2011) Changes in activities of nitrogen metabolism enzymes in cadmium stressed marrow seedlings. Asian Journal of Plant Science 10: 117–124.

Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahara T, Inanaga S, Tanaka K (2006) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta 225: 1255–1264.

Eltelib HA, Fujikawa Y, Esaka M (2012) Overexpression of the acerola (*Malpighia glabra*) monodehydroascorbate reductase gene in transgenic tobacco plants results in increased ascorbate levels and enhanced tolerance to salt stress. South African Journal of Botany 78: 295–301.

Emam MM, Khattab HE, Helal NM, Deraz AE (2014) Effect of selenium and silicon on yield quality of rice plant grown under drought stress. Australian Journal of Crop Science 8: 596.

Emamverdian A, Ding Y, Mokhberdoran F, Xie Y (2015) Heavy metal stress and some mechanisms of plant defense response. Scientific World Journal 2015: 756120.

Emamverdian A, Ding Y, Xie Y, Sangari S (2018) Silicon mechanisms to ameliorate heavy metal stress in plants. BioMed Research International 2018: 1-10.

Epstein E, Bloom AJ (2005) Mineral nutrition of plants: principles and perspectives, 2nd Edn. Sunderland: Sinauer Associates Inc.

Erb M, Kliebenstein DJ (2020) Plant secondary metabolites as defenses, regulators, and primary metabolites: the blurred functional trichotomy. Plant Physiology 184: 39–52.

Eremina M, Rozhon W, Poppenberger B (2016) Hormonal control of cold stress responses in plants. Cellular and Molecular Life Sciences 73: 797–810.

Ericsson Y (1970) Fluorides and human health. Lancet 2: 351–352.

Eyidogan F, Oz T (2007) Effect of salinity on antioxidant responses of chickpea seedlings. Acta Physiologiae Plantarum 29: 485–493.

Fang C, Fernie AR, Luo J (2019) Exploring the diversity of plant metabolism. Trends in Plant Science 24: 83–98.

Fang HH, Jing T, Liu ZQ, Zhang LP, Jin ZP, Pei YX (2014) Hydrogen sulfide interacts with calcium signaling to enhance the chromium tolerance in *Setaria italica*. Cell Calcium 56: 472–481.

Farhangi-Abriz S, Ghassemi-Golezani K (2022) The modified biochars influence nutrient and osmotic statuses and hormonal signaling of mint plants under fluoride and cadmium toxicities. Frontiers in Plant Sciences 13: 1064409.

Farouk S, Al-Amri SM (2019) Exogenous melatonin-mediated modulation of arsenic tolerance with improved accretion of secondary metabolite production, activating antioxidant capacity and improved chloroplast ultrastructure in rosemary herb. Ecotoxicology and Environmental Safety 180: 333–347.

Feng D, Wang X, Gao J, Zhang C, Liu H, Liu P, Sun X (2023) Exogenous calcium: Its mechanisms and research advances involved in plant stress tolerance. Frontiers in Plant Science 14: 1143963.

Feng X, Liu WX, Zeng FR, Chen ZH, Zhang GP, Wu FB (2016) K^+ Uuptake, H^+-ATPase pumping activity and Ca^{2+} efflux mechanism are involved in drought tolerance of barley. Environmental and Experimental Botany 129: 57–66.

Finkelstein R (2013) Abscisic Acid synthesis and response. Arabidopsis Book 11: e0166.

Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. Journal of Biological Chemistry 66: 375.

Fleet CM, Sun TP (2005) A DELLAcate balance: The role of gibberellin in plant morphogenesis. Current Opinion in Plant Biology 8: 77–85.

Footitt S, Douterelo-Soler I, Clay H, Finch-Savage WE (2011) Dormancy cycling in *Arabidopsis* seeds is controlled by seasonally distinct hormone-signaling pathways. Proceedings of the National Academy of Sciences USA 108: 20236–20241.

Fu J, Wu Y, Miao Y, Xu Y, Zhao E, Wang J, Sun H, Liu Q, Xue Y, Xu Y, Hu T (2017) Improved cold tolerance in *Elymus nutans* by exogenous application of melatonin may involve ABA-dependent and ABA-independent pathways. Scientific Reports 7: 1–11.

Gadi BR, Verma P, Amra R (2012) Influence of NaF on seed germination, membrane stability and some biochemical content in *Vigna* seedlings. Journal of Chemical, Biological and Physical Sciences 2: 1371-1378.

Gaitonde MK (1967) A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. Biochemical Journal 104: 627-633.

Galvez L, Clark RB (1991) Effects of silicon on growth and mineral composition of sorghum (*Sorghum bicolor*) grown with toxic levels of aluminium. Plant-Soil Interactions at Low PH. doi:10.1007/978-94-011-3438-5_92.

Gangwar S, Singh VP (2011) Indole acetic acid differently changes growth and nitrogen metabolism in *Pisum sativum* L. seedlings under chromium (VI) phytotoxicity: implication of oxidative stress. Scientia Horticulturae 129: 321–328.

Gao J, Liu C, Zhang J, Zhu S, Shen Y, Zhang R (2018) Effect of fluoride on photosynthetic pigment content and antioxidant system of *Hydrilla verticillata*. International Journal of Phytoremediation 20: 1257-1263.

Gao SQ, Chen M, Xu ZS, Zhao CP, Li L, Xu HJ, Tang YM, Zhao X, Ma YZ (2011) The soybean GmbZIP1 transcription factor enhances multiple abiotic stress tolerances in transgenic plants. Plant Molecular Biology 75: 537-553.

Gao YL, Zhang GZ (2019) A calcium sensor calcineurin B-like 9 negatively regulates cold tolerance via calcium signaling in *Arabidopsis thaliana*. Plant Signaling and Behavior 14: 6.

Garg N, Bhandari P (2015) Silicon nutrition and mycorrhizal inoculations improve growth, nutrient status, K^+/Na^+ ratio and yield of *Cicer arietinum* L. genotypes under salinity stress. Plant Growth Regulation 78: 371–387.

Garg N, Bhandari P (2016) Interactive effects of silicon and arbuscular mycorrhiza in modulating ascorbate-glutathione cycle and antioxidant scavenging capacity in differentially salt-tolerant *Cicer arietinum* L. genotypes subjected to long-term salinity. Protoplasma 253: 1325–1345.

Geigenberger P, Stitt M (1993) Sucrose synthase catalyses a readily reversible reaction in vivo in developing potato tubers and other plant tissues. Planta 189: 329–339.

Ghareeb H, Bozso Z, Ott PG, Repenning C, Stahl F, Wydra K (2011) Transcriptome of silicon-induced resistance against *Ralstonia solanacearum* in the silicon non-accumulator tomato implicates priming effect. Physiological and Molecular Plant Pathology 75: 83–89.

Ghassemi-Golezani K, Farhangi-Abriz S (2019) Biochar alleviates fluoride toxicity and oxidative stress in safflower (*Carthamus tinctorius* L.) seedlings. Chemosphere 223: 406–415.

Ghosh P, Roychoudhury A (2018) Differential levels of metabolites and enzymes related to aroma formation in aromatic indica rice varieties: comparison with non-aromatic varieties. 3 Biotech 8: 25.

Ghosh S, Maiti TK, Basu PS (2008) Bioproduction of ascorbic acid in root nodule and root of the legume pulse *Phaseolus mungo*. Current Microbiology 56: 495–498.

Ghosh S, Saha J, Biswas AK (2013) Interactive influence of arsenate and selenate on growth and nitrogen metabolism in wheat (*Triticum aestivum* L.) seedlings. Acta Physiologiae Plantarum 35: 1873–1885.

Gill PK, Sharma AD, Singh P, Bhullar SS (2001) Effect of various abiotic stresses on the growth, soluble sugars and water relations of sorghum seedlings grown in light and darkness. Bulgarian Journal of Plant Physiology 27: 72–84.

Gill SS, Anjum NA, Hasanuzzaman M, Gill R, Trivedi DK, Ahmad I, Pereira E, Tuteja N (2013) Glutathione and glutathione reductase: a boon in disguise for plant abiotic stress defense operations. Plant Physiology and Biochemistry 70: 204-212.

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48: 909-930.

Gill SS, Tuteja N (2011) Cadmium stress tolerance in crop plants: probing the role of sulfur. Plant Signaling and Behavior 6: 215–222.

Gomez-Cadenas A, Zentella R, Simmons MKW, Ho THD (2001) Gibberellin/ abscisic acid antagonism in barley aleurone cells: site of action of the protein kinase PKABA1 in relation to gibberellins signaling molecules. Plant Cell 13: 667–679.

Gong H, Zhu X, Chen K, Wang S, Zhang C (2005) Silicon alleviates oxidative damage of wheat plants in pots under drought. Plant Science 169: 313–321.

Gou T, Yang L, Hu W, Chen X, Zhu Y, Guo J, Gong H (2020) Silicon improves the growth of cucumber under excess nitrate stress by enhancing nitrogen assimilation and chlorophyll synthesis. Plant Physiology and Biochemistry 152: 53–61.

Govind G, Seiler C, Wobus U, Sreenivasulu N (2011) Importance of ABA homeostasis under terminal drought stress in regulating grain filling events. Plant Signaling and Behavior 6: 1228-1231.

Graham HD, Thomas LD (1961) Rapid, simple colorimetric method for the determination of micro quantities of gibberellic acid. Journal of Pharmaceutical Sciences 50: 44–48.

Green JD, Narahara HT (1980) Assay of succinate dehydrogenase activity by the tetrazolium method: evaluation of an improved technique in skeletal muscle fractions. Journal of Histochemistry and Cytochemistry 28: 408–412.

Grieve CM, Grattan SR (1983) Rapid assay for determination of water soluble quaternary ammonium compounds. Plant Soil 70: 303–307.

Gu HH, Qiu H, Tian T, Zhan SS, Deng TH, Chaney RL, Wang SZ, Tang YT, Morel JL, Qiu RL (2011) Mitigation effects of silicon rich amendments on heavy metal accumulation in rice (*Oryza sativa* L.) planted on multi-metal contaminated acidic soil. Chemosphere 83: 1234-1240.

Guilfoyle T, Hagen G, Gazzarrini S, Tsai AYL (2015) Hormone crosstalk during seed germination. Essays in Biochemistry 58: 151–164.

Gunes A, Pilbeam DJ, Inal A, Bagci EG, Coban S (2007a) Influence of silicon on antioxidant mechanisms and lipid peroxidation in chickpea (Cicer arietinum L.) cultivars under drought stress. Journal of Plant Interactions 2: 105–113.

Gunes A, Inal A, Bagci EG, Pilbeam DJ (2007b) Silicon-mediated changes of some physiological and enzymatic parameters symptomatic for oxidative stress in spinach and tomato grown in sodic-B toxic soil. Plant and Soil 290: 103-114.

Gunes A, Pilbeam DJ, Inal A, Coban S (2008) Influence of silicon on sunflower cultivars under drought stress, I: growth, antioxidant mechanisms, and lipid peroxidation. Communications in Soil Science and Plant Analysis 39: 1885–1903.

Guo Y, Song Y, Zheng H, Zhang Y, Guo J, Sui N (2018) NADP-malate dehydrogenase of sweet sorghum improves salt tolerance of *Arabidopsis thaliana*. Journal of Agricultural and Food Chemistry 66: 5992–6002.

Gupta B, Huang B (2014) Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. International Journal of Genomics 2014: 701596.

Gupta S, Banerjee S (2011) Fluoride accumulation in crops and vegetables and dietary intake in a fluoride-endemic area of West Bengal. Fluoride 44: 153–157.

Gupta S, Banerjee S, Mondal S (2009). Phytotoxicity of fluoride in the germination of paddy (Oryza sativa) and its effect on the physiology and biochemistry of germinated seedlings, Fluoride 42: 142-146.

Gupta SK, Deshpande RD, Agarwal M, Raval BR (2005) Origin of high fluoride in groundwater in the North Gujarat-Cambay region, India. Hydrogeology Journal 13: 596–605.

Habibi G (2015) Effects of soil-and foliar-applied silicon on the resistance of grapevine plants to freezing stress. Acta Biologica Szegediensis 59: 109–117.

Hageman RH, Reed AJ (1980) Nitrate reductase from higher plants. Methods in Enzymology 69: 270–280.

Haghighi M, Afifipour Z, Mozafarian M (2012) The alleviation effect of silicon on seed germination and seedling growth of tomato under salinity stress. Vegetable Crops Research Bulletin 76: 119-126.

Hajam YA, Lone R, Kumar R (2023) Role of plant phenolics against reactive oxygen species (ROS) induced oxidative stress and biochemical alterations. In: Lone R, Khan S, Mohammed Al-Sadi A (eds) Plant Phenolics in Abiotic Stress Management. Springer, Singapore, pp 125-147.

Halfter U, Ishitani M, Zhu J-K (2000) The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium binding protein SOS3. Proceedings of the National Academy of Sciences USA 97: 3735–3740.

Hancock JT (2019) Hydrogen sulfide and environmental stresses. Environmental and Experimental Botany 161: 50-56.

Hasanuzzaman M, Alam MM, Nahar K, Mohsin SM, Bhuyan MHMB, Parvin K, Nowak-Hawrylak B, Fujita M (2019) Silicon-induced antioxidant defense and methylglyoxal detoxification works coordinately in alleviating nickel toxicity in *Oryza sativa* L. Ecotoxicology 28: 261–276.

Hasanuzzaman M, Fujita M (2012) Heavy metals in the environment: current status, toxic effects on plants and possible phytoremediation. In: Anjum NA, Pereira MA, Ahmad I, Duarte AC, Umar S Khan NA (eds) Phyto-technologies: Remediation of Environmental Contaminants. Boca Raton, FL, USA: CRC Press, pp 7-73.

Hasanuzzaman M, Hossain MA, da Silva JAT, Fujita M (2012). Plant response and tolerance to abiotic oxidative stress: Antioxidant defense is a key factor. In Bandi V, Shanker AK, Shanker C, Mandapaka M (eds) Crop Stress and Its Management: Perspectives and Strategies, Berlin, Germany: Springer, pp 261–316.

Hasanuzzaman M, Hossain MS, Bhuyan MHMB, Al Mahmud J, Nahar K, Fujita M (2018) The role of sulfur in plant abiotic stress tolerance: molecular interactions and defense mechanisms. In: Hasanuzzaman M, Fujita M, Oku H, Nahar K, Hawrylak-Nowak B (eds) Plant Nutrients and Abiotic Stress Tolerance. Springer, Singapore, pp 221-252.

Hasanuzzaman M, Nahar K, Alam M, Roychowdhury R, Fujita M (2013) Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. International Journal of Molecular Sciences 14: 9643–9684.

Hasthanasombut S, Paisarnwipatpong N, Triwitayakorn K, Kirdmanee C, Supaibulwatana K (2011) Expression of *OsBADH1* gene in Indica rice (*Oryza sativa* L.) in correlation with salt, plasmolysis, temperature and light stresses. Plant Omics Journal 4: 400–407.

He L, Li B, Lu X, Yuan L, Yang Y, Yuan Y, Du J, Guo S (2015) The effect of exogenous calcium on mitochondria, respiratory metabolism enzymes and ion transport in cucumber roots under hypoxia. Scientific Reports 5: 11391.

He M, He C-Q, Ding N-Z (2018) Abiotic stresses: general defenses of land plants and chances for engineering multistress tolerance. Frontiers in Plant Science 9: 1771.

Hem JD (1985) Study and Interpretation of the Chemical Characteristics of Natural Water; Department of the Interior, US Geological Survey: Washington, DC, USA, Volume 2254.

Hirschi KD (2004) The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiology 136: 2938–2492.

Hong BD, Joo RN, Lee KS, Lee DS, Rhie JH, Min SW, Song SG, Chung DY (2016) Fluoride in soil and plant. Korean Journal of Agricultural Science 43: 522–536.

Hoshida H, Tanaka Y, Hibino T, Hayashi Y, Tanaka A, Takabe T, Takabe T (2000) Enhanced tolerance to stress tolerance in transgenic rice that overexpresses chloroplast glutamine synthetase. Plant Molecular Biology 43: 103–111.

Hosseini SA, Rethore E, Pluchon S, Ali N, Billiot B, Yvin JC (2019) Calcium application enhances drought stress tolerance in sugar beet and promotes plant biomass and beetroot sucrose concentration. International Journal of Molecular Sciences 20: 3777.

Hryvusevich P, Navaselsky I, Talkachova Y, Straltsova D, Keisham M, Viatoshkin A, Samokhina V, Smolich I, Sokolik A, Huang X, Yu M, Bhatla SC, Demidchik V (2021) Sodium influx and potassium efflux currents in sunflower root cells under high salinity. Frontiers in Plant Science 11: 613936.

Hu W, Su Y, Zhou J, Zhu H, Guo J, Huo H, Gong H (2022) Foliar application of silicon and selenium improves the growth, yield and quality characteristics of cucumber in field conditions. Scientia Horticulturae 294: 110776.

Hu ZJ, Li JX, Ding ST, Cheng F, Li X, Jiang YP, Yu JQ, Foyer CH, Shi K (2021) The protein kinase CPK28 phosphorylates ascorbate peroxidase and enhances thermotolerance in tomato. Plant Physiology 186: 1302–1317.

Huang TL, Huang HJ (2008) ROS and CDPK-like kinase-mediated activation of MAP kinase in rice roots exposed to lead. Chemosphere 71: 1377–1385.

Hubbard NL, Huber SC, Pharr DM (1989) Sucrose phosphate synthase and acid invertase as determinant of sucrose concentration in developing muskmelon (*Cucumis melo* L.) fruits. Plant Physiology 91: 1527–1534.

Hummel JP (1949) The fluorometric determination of malic acid. Journal of Biological Chemistry 180: 1225–1228.

Hussain S, Khan F, Hussain HA, Nie L (2016) Physiological and biochemical mechanisms of seed priming-induced chilling tolerance in rice cultivars. Frontiers in Plant Science 7: 1–14.

Hussain TM, Hazara M, Sultan Z, Saleh BK, Gopal GR (2008) Recent advances in salt stress biology a review. Biotechnology and Molecular Biology Reviews 3: 8–13.

Iqbal MA (2015) Role of moringa, brassica and sorghum water extracts in increasing crops growth and yield: a review. American-Eurasian Journal of Agricultural & Environmental Science 14: 1150–1158.

Iqbal Z, Iqbal MS, Singh SP, Buaboocha T (2020) Ca^{2+}/Calmodulin complex triggers CAMTA transcriptional machinery under stress in plants: Signaling cascade and molecular regulation. Frontiers in Plant Science 11: 16.

Islam T, Manna M, Kaul T, Pandey S, Reddy CS, Reddy MK (2015) Genome-wide dissection of Arabidopsis and rice for the identification and expression analysis of glutathione peroxidases reveals their stress-specific and overlapping response patterns. Plant Molecular Biology Reporter 33: 1413–1427.

Iwasaki K, Maier P, Fecht M, Horst WJ (2002) Leaf apoplastic silicon enhances manganese tolerance of cow-pea (*Vigna unguiculata*) Journal of Plant Physiology 159: 167–173.

Jacoby RP, Millar AH, Taylor NL (2013) Investigating the role of respiration in plant salinity tolerance by analyzing mitochondrial proteomes from wheat and a salinity tolerant amphiploid (Wheat × *Lophopyrum elongatum*). Journal of Proteome Research 12: 4807–4829.

Jiang M, Zhang J (2002) Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. Journal of Experimental Botany 53: 2401–2410.

Jiang XY, Omarov T, Yesbergenova SZ, Sagi M (2004) The effect of molybdate and tungstate in the growth medium on abscisic acid content and the Mo-hydroxylases activities in barley (*Hordeum vulgare* L.). Plant Sci 167: 297–300.

Jiang Y, Huang B (2001) Effects of calcium on antioxidant activities and water relations associated with heat tolerance in two cool-season grasses. Journal of Experimental Botany 52: 341–349.

Jing X, Cai CJ, Fan SH, Wang LJ, Zeng XL (2019) Spatial and temporal calcium signaling and its physiological effects in Moso Bamboo under drought stress. Forests 10: 224.

Joudmand A, Hajiboland R (2019) Silicon mitigates cold stress in barley plants via modifying the activity of apoplasmic enzymes and concentration of metabolites. Acta Physiologiae Plantarum 41: 29.

Kashyap SJ, Sankannavar R, Madhu GM (2021) Fluoride sources, toxicity and fluorosis management techniques- A brief review. Journal of Hazardous Materials Letters 2: 100033.

Katz O (2018) Plant silicon and phytolith research and the Earth-life super discipline. Frontiers in Plant Science 9: 1281.

Keller C, Rizwan M, Davidian JC, Pokrovsky OS, Bovet N, Chaurand P, Meunier JD (2015) Effect of silicon on wheat seedlings (*Triticum turgidum* L.) grown in hydroponics and exposed to 0 to 30 µM Cu. Planta 241: 847–860.

Khan A, Kamran M, Imran M, Al-Harrasi A, Al-Rawahi A, Al-Amri I, In-Jung L, Khan AL (2019) Silicon and salicylic acid confer high-pH stress tolerance in tomato seedlings. Scientific Reports 9.

Khan A, Khan AL, Imran M, Asaf S, Kim YH, Bilal S, Numan M, Al-Harrasi A, Al-Rawahi A, Lee IJ (2020) Silicon-induced thermotolerance in *Solanum lycopersicum* L. via activation of antioxidant system, heat shock proteins, and endogenous phytohormones. BMC Plant Biology 20: 248.

Kim YH, Khan AL, Lee IJ (2016) Silicon: a duo synergy for regulating crop growth and hormonal signaling under abiotic stress conditions. Critical Reviews in Biotechnology 36: 1099–1109.

Kim YH, Khan AL, Waqas M, Lee IJ (2017) Silicon regulates antioxidant activities of crop plants under abiotic-induced oxidative stress: a review. Frontier in Plant Science 8: 510.

Kim YH, Khan AL, Waqas M, Shim JK, Kim DH, Lee KY, Lee IJ (2014) Silicon application to rice root zone influenced the phytohormonal and antioxidant responses under salinity stress. Journal of Plant Growth Regulation 33:137–149.

Kinraide TB (1998) Three mechanisms for the calcium alleviation of mineral toxicities. Plant Physiology 118: 513–520.

Kocsy G, Szalai G, Galiba G (2002) Induction of glutathione synthesis and glutathione reductase activity by abiotic stresses in maize and wheat. The Scientific World Journal 2: 1726–1732.

Kollárová K, Kusa Z, Vatehova-Vivodova Z, Liskova D (2019) The response of maize protoplasts to cadmium stress mitigated by silicon. Ecotoxicology and Environmental Safety 170: 488–494.

Kolupaev YE, Akinina GE, Mokrousov AV (2005) Induction of heat tolerance in wheat coleoptiles by calcium ions and its relation to oxidative stress. Russian Journal of Plant Physiology 52: 199–204.

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. Journal of Experimental Botany 63: 1593–1608.

Kuk YI, Shin JS, Burgos NR, Hwang TE, Han O, Cho BH, Jung S, Guh JO (2003) Antioxidative Enzymes offer protection from chilling damage in rice plants. Crop Science 43: 2109-2117.

Kukreja S, Nandwal AS, Kumar N, Sharma SK, Sharma SK, Unvi V, Sharma PK (2005) Plant water status, H_2O_2 scavenging enzymes, ethylene evolution and membrane integrity of *Cicer arietinum* roots as affected by salinity. Biologia Plantarum 49: 305–308.

Kumar A, Kumari S (2011) Fluoride toxicity enhances phagocytic activity of macrophages in spleen of rats. Society of Applied Sciences 2: 283–287.

Kumar KA, Rao AVB (2008) Physiological responses to fluoride in two cultivars of Mulberry. World Journal of Agricultural Sciences 4: 463–466.

Kumar KA, Varaprasad P, Rao AVB (2009) Effect of fluoride on catalase, guiacol peroxidase and ascorbate oxidase activities in two verities of mulberry leaves (*Morus alba* L.). Research Journal of Earth Sciences 1: 69–73.

Kumar R, Sinha R, Sharma PK, Ivy N, Kumar P, Kant N, Jha A, Jha PK, Gupta PK, Sharma P, Singh RK, Singh RP, Ghosh A, Vara Prasad PV (2021) Bioaccumulation of fluoride in plants and its microbially assisted remediation: A review of biological processes and technological performance. Processes 9: 2154.

Kumar RG, Shah K, Dubey RS (2000) Salinity induced behavioural changes in malate dehydrogenase and glutamate dehydrogenase activities in rice seedlings of differing salt tolerance. Plant Science 156: 23–34.

Kumar S, Singh M (2015) Effect of fluoride contaminated irrigation water on eco-physiology, biomass and yield in *Gossypium hirsutum* L. Tropical Plant Research 2: 134–142.

Kumaran S, Francois JA, Krishnan HB, Jez JM (2008) Regulatory protein-protein interactions in primary metabolism: the case of the cysteine synthase complex. In: Khan NA, Singh S, Umar S (eds) Sulfur assimilation and abiotic stresses in plants. Springer, The Netherlands, pp 97-109.

Kundu MC, Mandal B (2009) Assessment of potential hazards of fluoride contamination in drinking groundwater of an intensively cultivated district in West Bengal, India. Environmental Monitoring and Assessment 152: 97–103.

Kundu N, Panigrahi MK, Tripathy S, Munshi S, Powell MA, Hart BR (2001) Geochemical appraisal of fluoride contamination of groundwater in the Nayagarh district of Orissa, India. Environmental Geology 41: 451–460.

Kuromori T, Fujita M, Takahashi F, Yamaguchi-Shinozaki K, Shinozaki K (2022) Inter-tissue and inter-organ signaling in drought stress response and phenotyping of drought tolerance. Plant Journal 109: 342–358.

Kusano M, Fukushima A, Redestig H, Saito K (2011) Metabolomic approaches toward understanding nitrogen metabolism in plants. Journal of Experimental Botany 62: 1439–1453.

Kusano T, Yamaguchi K, Berberich T, Takahashi Y (2007) Advances in polyamine research in 2007. Journal of Plant Research 120: 345–350.

Kushwaha BK, Singh VP (2020) Glutathione and hydrogen sulfide are required for sulfur-mediated mitigation of Cr(VI) toxicity in tomato, pea and brinjal seedlings. Physiologia Plantarum 168: 406–421.

Lalhminghlui K, Jagetia GC (2018) Evaluation of the free-radical scavenging and antioxidant activities of Chilauni, *Schima wallichii* Korth in vitro. Future Science OA 4: FSO272.

Larkindale J, Knight MR (2002) Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiology 128: 682–695.

Leustek T, Murillo M, Cervantes M (1994) Cloning of a cDNA encoding ATP sulfurylase from *Arabidopsis thaliana* by functional expression in *Saccharomyces cerevisiae*. Plant Physiology 105: 897-902.

Levine RL, Williams JA, Stadtman ER, Shacter E (1994) Carbonyl assays for determination of oxidatively modified proteins. Methods in Enzymology 233: 346-357.

Li C, Liang D, Chang C, Jia D, Ma F (2015) Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behavior in two *Malus* species under drought stress. Journal of Experimental Botany 66: 669–680.

Li H, Mo Y, Cui Q (2019) Transcriptomic and physiological analyses reveal drought adaptation strategies in drought-tolerant and susceptible watermelon genotypes. Plant Science 278: 32–43.

Li Q, Gao Y, Yang A (2020) Sulfur Homeostasis in Plants. International Journal of Molecular Sciences 21: 8926.

Li R, Zhang J, Wei J, Wang H, Wang Y, Ma R (2009a) Functions and mechanisms of the CBL-CIPK signalling system in plant response to abiotic stress. Proceedings of the National Academy of Sciences USA 19: 667–676.

Li T-L, Li M, Sun Z-P (2009b) Regulation effect of calcium and salicylic acid on defense enzyme activities in tomato leaves under sub-high temperature stress. Yingyong Shengtai Xuebao 20: 586–590.

Li W, Sun Z-H, Zhang W-C, Ma X-T, Liu D-H (1997) Role of Ca^{2+} and calmodulin on freezing tolerance of citrus protoplasts. Acta Biochimica et Biophysica Sinica 23: 262–266.

Li Y, Liu Y, Jin L, Peng R (2022) Crosstalk between Ca^{2+} and other regulators assists plants in responding to abiotic stress. Plants 11: 1351.

Li ZG (2015) Quantification of hydrogen sulfide concentration using methylene blue and 5, 5-dithiobis (2-NitrobenzoicAcid) methods in plants. Methods in Enzymology 554: 101-110.

Li ZG, Gong M, Liu P (2012) Hydrogen sulfide is a mediator in H_2O_2-induced seed germination in *Jatropha curcas*. Acta Physiologiae Plantarum 34: 2207–2213.

Li ZG, Gong M, Xie H, Yang L, Li J (2012) Hydrogen sulfide donor sodium hydrosulfide-induced heat tolerance in tobacco (*Nicotiana tabacum* L.) suspension cultured cells and involvement of Ca^{2+} and calmodulin. Plant Science 185: 185–189.

Liang Y, Nikolic M, Bélanger R, Gong H, Song A (2015) Silicon in agriculture: from theory to practice. Dordrecht: Springer.

Liang Y, Sun W, Zhu YG, Christie P (2007) Mechanisms of silicon-mediated alleviation of abiotic stresses in higher plants: a review. Environmental Pollution 147: 422–428.

Lin C-L, Chen H-J, Hou W-C (2002) Activity staining of glutathione peroxidase after electrophoresis on native and sodium dodecyl sulfate polyacrylamide gels. ELECTROPHORESIS 23: 513–516.

Liu J, Shabala S, Zhang J, Ma G, Chen D, Shabala L, Zeng F, Chen ZH, Zhou M, Venkataraman G, Zhao Q (2020) Melatonin improves rice salinity stress tolerance by NADPH oxidase-dependent control of the plasma membrane K^+ transporters and K^+ homeostasis. Plant Cell and Environment 43: 2591–2605.

Liu J-J, Lin S-H, Xu P-L, Wang X-J, Bai J-G (2009) Effects of exogenous silicon on the activities of antioxidant enzymes and lipid peroxidation in chilling-stressed cucumber leaves. Agricultural Science in China 8: 1075–1086.

Liu Q, Yan S, Huang W, Yang J, Dong J, Zhang S, Zhao J, Yang T, Mao X, Zhu X, Liu B (2018) NAC transcription factor ONAC066 positively regulates disease resistance by suppressing the ABA signaling pathway in rice. Plant Molecular Biology 98: 289-302.

López M, Tejera NA, Iribarne C, Lluch C, Herrera-Cervera JA (2008) Trehalose and trehalase in root nodules of *Medicago truncatula* and *Phaseolus vulgaris* in response to salt stress. Physiologia Plantarum 134: 575–582.

Lopez-Bucio J, Nieto-Jacobo MF, Ramırez-Rodrıguez V, Herrera-Estrella L (2000) Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Science 160: 1–13.

Lu J, Zhang L, Lewis RS, Bovet L, Goepfert S, Jack AM, Crutchfield JD, Ji H, Dewey RE (2016) Expression of a constitutively active nitrate reductase variant in tobacco reduces tobacco-specific nitrosamine accumulation in cured leaves and cigarette smoke. Plant Biotechnology Journal 14: 1500–1510.

Lukacova Z, Svubova R, Kohanova J, Lux A (2013) Silicon mitigates the Cd toxicity in maize in relation to cadmium translocation, cell distribution, antioxidant enzymes stimulation and enhanced endodermal apoplasmic barrier development. Plant Growth Regulation 70: 89–103.

Luo Q, Wei Q, Wang R, Zhang Y, Zhang F, He Y, Yang G, He G (2018) Ectopic expression of BdCIPK31 confers enhanced low-temperature tolerance in transgenic tobacco plants. Acta Biochimica et Biophysica Sinica 50: 199–208.

Luyckx M, Hausman J-F, Lutts S, Guerriero G (2017) Silicon and plants: current knowledge and technological perspectives. Frontiers in Plant Science 8: 411.

Ma D, Sun D, Wang C, Qin H, Ding H, Li Y, Guo T (2016) Silicon application alleviates drought stress in wheat through transcriptional regulation of multiple antioxidant defense pathways. Journal of Plant Growth Regulation 35: 1–10.

Ma D-M, Xu W-R, Li H-W, Jin F-X, Guo L-N, Wang J, Dai H-J, Xu X (2014) Co-expression of the *Arabidopsis* SOS genes enhances salt tolerance in transgenic tall fescue (*Festuca arundinacea* Schreb.). Protoplasma 251: 219–231.

Ma JF, Tamai K, Yamaji N, Mitani N, Konishi S, Katsuhara M, Ishiguro M, Murata Y, Yano M (2006) A silicon transporter in rice. Nature 440: 688–691.

Ma JF, Yamaji N, Mitani-Ueno N (2011) Transport of silicon from roots to panicles in plants. Proceedings of the Japan Academy (Series B: Physical and Biological Sciences) 87: 377–385.

Ma L, Ye J, Yang Y, Lin H, Yue L, Luo J, Long Y, Fu H, Liu X, Zhang Y, Wang Y, Chen L, Kudia J, Wang Y, Han S, Song C-P, Guo Y (2019) The SOS2-SCaBP8 complex generates and fine-tunes an AtANN4-dependent calcium signature under salt stress. Developmental Cell 48: 697–709.

Ma X, Li QH, Yu YN, Qiao YM, Haq SU, Gong ZH (2020) The CBL-CIPK pathway in plant response to stress signals. International Journal of Molecular Sciences 21: 5668.

Mabagala FS, Geng Y, Cao G, Wang L, Wang M, Zhang M (2020) Silicon accumulation, partitioning and remobilisation in spring maize (*Zea mays* L.) under silicon supply with straw return in Northeast China. Journal of Plant Nutrition 44: 1498–1514.

Macnee W, Rahman I (2001) Is oxidative stress central to the pathogenesis of chronic obstructive pulmonary disease? Trends in Molecular Medicine 7: 55–62.

Magome H, Kamiya Y (2018) Inactivation processes. Annual Plant Review Online 73–93.

Mahajan S, Pandey GK, Tuteja N (2008) Calcium- and salt-stress signaling in plants: Shedding light on SOS pathway. Archives of Biochemistry and Biophysics 471: 146–158.

Mahdieh M, Habibollahi N, Amirjani MR, Abnosi MH, Ghorbanpour M (2015) Exogenous silicon nutrition ameliorates salt-induced stress by improving growth and efficiency of PSII in *Oryza sativa* L. cultivars. Journal of Soil Science and Plant Nutrition 15: 1050–1060.

Maheshwari DK, Dheeman S, Agarwal M (2015) Phytohormone-Producing PGPR for Sustainable Agriculture. In: Maheshwari D (eds) Bacterial Metabolites in Sustainable Agroecosystem. Sustainable Development and Biodiversity, vol 12. Springer, Cham, pp 159–182.

Maksimovic JD, Mojović M, Maksimović V, Römheld V, Nikolic M (2012) Silicon ameliorates manganese toxicity in cucumber by decreasing hydroxyl radical accumulation in the leaf apoplast. Journal of Experimental Botany 63: 2411–2420.

Maksymiec W, Baszynski T (1999) Are calcium ions and calcium channels involved in the mechanisms of Cu^{2+} toxicity in bean plants? The influence of leaf age. Photosynthetica 36: 267–278.

Malayeri BE, Noori M, Jafari M (2012) Using the pollen viability and morphology for fluoride pollution biomonitoring. Biological Trace Element Research 147: 315–319.

Mao X, Chen S, Li A, Zhai C, Jing R (2014) Novel NAC transcription factor TaNAC67 confers enhanced multi-abiotic stress tolerances in *Arabidopsis*. PLoS One 9: e84359.

Marbaniang DG, Diengdoh DF, Nongpiur CL (2014) Preliminary investigation of the physico-chemical properties of a geothermal spring (hot spring) located at Jakrem, Meghalaya, India. Keanean Journal of Science 3: 35–42.

Margaretha JA, Irawan C, Lestari PS, Rahma A, Tanjung E (2018) The effectiveness of calcium hydroxide and sodium hydroxide as neutralizer in coagulation for reducing fluoride in hazardous wastewater. International Journal of Chemical Science 6: 786–791.

Marxen A, Klotzbucher T, Jahn R, Kaiser K, Nguyen VS, Schmidt A, Schadler M, Vetterlein D (2015) Interaction between silicon cycling and straw decomposition in a silicon deficient rice production system. Plant and Soil 398: 153–163.

Matos IF, Morales LMM, Santana DB, Silva GMC, Gomes MMdA, Ayub RA, Costa JH and Oliveira JGd (2022) Ascorbate synthesis as an alternative electron source for mitochondrial respiration: Possible implications for the plant performance. Frontier in Plant Science 13: 987077.

Matthus E, Wilkins KA, Swarbreck SM, Doddrell NH, Doccula FG, Costa A, Davies JM (2019) Phosphate starvation alters abiotic-stress-induced cytosolic free calcium increases in roots. Plant Physiology 179: 1754–1767.

Mauad M, Crusciol CAC, Nascente AS, Filho HG, Lima GPP (2016) Effects of silicon and drought stress on biochemical characteristics of leaves of upland rice cultivars. RCA 47: 532–539.

McCready RM, Guggolz J, Silviera V, Owens HS (1950) Determination of starch and amylase in vegetables. Analytical Chemistry 22: 1156–1158.

Medvedev SS (2005) Calcium signaling system in plants. Russian Journal of Plant Physiology 52: 249–270.

Mehrotra R, Bhalothia P, Bansal P, Basantani MK, Bharti V, Mehrotra S (2014) Abscisic acid and abiotic stress tolerance- different tiers of regulation. Journal of Plant Physiology 171: 486-496.

Meng H, Zhang T, Liu W, Wang H, Wang C, Zhao Z, Liu N, Wang W (2014) Sodium fluoride induces apoptosis through the downregulation of hypoxia-inducible factor-1α in primary cultured rat chondrocytes. International Journal of Molecular Medicine 33: 351–358.

Metwally AM, Radi AA, El-Shazoly RM, Hamada AM (2018) The role of calcium, silicon and salicylic acid treatment in protection of canola plants against boron toxicity stress. Journal of Plant Research 131: 1015-1028.

Millar AH, Mittova V, Kiddle G, Heazlewood JL, Bartoli CG, Theodoulou FL, Foyer CH (2003) Control of ascorbate synthesis by respiration and its implications for stress responses. Plant Physiology 133: 443–447.

Mir RA, Bhat BA, Yousuf H, Islam ST, Raza A, Rizvi MA, Charagh S, Albaqami M, Sofi PA, Zargar SM (2022) Multidimensional role of silicon to activate resilient plant growth and to mitigate abiotic stress. Frontiers in Plant Science 13: 819658.

Miron D, Schaffer AA (1991) Sucrose phosphate synthase, sucrose synthase and invertase activities in developing fruit of *Lycopersicon esculentum* mill. and the sucrose accumulating *Lycopersicon hirsutum* humb. and bompl. Plant Physiology 95: 623–627.

Mishra M, Wungrampha S, Kumar G, Singla-Pareek SL, Pareek A (2020) How do rice seedlings of landrace Pokkali survive in saline fields after transplantation? Physiology, biochemistry, and photosynthesis. Photosynthesis Research 150: 117-135.

Misra N, Gupta AK, Dwivedi UN (2006) Changes in free amino acids and stress protein synthesis in two genotypes of green gram under salt stress. Journal of Plant Sciences 1: 56–66.

Molla MR, Ali MR, Hasanuzzaman M, Al-Mamun MH, Ahmed A, Nazim-Ud-Dowla M, Rohman MM (2014) Exogenous proline and betaine-induced upregulation of glutathione transferase and glyoxalase i in lentil (*Lens culinaris*) under drought stress. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 42: 73–80.

Moller IM, Jensen PE, Hansson A (2007) Oxidative modifications to cellular components in plants. Annual Review of Plant Biology 58: 459–481.

Moradtalab N, Weinmann M, Walker F, Höglinger B, Ludewig U, Neumann G (2018) Silicon improves chilling tolerance during early growth of maize by effects on micronutrient homeostasis and hormonal balances. Frontiers in Plant Science 9: 420.

Mortensen A, Skibsted LH, Truscott TG (2001) The interaction of dietary carotenoids with radical species. Archives of Biochemistry and Biophysics 385: 13–19.

Mukherjee I, Singh UK (2018) Groundwater fluoride contamination, probable release, and containment mechanisms: a review on Indian context. Environmental Geochemistry and Health 40: 2259–2301.

Muneer S, Jeong BR (2015) Proteomic analysis of salt-stress responsive proteins in roots of tomato (*Lycopersicon esculentum* L.) plants towards silicon efficiency. Plant Growth Regulation 77: 133–146.

Nageshbabu R, Jyothi MN (2013) Profile of small interfering RNAs from french bean *Phaseolus vulgaris* under abiotic stress conditions. International Journal of Pharma and Bio Sciences 4: 176–185.

Nahar K, Hasanuzzaman M, Rahman A, Alam MM, Mahmud JA, Suzuki T, Fujita M (2016) Polyamines confer salt tolerance in mung bean (*Vigna radiata* L.) by reducing sodium uptake, improving nutrient homeostasis, antioxidant defense, and methylglyoxal detoxification systems. Frontiers in Plant Science 7: 1104.

Nakamura A, Fukuda A, Sakai S, Tanaka Y (2006) Molecular cloning, functional expression and subcellular localization of two putative vacuolar voltage-gated chloride channels in rice (*Oryza sativa* L.). Plant and Cell Physiology 47: 32–42.

Nakano Y, Asada K (1981) Hydrogen-peroxide is scavenged by ascorbate-specific peroxidase in spinach-chloroplasts. Plant Cell and Physiology 22: 867–880.

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. Plant Physiology 149: 88–95.

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annual Review of Plant Biology 56: 165–185.

Nawaz M, Wang Z (2020) Abscisic acid and glycine betaine mediated tolerance mechanisms under drought stress and recovery in *Axonopus compressus*: a new insight. Scientific Reports 10: 6942.

Ndayiragije A, Lutts S (2006) Do exogenous polyamines have an impact on the response of a salt-sensitive rice cultivar to NaCl? Journal of Plant Physiology 163: 506–516.

Nikolic M, Nikolic N, Liang Y, Kirkby EA, Romheld V (2007) Germanium-68 as an adequate tracer for silicon transport in plants. Characterization of silicon uptake in different crop species. Plant Physiology 143: 495–503.

North HM, De Almeida A, Boutin JP, Frey A, To A, Botran Sotta B, Marion-Poll A (2007) The *Arabidopsis* ABA-deficient mutant aba4 demonstrates that the major route for stress-induced ABA accumulation is via neoxanthin isomers. Plant Journal 50: 810–824.

Nunes-Nesi A, Araújo WL, Obata T, Fernie AR (2013) Regulation of the mitochondrial tricarboxylic acid cycle. Current Opinion in Plant Biology 16: 335–343.

Oshino N, Jamieson D, Sugano T, Chance B (1975) Optical measurement of the catalase-hydrogen peroxide intermediate (Compound I) in the liver of anaesthetized rats and its implication to hydrogen peroxide production in situ. Biochemical Journal 146: 67–77.

Ozsvath DL (2009) Fluoride and environmental health: A review. Reviews in Environmental Science & Biotechnology 8: 59–79.

Pal M, Tajti J, Szalai G, Peeva V, Vegh B, Janda T (2018) Interaction of polyamines, abscisic acid and proline under osmotic stress in the leaves of wheat plants. Scientific Reports 8: 12839.

Panda D (2015) Fluoride toxicity stress: physiological and biochemical consequences on plants. International Journal of Bio-resource, Environment and Agricultural Sciences 1: 70-84.

Parida AK, Das AB, Sanada Y, Mohanty P (2004) Effects of salinity on biochemical components of the mangrove, *Aegiceras corniculatum*. Aquatic Botany 80: 77-87.

Parkhey S, Naithani SC, Keshavkant S (2014) Protein metabolism during natural ageing in desiccating recalcitrant seeds of *Shorea robusta*. Acta Physiologiae Plantarum 36: 1649-1659.

Paschalidis KA, Roubelakis-Angelakis KA (2005) Sites and regulation of polyamine catabolism in the tobacco plant. Correlations with cell division/expansion, cell cycle progression, and vascular development. Plant Physiology 138: 2174–2184.

Passaia G, Fonini LS, Caverzan A, Jardim-Messeder D, Christoff AP, Gaeta ML, Je DAM, Margis R, Margis-Pinheiro M (2013) The mitochondrial glutathione peroxidase GPX3 is essential for H_2O_2 homeostasis and root and shoot development in rice. Plant Science 208:93–101.

Patra N, Hariharan S, Gain H, Maiti MK, Das A, Banerjee J (2021) Typical but delicate Ca^{++}re: dissecting the essence of calcium signaling network as a robust response coordinator of versatile abiotic and biotic stimuli in plants. Frontiers in Plant Science 12: 752246.

Paul S, Guha T, Dey S, Paul S, Kundu R (2022) Amelioration of cadmium toxicity by enhancing nitrogen assimilation and photosynthetic activity by two different nitrogen supplements in rice (*Oryza sativa* L.) cv. Lalat. Plant Stress 4: 100082.

Paul S, Roychoudhury A (2017) Seed priming with spermine and spermidine regulates the expression of diverse groups of abiotic stress-responsive genes during salinity stress in the seedlings of indica rice varieties. Plant Gene 11: 124–132.

Paul S, Roychoudhury A (2019) Transcript analysis of abscisic acid inducible genes in response to different abiotic disturbances in two indica rice varieties. Theoretical and Experimental Plant Physiology 31: 249–272.

Paul S, Roychoudhury A, Banerjee A, Chaudhuri N, Ghosh P (2017) Seed pre-treatment with spermidine alleviates oxidative damages to different extent in the salt (NaCl)-stressed seedlings of three indica rice cultivars with contrasting level of salt tolerance. Plant Gene 11: 112–123.

Pemmaraju M, Rao SM (2011) De-fluoridation of groundwater using magnesium oxide. Environmentalist 31: 39–53.

Peng A, Yu K, Yu S, Li Y, Zuo H, Li P, Li J, Huang J, Liu Z, Zhao J (2023) Aluminum and fluoride stresses altered organic acid and secondary metabolism in tea (*Camellia sinensis*) plants: Influences on plant tolerance, tea quality and safety. International Journal of Molecular Sciences 24: 4640.

Pettenati M, Perrin J, Pauwels H, Ahmed S (2013) Simulating fluoride evolution in groundwater using a reactive multicomponent transient transport model: Application to a crystalline aquifer of southern India. Applied Geochemistry 29: 102–116.

Phimchan P, Chanthai S, Bosland PW, Techawongstien S (2004) Enzymatic changes in phenylalanine ammonia-lyase, cinnamic-4-hydroxylase, capsaicin synthase, and peroxidase activities in Capsicum under drought stress. Journal of Agricultural and Food Chemistry 62: 7057-7062.

Pickering W (1985) The mobility of soluble fluoride in soils. Environmental Pollution Series B, Chemical and Physical 9: 281–308.

Pignocchi C, Fletcher JM, Wilkinson JE, Barnes JD, Foyer CH (2003) The function of ascorbate oxidase in tobacco. Plant Physiology 132: 1631–1641.

Planchet E, Verdu I, Delahaie J, Cukier C, Girard C, Morère-Le Paven M-C, Limami AM (2014) Abscisic acid-induced nitric oxide and proline accumulation in independent pathways under water-deficit stress during seedling establishment in *Medicago truncatula*. Journal of Experimental Botany 65: 2161–2170.

Pontigo S, Godoy K, Jiménez H, Gutiérrez-Moraga A, Mora ML, Cartes P (2017) Silicon-mediated alleviation of aluminum toxicity by modulation of Al/Si uptake and antioxidant performance in ryegrass plants. Frontiers in Plant Science 8: 642.

Poovaiah BW, Reddy ASN, Leopold AC (1987) Calcium messenger system in plants. CRC Critical Reviews in Plant Sciences 6: 47–103.

Puchegger S, Redl B, Stöffler G (1990) Purification and properties of a thermostable fumarate hydratase from the archaeo bacterium *Sulfolobus solfataricus*. The Journal of General Microbiology 136: 1537–1541.

Quan NT, Anh LH, Khang DT, Tuyen PT, Toan NP, Minh TN, Minh LT, Bach DT, Ha PTT, Elzaawely AA, Khanh TD, Trung KH, Xuan TD (2016) Involvement of secondary metabolites in response to drought stress of rice (*Oryza sativa* L.). Agriculture 6: 23–37.

Radostits OM, Leslie KE, Fetrow J (1994) Herd health—food animal production medicine. Saunders, Philadelphia.

Rady MM (2011) Effect of 24-epibrassinolide on growth, yield, antioxidant system and cadmium 496 content of bean (*Phaseolus vulgaris* L.) plants under salinity and cadmium stress. Scientia Horticulturae 129: 232–237.

Raghavendra AS, Gonugunta VK, Christmann A, Grill E (2010) ABA perception and signalling. Trends in Plant Science 15: 395–401.

Ram A, Verma P, Gadi BR (2014) Effect of fluoride and salicylic acid on seedling growth and biochemical parameters of watermelon (*Citrullus lanatus*). Fluoride 47: 49-55.

Ranjan A, Sinha R, Bala M, Pareek A, Singla-Pareek SL, Singh AK (2021) Silicon-mediated abiotic and biotic stress mitigation in plants: Underlying mechanisms and potential for stress resilient agriculture. Plant Physiology and Biochemistry 163: 15–25.

Ranjan R, Ranjan A (2015) Fluoride toxicity in animals. SpringerBriefs in Animal Sciences (1st ed.). Berlin: Springer.

Rao NS (1997) The occurrence and behaviour of fluoride in the groundwater of the lower Vamsadhara river basin, India. Hydrological Sciences Journal 42: 877–892.

Rastogi A, Yadav S, Hussain S, Kataria S, Hajihashemi S, Kumari P, Yang X, Brestic M (2021) Does silicon really matter for the photosynthetic machinery in plants…? Plant Physiology and Biochemistry 169: 40-48.

Razavi F, Mahmoudi R, Rabiei V, Aghdam MS, Soleimani A (2018) Glycine betaine treatment attenuates chilling injury and maintains nutritional quality of hawthorn fruit during storage at low temperature. Scientia Horticulturae (Amsterdam) 2018: 233188–233194.

Repetto M, Semprine J, Boveris A (2012) Lipid peroxidation: chemical mechanism, biological implications and analytical determination. In Catala A (ed) Lipid peroxidation. Rijeka, Croatia, InTech, pp 3–30.

Reyes JL, Chua N-H (2007) ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. The Plant Journal 49: 592–606.

Rhimi N, Ben AH, Elloumi N, Athar HR, Noreen S, Ashraf M, Ben Abdallah F, Ben Nasri-Ayachi M (2016) Morpho-anatomical and physiological changes in grapevine leafs exposed to atmospheric fluoride and sulfur dioxide pollution. Applied Ecology and Environmental Research 14: 77–89.

Rice-Evans CA, Miller NJ, Paganga G (1997) Antioxidant properties of phenolic compounds. Trends in Plant Sciences 2: 152–159.

Rizwan M, Meunier JD, Miche H, Keller C (2012) Effect of silicon on reducing cadmium toxicity in durum wheat (*Triticum turgidum* L. cv. Claudio W.) grown in a soil with aged contamination. Journal of Hazardous Materials 209–210: 326–334.

Rodrigues FA, Benhamou N, Datnoff LE, Jones JB, Belanger RR (2003) Ultrastructural and cytochemical aspects of silicon-mediated rice blast resistance. Phytopathology 93: 535–546.

Rohanipoor A, Norouzi M, Moezzi A, Hassibi P (2013) Effect of silicon on some physiological properties of maize (*Zea mays*) under salt stress. Journal of Biological and Environmental Sciences 7: 71–79.

Rohilla P, Yadav JP (2019) Acute salt stress differentially modulates nitrate reductase expression in contrasting salt responsive rice cultivars. Protoplasma 256: 1267–1278.

Roychoudhury A, Banerjee A (2017) Abscisic acid signaling and involvement of mitogen activated protein kinases and calcium dependent protein kinases during plant abiotic stress. In: Pandey G (ed) Mechanism of plant hormone signaling under stress, vol 1. Wiley, Hoboken, pp 197–241.

Roychoudhury A, Basu S (2012) Ascorbate-glutathione and plant tolerance to various abiotic stresses. In: Anjum NA, Umar S, Ahmad A (Eds.), Oxidative stress in plants: causes, consequences and tolerance, IK International Publishers, New Delhi, pp. 177-258.

Roychoudhury A, Basu S, Sarkar SN, Sengupta DN (2008) Comparative physiological and molecular responses of a common aromatic indica rice cultivar to high salinity with non-aromatic indica rice cultivars. Plant Cell Reports 27: 1395–1410.

Roychoudhury A, Basu S, Sarkar SN, Sengupta DN (2008) Comparative physiological and molecular responses of a common indica rice cultivar to high salinity with non-aromatic indica rice cultivars. Plant Cell Reports 27: 1395–1410.

Roychoudhury A, Basu S, Sengupta DN (2011) Amelioration of salinity stress by exogenously applied spermidine or spermine in three varieties of indica rice differing in their level of salt tolerance. Journal of Plant Physiology 168: 317-328.

Roychoudhury A, Basu S, Sengupta DN (2012) Antioxidants and stress-related metabolites in the seedlings of two indica rice varieties exposed to cadmium chloride toxicity. Acta Physiologiae Plantarum 34: 835–847.

Roychoudhury A, Ghosh S (2013) Physiological and biochemical responses of mungbean (*Vigna radiata* l. wilczek) to varying concentrations of cadmium chloride or sodium chloride. Unique Journal of Pharmaceutical and Biological Sciences 1: 11–21.

Roychoudhury A, Ghosh S, Paul S, Mazumdar S, Das G, Das S (2016) Pre-treatment of seeds with salicylic acid attenuates cadmium chloride-induced oxidative damages in the seedlings of mungbean (*Vigna radiata* L. Wilczek). Acta Physiologiae Plantarum 38: 11.

Roychoudhury A, Paul S, Basu S (2013) Cross-talk between abscisic acid-dependent and abscisic acid-independent pathways during abiotic stress. Plant Cell Reports 32: 985–1006.

Roychoudhury A, Roy C, Sengupta DN (2007)Transgenic tobacco plants overexpressing the heterologous *lea* gene *Rab16A* from rice during high salt and water deficit display enhanced tolerance to salinity stress. Plant Cell Reports 26: 1839-1859.

Ruan J, Ma L, Shi Y, Han W (2004) The impact of pH and calcium on the uptake of fluoride by tea plants (*Camellia sinensis* L.). Annals of Botany 93: 97–105.

Ruan J, Wong MH (2001) Accumulation of fluoride and aluminium related to different varieties of tea plant. Environmental Geochemistry and Health 23: 53–63.

Sachan P, Lal N (2018) Effect of sodium fluoride on germination, seedling growth and photosynthetic pigments in *Cicer arietinum* L. and *Hordeum vulgare* L. Moj Ecology and Environmental Science 3: 300–304.

Sadasivam S, Manickam A (2008) Biochemical methods, 3rd edn. New Age International Publishers, New Delhi.

Sadeghipour O (2019) Polyamines protect mung bean [*Vigna radiata* (L.) Wilczek] plants against drought stress. Biologia Futura 70: 71–78.

Saeedipour S (2011) Salinity tolerance of rice lines related to endogenous abscisic acid (ABA) level synthesis under stress. African Journal of Plant Science 5: 628–633.

Saffran M, Denstedt O (1948) A rapid method for determination of citric acid. Journal of Biological Chemistry 175: 849–855.

Sagi M, Fluhr R (2001) Superoxide production by plant homologues of the gp91(phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiology 126: 1281–1290.

Saha G, Mostofa MG, Rahman MM, Tran L-S P (2021) Silicon-mediated heat tolerance in higher plants: a molecular outlook. Plant Physiology and Biochemistry 166: 341–347.

Saha P, Kunda P, Biswas AK (2012) Influence of sodium chloride on the regulation of Krebs cycle intermediates and enzymes of respiratory chain in mung bean (*Vigna radiata* L. Wilczek) seedlings. Plant Physiology and Biochemistry 60: 214–222.

Sahebi M, Hanafi MM, Siti Nor Akmar A, Rafii MY, Azizi P, Tengoua FF, Nurul Mayzaitul Azwa J, Shabanimofrad M (2015) Importance of silicon and mechanisms of biosilica formation in plants. Biomed Research International 2015: 396010.

Saito S, Hirai N, Matsumoto C, Ohigashi H, Ohta D, Sakata K, Mizutani M (2004) Arabidopsis CYP707As encode (+)-abscisic acid 8′-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. Plant Physiology 134: 1439–1449.

Saleh AH, Abdel-Kader Z (2003) Metabolic responses of two *Helianthus annus* cultivars to different fluoride concentrations during germination and seedling growth stages. Egyptian Journal of Biological 5: 43–54.

Samanta S, Banerjee A, Roychoudhury A (2021a) Arsenic toxicity is counteracted by exogenous application of melatonin to different extents in arsenic-susceptible and arsenic-tolerant rice cultivars. Journal of Plant Growth Regulation 41: 2210-2231.

Samanta S, Banerjee A, Roychoudhury A (2021b) Melatonin application differentially modulates the enzymes associated with antioxidative machinery and ascorbate-glutathione cycle during arsenate exposure in indica rice varieties. Plant Biology 1: 193-201.

Samanta S, Singh A, Banerjee A, Roychoudhury A (2020) Exogenous supplementation of melatonin alters representative organic acids and enzymes of respiratory cycle as well as sugar metabolism during arsenic stress in two contrasting indica rice cultivars. Journal of Biotechnology 324: 220–232.

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, New York.

Sanchez DH, Siahpoosh MR, Roessner U, Udvardi M, Kopka J (2008) Plant metabolomics reveals conserved and divergent metabolic responses to salinity. Physiologia Plantarum 132: 209–219.

Sanders D, Brownlee C, Harper JF (1999) Communication with calcium. The Plant Cell 11: 691–706.

Sanders D, Pelloux J, Brownlee C, Harper JF (2002) Calcium at the crossroads of signaling. The Plant Cell 14: 401–417.

Santiago FEM, Silva MLS, Cardoso AAS, Duan Y, Guilherme LRG, Liu J, Li L (2020) Biochemical basis of differential selenium tolerance in arugula (*Eruca sativa* Mill.) and lettuce (*Lactuca sativa* L.). Plant Physiology and Biochemistry 157: 328-338.

Sarker U, Oba S (2018) Drought stress enhances nutritional and bioactive compounds, phenolic acids and antioxidant capacity of *Amaranthus* leafy vegetable. BMC Plant Biology 18: 258–273.

Saruhan N, Terzi R, Saglam A, Kadioglu A (2009) The relationship between leaf rolling and ascorbate-glutathione cycle enzymes in apoplastic and symplastic areas of *Ctenanthe setosa* subjected to drought stress. Biological Research 42: 315–326.

Sattar A, Cheema MA, Basra SMA, Wahid A (2013) Optimization of source and rate of soil applied silicon for improving the growth of wheat. Pakistan Journal of Agricultural Science 50: 63–68.

Saxena A, Kulshrestha UC, Kumar N, Kumari KM, Srivastava SS (1994) Distribution of air-borne fluoride: Vapour phase, particulate, precipitation and dry deposition. Environmental Technology 15: 51–59.

Scheringer M (2009) Long-range transport of organic chemicals in the environment. Environmental Toxicology and Chemistry 28: 677–690.

Schnarrenberger C, Martin W (2002) Evolution of the enzymes of the citric acid cycle and the glyoxylate cycle of higher plants: a case study of endosymbiotic gene transfer. European Journal of Biochemistry 269: 868–883.

SCHER (Scientific Committee on Health and Environmental Risks) (2011) Opinion of critical review of any new evidence on the hazard profile, health effects, and human exposure to fluoride and the fluoridating agents of drinking water. Brussels, Belgium: Directorate General for Health and Consumers, European Commission; pp. 2-4.

Selinus O, Alloway B, Centeno JA, Finkelman RB, Fuge R, Lindh U, Smedley P (2016) Essentials of Medical Geology; Springer: Berlin/Heidelberg, Germany.

Selote DS, Chopra RK (2006) Drought acclimation confers oxidative stress tolerance by inducing coordinated antioxidant defense at cellular and subcellular level in leaves of wheat seedlings. Physiologia Plantarum 127: 494–450.

Seo M, Aoki H, Koiwai H, Kamiya Y, Nambara E, Koshiba T (2004) Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. Plant Cell Physiology 45: 1694–1703.

Shahab S, Mustafa G, Khan I, Zahid M, Yasinzai M, Ameer N, Asghar N, Ullah I, Nadhman A, Ahmad A, Munir I, Mujahid A, Hussain T, Ahmad MN, Ahmad SS (2017) Effects of fluoride ion toxicity on animals, plants and soil health: A review. Fluoride 50: 393-408.

Shahid MA, Balal RM, Pervez MA, Abbas T, Aqeel MA, Javaid MM, Garcia-Sanchez F (2014) Exogenous proline and proline-enriched *Lolium perenne* leaf extract protects against phytotoxic effects of nickel and salinity in *Pisum sativum* by altering polyamine metabolism in leaves. Turkish Journal of Botany 38: 914–926.

Shankar PSV, Kulkarni H, Krishnan S (2011) India's groundwater challenge and the way forward. Economic and Political Weekly, xlvi, 37–45.

Shao HB, Chu LY, Lu ZH, Kang CM (2007) Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. International Journal of Biological Sciences 4: 8–14.

Shariat Jafari H, Kafi M, Astaraei AR (2009) Interactive effects of NaCl induced salinity, calcium and potassium on physiomorphological traits of sorghum (*Sorghum bicolor* L.). Pakistan Journal of Botany 41: 3053–3063.

Sharma L, Priya M, Kaushal N, Bhandhari K, Chaudhary S, Dhankher OP, Prasad PVV, Siddique KHM, Nayyar H (2020) Plant growth-regulating molecules as thermoprotectants: functional relevance and prospects for improving heat tolerance in food crops. Journal of Experimental Botany 71: 569-594.

Sharma P and Dubey SR (2005) Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: Role of osmolytes as enzyme protectant. Journal of Plant Physiology 162: 854–864.

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany 2012: 1–26.

Sharma R, Kaur R (2019) Fluoride toxicity triggered oxidative stress and the activation of antioxidative defence responses in *Spirodela polyrhiza* L. Schleiden. Journal of Plant Interactions 14: 440-452.

Sharma R, Kumari A, Rajput S, Nishu Arora S, Rampal R, Kaur R (2019). Accumulation, morpho-physiological and oxidative stress induction by single and binary treatments of fluoride and low molecular weight phthalates in *Spirodela polyrhiza* L. Schleiden. Scientific Reports 9: 20006.

Sheng H, Zeng J, Liu Y, Wang X, Wang Y, Kang H, Fan X, Sha L, Zhang H, Zhou Y (2016) Sulfur mediated alleviation of Mn toxicity in polish wheat relates to regulating Mn allocation and improving antioxidant system. Frontiers in Plant Science 7: 1382.

Shevyakova NI, Musatenko LI, Stetsenko LA, Rakitin VY, Vedenicheva NP, Kuznetsov VV (2013) Effect of ABA on the contents of proline, polyamines, and cytokinins in the common ice plants under salt stress. Russian Journal of Plant Physiology 60: 741–748.

Shi G-Q, Fu J-Y, Rong L-J, Zhang P-Y, Guo C-J, Xiao K (2018) TaMIR1119, a miRNA family member of wheat (*Triticum aestivum*), is essential in the regulation of plant drought tolerance. Journal of Integrative Agriculture 17: 2369–2378.

Shi H, Ye T, Zhong B, Liu X, Chan Z (2014a) Comparative proteomic and metabolomic analyses reveal mechanisms of improved cold stress tolerance in bermudagrass (*Cynodon dactylon* (L.) Pers.) by exogenous calcium. Journal of Integrative Plant Biology 56: 1064-1079.

Shi Y, Zhang Y, Yao H, Wu J, Sun H, Gong H (2014b) Silicon improves seed germination and alleviates oxidative stress of bud seedlings in tomato under water deficit stress. Plant Physiology and Biochemistry 78: 27–36.

Shi X, Zhang C, Wang H, Zhang F (2005) Effect of Si on the distribution of Cd in rice seedlings. Plant and Soil 272: 53–60.

Shivarajashankara YM, Shivashankara AR, Bhat PG, Rao SH (2002) Brain lipid peroxidation and antioxidant systems of young rats in chronic fluoride intoxication. Fluoride 35: 197–203.

Shoresh M, Spivak M, Bernstein N (2011) Involvement of calcium-mediated effects on ROS metabolism in the regulation of growth improvement under salinity. Free Radical Biology & Medicine 51: 1221–1234.

Shu K, Zhou W, Chen F, Luo X, Yang W (2018) Abscisic acid and gibberellins antagonistically mediate plant development and abiotic stress responses. Frontiers in Plant Science 9: 416.

Siddiqui ZS (2013) Effects of double stress on antioxidant enzyme activity in *Vigna radiata* (L.) wilczek. Acta Botanica Croatica 72: 145–156.

Sil P, Das P, Biswas AK (2018) Silicon induced mitigation of TCA cycle and GABA synthesis in arsenic stressed wheat (*Triticum aestivum* L.) seedlings. South African Journal of Botany 119: 340–352.

Sil P, Das P, Biswas AK (2020) Impact of exogenous silicate amendments on nitrogen metabolism in wheat seedlings subjected to arsenate stress. SILICON 12: 535–545.

Singh A, Roychoudhury A (2023) Abscisic acid in plants under abiotic stress: crosstalk with major phytohormones. Plant Cell Reports 42: 961–974.

Singh G, Kumari B, Sinam G, Kriti Kumar N, Mallick S (2018) Fluoride distribution and contamination in the water, soil and plants continuum and its remedial technologies, an Indian perspective– a review. Environmental Pollution 239: 95–108.

Singh K, Chandra A (2021) DREBs-potential transcription factors involve in combating abiotic stress tolerance in plants. Biologia 76: 3043–3055.

Singh N, Ma LQ, Vu JC, Raj A (2009) Effects of arsenic on nitrate metabolism in arsenic hyperaccumulating and non-hyperaccumulating ferns. Environmental Pollution 157: 2300–2305.

Singh VP, Tripathi DK, Kumar D, Chauhan DK (2011) Influence of exogenous silicon addition on aluminium tolerance in rice seedlings. Biological Trace Element Research144: 1260–1274.

Singhal RK, Saha D, Skalicky M, Mishra UN, Chauhan J, Behera LP, Lenka D, Chand S, Kumar V, Dey P, Indu, Pandey S, Vachova P, Gupta A, Brestic M, El Sabagh A (2021) Crucial Cell Signaling Compounds Crosstalk and Integrative Multi-Omics Techniques for Salinity Stress Tolerance in Plants. Frontiers in Plant Science 12: 670369.

Sivanesan I, Son MS, Soundararajan P, Jeong BR (2014) Effect of silicon on growth and temperature stress tolerance of *Nephrolepis exaltata* 'Corditas'. Horticultural Science and Technology 32: 142–148.

Skjelkvale BL (1994) Factors influencing fluoride concentrations in Norwegian lakes. Water, Air, and Soil pollution 77: 151–167.

Smirnoff N, Colombe SV (1988) Drought influences the activity of enzymes of the chloroplast hydrogen peroxide scavenging system. Journal of Experimental Botany 39: 1097–1108.

Smolik B, Telesinski A, Szymczak J, Zakrzewska H (2011) Assessing of humus usefulness in limiting of soluble fluoride content in soil. Environmental Protection and Natural Resources 49: 202–208.

Snell FD, Snell CT (1953) Colorimetric Methods of Analysis, including Some Turbidimetric and Nephelometric Methods, vol III. D. Van Nostrand Co., Inc, Princeton, Jersey, Toronto, New York and London, p 606.

Sogarwal A, Kumari N, Sharma V (2023) Silicon alleviates fluoride induced oxidative damage in wheat cultivars by improving antioxidants defense system and reducing fluoride uptake. Silicon. doi: 10.1007/s12633-023-02412-8.

Søyseth V, Kongerud J (1992) Prevalence of respiratory disorders among aluminium potroom workers in relation to exposure to fluoride. British Journal of Industrial Medicine 49: 125–130.

Spadaro D, Yun BW, Spoel SH, Chu C, Wang YQ, Loake GJ (2010) The redox switch: dynamic regulation of protein function by cysteine modifications. Physiologia Plantarum 138: 360-371.

Sreenivasulu N, Harshavardhan VT, Govind G, Seiler C, Kohli A (2012) Contrapuntal role of ABA: does it mediate stress tolerance or plant growth retardation under long-term drought stress? Gene 506: 265-273.

Srere PA (1969) Citrate synthase: [EC 4.1.3.7. Citrate oxaloacetate-lyase (CoAacetylating)]. In: Methods in enzymology, vol 13. Academic Press, pp 3–11.

Srivastava RK, Pandey P, Rajpoot R, Rani A, Gautam A, Dubey RS (2014) Exogenous application of calcium and silica alleviates cadmium toxicity by suppressing oxidative damage in rice seedlings. Protoplasma 252: 959–975.

Strand A, Hurry V, Henkes S, Huner N, Gustafsson P, Gardeström P, Stitt M (1999) Acclimation of Arabidopsis leaves developing at low temperatures. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiology 119: 1387–1398.

Sulzbach CW, Pack MR (1972) Effect of fluoride on pollen germination, pollen tube growth, and fruit development in tomato and cucumber. Phytopathology 62: 1246–1253.

Susheela AK, Kumar A, Bhatnagar M, Bahudur R (1993) Prevalence of endemic fluorosis with gastro-intestinal manifestations in people living in some north-Indian villages. Fluoride 26: 97–104.

Suzuki N (2005) Alleviation by calcium of cadmium-induced root growth inhibition in *Arabidopsis* seedlings. Plant Biotechnology Journal 22: 19–25.

Suzuki N, Katano K (2018) Coordination between ROS regulatory systems and other pathways under heat stress and pathogen attack. Frontiers in Plant Science 9: 8.

Suzuki N, Mittler R (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiologia Plantarum 126: 45–51.

Szalai G, Kellos T, Galiba G, Kocsy G (2009) Glutathione as an antioxidant and regulatory molecule in plantsunder abiotic stress conditions. Plant Growth Regulation 28: 66–80.

Tak Y, Asthir B (2017) Fluoride-induced changes in the antioxidant defence system in two contrasting cultivars of *Triticum aestivum* L. Fluoride 50: 324–333.

Tale Ahmad S, Haddad R (2011) Study of silicon effects on antioxidant enzyme activities and osmotic adjustment of wheat under drought stress. Czech Journal of Genetics and Plant Breeding 47: 17–27.

Talukdar D (2012) Exogenous calcium alleviates the impact of cadmium-induced oxidative stress in *Lens culinaris* medic. Seedlings through modulation of

antioxidant enzyme activities. Journal of Crop Science and Biotechnology 15: 325–334.

Tan BC, Joseph LM, Deng WT, Liu L, Li QB, Cline K, McCarty DR (2003) Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. Plant Journal 35: 44–56.

Tan X, Fan Z, Kuang J, Lu W, Reiter RJ, Prakash L, Su X-G, Zhou J, Chen J-Y, Shan W (2019) Melatonin delays leaf senescence of Chinese flowering cabbage by suppressing ABFs-mediated abscisic acid biosynthesis and chlorophyll degradation. Journal of Pineal Research 67: e12570.

Tavener SJ, Clark JH (2006) Fluorine: Friend or Foe? A green chemist's perspective. In Tressaud A (ed.), Fluorine and the environment: Agrochemicals, archaeology, green chemistry and water. Amsterdam, Elsevier, pp. 177–202.

Teixeira FK, Menezes-Benavente L, Galvão VC, Margis Pinheiro M (2005) Multigene families encode enzymes of antioxidant metabolism in *Eucalyptus grandis* L. Genetics and Molecular Biology 28: 529–538.

Thomas DM, Hards DK, Rogers SD, Ng KW, Best JD (1996) Insulin receptor expression in bone. Journal of Bone and Mineral Research 11: 1312–1320.

Toh S, Imamura A, Watanabe A, Nakabayashi K, Okamoto M, Jikumaru Y, Hanada A, Aso Y, Ishiyama K, Tamura N, Iuchi S, Kobayashi M, Yamaguchi S, Kamiya Y, Nambara E, Kawakami N (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in *Arabidopsis* seeds. Plant Physiology 146: 1368–1385.

Trewavas AJ, Malho R (1998) Ca^{2+} signalling in plant cells: The big network! Current Opinion in Plant Biology 1: 428–433.

Tripathi DK, Singh VP, Prasad SM, Chauhan DK, Dubey NK, Rai AK (2015) Silicon-mediated alleviation of Cr (VI) toxicity in wheat seedlings as evidenced by chlorophyll florescence, laser induced breakdown spectroscopy and anatomical changes. Ecotoxicology and Environmental Safety 113:133–144.

Trivedi DK, Gill SS, Tuteja N (2016) Abscisic Acid (ABA): Biosynthesis, regulation, and role in abiotic stress tolerance. In Tuteja N, Gill SS (eds) Abiotic Stress Response in Plants. Wiley Wiley-VCH Verlag GmbH & Co. Weinheim, Germany, pp 311-322.

Trivedi N, Mithal A, Gupta SK, Godbole MM (1993) Reversible impairment of glucose tolerance in patients with endemic fluorosis, Diabetologia 36: 826–828.

Tubana BS, Heckman JR (2015) Silicon in soils and plants. In: Rodrigue FA, Datnoff LE (eds.), Silicon and plant diseases. Cham: Springer International Publishing, pp 7-51.

Tuna AL, Kaya C, Higgs D, Murillo-Amador B, Aydemir S, Girgin AR (2008) Silicon improves salinity tolerance in wheat plants. Environmental and Experimental Botany 62: 10–16.

Tuteja N, Mahajan S (2007) Calcium signaling network in plants: an overview. Plant Signalling and Behaviour 2: 79–85.

Tylenda CA (2003) Toxicological Profile for Fluorides, Hydrogen Fluoride, and Fluorine; Agency for Toxic Substances and Disease Registry: Atlanta, GA, USA.

Um TY, Lee S, Kim JK, Jang G, Choi YD (2018) CHLORIDE CHANNEL 1 promotes drought tolerance in rice, leading to increased grain yield. Plant Biotechnology Reports 12: 283–293.

Usadel B, Blasing OE, Gibon Y, Poree F, Hohne M, Gunter M, Trethewey R, Kamlage B, Poorter H, Stitt M (2008) Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in *Arabidopsis* rosettes to a progressive decrease of temperature in the non-freezing range. Plant Cell and Environment 31: 518–547.

Vaculík M, Landberg T, Greger M, Luxová M, Stoláriková M, Lux A (2012) Silicon modifies root anatomy, and uptake and subcellular distribution of cadmium in young maize plants. Annals of Botany 110: 433–443.

Vaghela PM, Patel AD, Pandey IB, Pandey AN (2009) Implications of calcium nutrition on the response of *Salvadora oleoides* (Salvadoraceae) to Soil Salinity. Arid Land Research and Management 23: 311–326.

Vaistij FE, Barros-Galvao T, Cole AF, Gilday AD, He Z, Li Y, Harvey D, Larson TR, Graham IA (2018) MOTHER-OF-FT-AND-TFL1 represses seed germination under far-red light by modulating phytohormone responses in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences USA 115: 8442–8447.

Vassey TL, Quick WP, Sharkley TD, Stitt M (1991) Water stress, CO_2 and light effects on sucrose phosphate synthase activity in *Phaseolus vulgaris*. Physiologia Plantarum 81: 37–44.

Vega JM, Jacobo C, Manuel M (1980) Purification and molecular properties of nitrite reductase from *Anabaena* sp. 7119. Methods in Enzymology 69: 255–270.

Vike E (2005) Uptake, deposition and wash off of fluoride and aluminium in plant foliage in the vicinity of an aluminium smelter in Norway. Water, Air, and Soil Pollution, 160: 145–159.

Vishal B, Kumar PP (2018) Regulation of seed germination and abiotic stresses by gibberellins and abscisic acid. Frontiers in Plant Science 9: 838.

Vithanage M, Bhattacharya P (2015) Fluoride in the environment: Sources, distribution and defluoridation. Environmental Chemistry Letters 13: 131–147.

Vivancos J, Deshmukh R, Gregoire C, Remus-Borel W, Belzile F, Belanger RR (2016) Identification and characterization of silicon efflux transporters in horsetail (*Equisetum arvense*). Journal of Plant Physiology 200: 82-89.

Vogel AI (1961) Colorimetric estimation of nitrogen by Nessler's reagent. a textbook of quantitative inorganic analysis. Langman and Green, India, pp 97–108.

Vojtovič D, Luhová L, Petřivalský M (2020) Something smells bad to plant pathogens: Production of hydrogen sulfide in plants and its role in plant defence responses. Journal of Advanced Research 27: 199-209.

Wang AG, Xia T, Chu QL, Zhang M, Liu F, Chen XM, Yang KD (2004) Effects of fluoride on lipid peroxidation, DNA damage and apoptosis in human embryo hepatocytes. Biomedical and Environmental Sciences 17: 217–222.

Waskiewicz A, Beszterda M, Goliński P (2014) Nonenzymatic antioxidants in plants. In: Ahmad P (ed) Oxidative damage to plants. Elsevier, Amsterdam, pp 201–234.

Wegner LH, de Boer AH (1997) Properties of two outward-rectifying channels in root xylem parenchyma cells suggest a role in K^+ homeostasis and long-distance signaling. Plant Physiology 115: 1707–1719.

WHO (1984) Fluorine and fluoride: Geneva: Environmental Health Criteria. Volume 36.

WHO (1997) Surveillance and control of community supplies. In Surveillance and Control of Community Supplies; WHO: Geneva, Switzerland, 1997; p. 238.

WHO (2002) Fluorides. Environmental Health Criteria 227. Geneva.

WHO (2004) Guidelines for Drinking-Water Quality; World Health Organization: Geneva, Switzerland, Volume 1.

Widodo, Patterson JH, Newbigin E, Tester M, Bacic A, Roessner U (2009) Metabolic responses to salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. Journal of Experimental Botany 60: 4089–4103.

Wild R, Ooi L, Srikanth V, Münch G (2012) A quick, convenient and economical method for the reliable determination of methylglyoxal in millimolar concentrations: the N-acetyl-l-cysteine assay. Analytical and Bioanalytical Chemistry 403: 2577–2581.

Williams M, Randall DD (1979) Pyruvate dehydrogenase complex from chloroplasts of *Pisum sativum* L. Plant Physiology 64: 1099–1103.

World Bank (2010) Deep wells and prudence: Towards pragmatic action for addressing groundwater overexploitation in India. International Bank for Reconstruction and Development. Washington, DC: The World Bank.

Wu G, Feng R, Li S, Du Y (2017) Exogenous application of proline alleviates salt-induced toxicity in sainfoin seedlings. Journal of Animal and Plant Sciences 27: 246–251.

Wu J, Mock HP, Giehl RFH, Pitann B, Mühling KH (2019) Silicon decreases cadmium concentrations by modulating root endodermal suberin development in wheat plants. Journal of Hazardous Materials 364: 581-590.

Xing XJ, Ding YR, Jin JY, Song AP, Chen SM, Chen FD, Fang WM, Jiang JF (2021) Physiological and transcripts analyses reveal the mechanism by which melatonin alleviates heat stress in chrysanthemum seedlings. Frontiers in Plant Science 12: 17.

Xu C, He CG, Wang YJ, Bi YF, Jiang H (2020) Effect of drought and heat stresses on photosynthesis, pigments, and xanthophyll cycle in alfalfa (*Medicago sativa* L.). Photosynthetica 58: 1226-1236.

Xu ZY, Lee KH, Dong T, Jeong JC, Jin JB, Kanno Y, Kim DH, Kim SY, Seo M, Bressan RA, Yun DJ, Hwang I (2012) A vacuolar b-glucosidase homolog that possesses glucose-conjugated abscisic acid hydrolyzing activity plays an important role in osmotic stress responses in *Arabidopsis*. The Plant Cell 24: 2184–2199.

Yadu B, Chandrakar V, Keshavkanta S (2016) Responses of plants to fluoride: an overview of oxidative stress and defence mechanisms. Fluoride 49: 293-302.

Yadu B, Chandrakar V, Meena R, Sahu K (2017) Glycine betaine reduces oxidative injury and enhances fluoride stress tolerance via improving antioxidant enzymes, proline and genomic template stability in *Cajanus cajan* L. South African Journal of Botany 111: 68–75.

Yamaji N, Sakurai G, Mitani-Ueno N, Ma JF (2015) Orchestration of three transporters and distinct vascular structures in node for intervascular transfer of silicon in rice. Proceedings of the National Academy of Sciences USA 112: 11401–11406.

Yang H, Gu X, Ding M, Lu W, Lu D (2019) Activities of starch synthetic enzymes and contents of endogenous hormones in waxy maize grains subjected to post-silking water deficit. Scientific Reports 9(1).

Yang J, Zhang J, Wang Z, Zhu Q (2001) Activities of starch hydrolytic enzymes and sucrose-phosphate synthase in the stems of rice subjected to water stress during grain filling. Journal of Experimental Botany 52: 2169–2179.

Yang L, Zhang J, He J, Qin Y, Hua D, Duan Y, Chen Z, Gong Z (2014) ABA mediated ROS in mitochondria regulate root meristem activity by controlling PLETHORA expression in *Arabidopsis*. PLoS Genetics 10: e1004791.

Yin J, Jia J, Lian Z, Hu Y, Guo J, Huo H, Zhu Y, Gong H (2019) Silicon enhances the salt tolerance of cucumber through increasing polyamine accumulation and decreasing oxidative damage. Ecotoxicology and Environmental Safety 169: 8–17.

Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Plant Science 151: 59–66.

Yoshimoto N, Inoue E, Saito K, Yamaya T, Takahashi H (2003) Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in *Arabidopsis*. Plant Physiology 131: 1511–1517.

Yu J, Lai Y, Wu X, Wu G, Guo C (2016) Overexpression of *OsEm1* encoding a group I LEA protein confers enhanced drought tolerance in rice. Biochemical and Biophysical Research Communications 478: 703–709.

Yuan F, Lyu MJ, Leng BY, Zhu XG, Wang BS (2016) The transcriptome of NaCl-treated *Limonium bicolor* leaves reveals the genes controlling salt secretion of salt gland. Plant Molecular Biology 91: 241-256.

Zafar SA, Hameed A, Ashraf M, Khan AS, Qamar Z-U, Li X, Siddique KHM (2020) Agronomic, physiological and molecular characterisation of rice mutants revealed the key role of reactive oxygen species and catalase in high-temperature stress tolerance. Functional Plant Biology 47: 440–453.

Zahedi SM, Hosseini MS, Abadía J, Marjani M (2020) Melatonin foliar sprays elicit salinity stress tolerance and enhance fruit yield and quality in strawberry (*Fragaria* × *ananassa Duch.*). Plant Physiology and Biochemistry 149: 313–323.

Zargar SM, Agnihotri A (2013) Impact of silicon on various agromorphological and physiological parameters in maize and revealing its role in enhancing water stress tolerance. Emirates Journal of Food and Agriculture 25: 138–141.

Zargar SM, Macha MA, Nazir M, Agrawal GK, Rakwal R (2012) Silicon: a multitalented micronutrient in OMICS perspective-an update. Current Proteomics 9: 245–254.

Zargar SM, Mahajan R, Bhat JA, Nazir M, Deshmukh R (2019) Role of silicon in plant stress tolerance: opportunities to achieve a sustainable cropping system. 3 Biotech 9: 73.

Zeb A, Ullah F (2016) A simple spectrophotometric method for the determination of thiobarbituric acid reactive substances in fried fast foods. Journal of Analytical Methods in Chemistry 2016: 9412767.

Zeng H, Zhao B, Wu H, Zhu Y, Chen H (2020) Comprehensive in silico characterization and expression profiling of nine gene families associated with calcium transport in soybean. Agronomy 10: 1539.

Zhai CZ, Zhao L, Yin LJ, Chen M, Wang QY, Li LC, Xu ZS, Ma YZ (2013) Two wheat glutathione peroxidase genes whose products are located in chloroplasts improve salt and H_2O_2 tolerances in Arabidopsis. PLoS ONE 8: e73989.

Zhang C, Wang L, Nie Q, Zhang W, Zhang F (2008) Long-term effects of exogenous silicon on cadmium translocation and toxicity in rice (*Oryza sativa* L.) Environmental and Experimental Botany 62: 300–307.

Zhang H-J, Zhang N, Yang R-C, Wang L, Sun Q-Q, Li D-B, Cao YY, Weeda S, Zhao B, Ren S, Guo Y-D (2014) Melatonin promotes seed germination under high salinity by regulating antioxidant systems, ABA and GA4 interaction in cucumber (*Cucumis sativus* L.). Journal of Pineal Research 57: 269–279.

Zhang J, Shi Y, Zhang X, Du H, Xu B, Huang B (2017) Melatonin suppression of heat-induced leaf senescence involves changes in abscisic acid and cytokinin biosynthesis and signaling pathways in perennial ryegrass (*Lolium perenne* L.). Environmental and Experimental Botany 138: 36–45.

Zhang L, Becker DF (2015) Connecting proline metabolism and signaling pathways in plant senescence. Frontiers in Plant Science 6: 552.

Zhang L, Gao M, Hu J, Zhang X, Wang K, Ashraf M (2012) Modulation role of abscisic acid (ABA) on growth, water relations and glycine betaine metabolism in two maize (*Zea mays* L.) cultivars under drought stress. International Journal of Molecular Science 13: 3189–3202.

Zhang S, Zhang X, Liu H, Qu W, Guan Z, Zeng Q, Jiang C, Gao H, Zhang C, Lei R (2015) Modifying effect of COMT gene polymorphism and a predictive role for proteomics analysis in children's intelligence in endemic fluorosis area in Tianjin, China. Toxicological Sciences 144: 238–245.

Zhang T, Peng JT, Klair A, Dickinson AJ (2023) Non-canonical and developmental roles of the TCA cycle in plants. Current Opinion in Plant Biology 74: 102382.

Zhang Y, Hu XH, Shi Y, Zou ZR, Yan F, Zhao YY, Zhang H, Zhao JZ (2013) Beneficial role of exogenous spermidine on nitrogen metabolism in tomato seedlings exposed to saline-alkaline stress. Journal of the American Society for Horticultural Science 138: 38–49.

Zhao Y, Pan Z, Zhang Y, Qu XL, Zhang YG, Yang YQ, Jiang XN, Huang SJ, Yuan M, Schumaker KS, Guo Y (2013) The actin-related protein2/3 complex regulates mitochondrial-associated calcium signaling during salt stress in *Arabidopsis*. The Plant Cell 25: 4544–4559.

Zheng HY, Wang WL, Xu K, Xu Y, Ji DH, Chen CS, Xie CT (2020) Ca^{2+} influences heat shock signal transduction in *Pyropia haitanensis*. Aquaculture 516: 8.

Zhou J, Tian X, Qiao L, Qin P (2012) Respiratory enzyme activity and regulation of respiration pathway in seashore mallow (*Kosteletzkya virginica*) seedlings under waterlogging conditions. Austrian Journal of Crop Science 6: 756–762.

Zhou R, Silcher RC, Quebedeaux B (2002) Apple leaf sucrose phosphate synthase is inhibited by sorbitol-6-phosphate. Functional Plant Biology 29: 569–574.

Zhu X, Feng Y, Liang G, Liu N, Zhu JK (2013) Aequorin-based luminescence imaging reveals stimulus- and tissue-specific Ca^{2+} dynamics in *Arabidopsis* plants. Molecular Plant 6: 444-455.

Zlatev ZS, Lidon FC, Ramalho JC, Yordanov IT (2006) Comparison of resistance to drought of three bean cultivars. Biologia plantarum 50: 389–394.

Zuo H, Chen L, Kong M, Qiu L, Lü P, Wu P, Yang Y, Chen, K (2018) Toxic effects of fluoride on organisms. Life Sciences 198: 18–24.

Annexure

List of Publication

➢ **Research Articles**

1. **Singh A**, Roychoudhury A (2023) Deciphering the biochemical regulation of metabolic pathways in abating stress-induced damages during co-exposure of arsenic and fluoride in a non-aromatic and an aromatic rice variety. Physiology and Molecular Biology of Plants. doi: 10.1007/s12298-023-01355-z
2. **Singh A**, Chakraborty S, Roychoudhury A (2023) Activated charcoal alleviates fluoride stress by restricting fluoride uptake and counteracting oxidative damages in the rice cultivar MTU1010. Turkish Journal of Botany 47: 97-110.
3. **Singh A**, Roychoudhury A (2023) Salicylic acid-mediated alleviation of fluoride toxicity in rice by restricting fluoride bioaccumulation and strengthening the osmolyte, antioxidant and glyoxalase systems. Environmental Science and Pollution Research 30: 25024-25036.
4. Chakraborty S, **Singh A**, Roychoudhury A (2022) Extensive cross-talk among stress-regulated protective metabolites, biogenic-amines and phytohormone-signalling, co-ordinated by dopamine-mediated seed-priming, governs tolerance against fluoride stress in rice. Plant Cell Reports 41: 2261-2278.
5. **Singh A**, Roychoudhury A (2022) Fluoride toxicity imposes differential reprogramming of the representative intermediates and enzymes belonging to nitrogen metabolism in two indica rice varieties, varying in their pattern of fluoride stress response. Journal of Plant Growth Regulation. doi:10.1007/s00344-022-10780-5.
6. **Singh A**, Banerjee A, Roychoudhury A (2022) Fluoride tolerance in rice is negatively regulated by the 'stress-phytohormone' abscisic acid (ABA), but promoted by ABA-antagonist growth regulators, melatonin, and gibberellic acid. Protoplasma 259: 1331-1350.
7. Roychoudhury A, **Singh A**, Aftab T, Ghosal P, Banik N (2021) Seedling priming with sodium nitroprusside rescues *Vigna radiata* from salinity stress-induced oxidative damages. Journal of Plant Growth Regulation 40: 2454–2464.
8. **Singh A**, Banerjee A, Roychoudhury A (2021) Differential responses of *Vigna radiata* and *Vigna mungo* to fluoride-induced oxidative stress and amelioration via exogenous application of sodium nitroprusside. Journal of Plant Growth Regulation 40: 2342–2357.

9. **Singh A**, Roychoudhury A, Samanta S, Banerjee A (2021) Fluoride stress-mediated regulation of tricarboxylic acid cycle and sugar metabolism in rice seedlings in absence and presence of exogenous calcium. Journal of Plant Growth Regulation 40: 1579–1593.
10. Banerjee A, **Singh A**, Roychoudhury A (2021) Fluoride toxicity variably affects overall physiology and grain development in three contrasting rice genotypes, representing a potential biohazard. Environmental Science and Pollution Research 28: 40220-40232.
11. Banerjee A, **Singh A**, Sudarshan M, Roychoudhury A (2021) Silicon nanoparticle-pulsing mitigates fluoride stress in rice by fine-tuning the ionomic and metabolomic balance and refining agronomic traits. Chemosphere 262: 127826.
12. Samanta S, **Singh A**, Banerjee A, Roychoudhury A (2020) Exogenous supplementation of melatonin alters representative organic acids and enzymes of respiratory cycle as well as sugar metabolism during arsenic stress in two contrasting indica rice cultivars. Journal of Biotechnology 324: 220-232.
13. Banerjee A, **Singh A**, Roychoudhury A (2020) De novo RNA-Seq analysis in sensitive rice cultivar and comparative transcript profiling in contrasting genotypes reveal genetic biomarkers for fluoride-stress response. Environmental Pollution 267: 115378.
14. **Singh A**, Banerjee A, Roychoudhury A (2020) Alleviation of fluoride-mediated toxicity via seed priming with calcium oxide in *Oryza sativa* L. cv. Khitish. Science and Culture 86: 317-321.
15. **Singh A**, Roychoudhury A (2020) Silicon-regulated antioxidant and osmolyte defense and methylglyoxal detoxification functions co-ordinately in attenuating fluoride toxicity and conferring protection to rice seedlings. Plant Physiology and Biochemistry 154: 758-769.
16. Banerjee A, Samanta S, **Singh A**, Roychoudhury A (2020) Deciphering the molecular mechanism behind stimulated co-uptake of arsenic and fluoride from soil, associated toxicity, defence and glyoxalase machineries in arsenic-tolerant rice. Journal of Hazardous Materials 390: 121978.
17. **Singh A**, Banerjee A, Roychoudhury A (2020) Seed priming with calcium compounds abrogate fluoride-induced oxidative stress by upregulating defence pathways in an indica rice variety. Protoplasma 257: 767-782.

18. Banerjee A, **Singh A**, Roychoudhury A (2019) Spermidine application reduces fluoride uptake and ameliorates physiological injuries in a susceptible rice cultivar by activating diverse regulators of the defense machinery. Environmental Science and Pollution Research 26: 36598-36614.

➢ **Review Articles**

1. **Singh A**, Roychoudhury A (2023) Abscisic acid in plants under abiotic stress: crosstalk with major phytohormones. Plant Cell Reports 42: 961-974.
2. Chakraborty S, **Singh A**, Roychoudhury A (2022) Biogenic nanoparticles and generation of abiotic stress-resilient plants: A new approach for sustainable agriculture. Plant Stress 6: 100117.
3. **Singh A**, Roychoudhury A (2021) Gene regulation at transcriptional and post-transcriptional levels to combat salt stress in plants. Physiologia Plantarum 173: 1556-1572.

➢ **Book Chapters**

1. **Singh A**, Roychoudhury A (2023) RNA Interference (RNAi) technology: An effective tool in plant breeding. In book: Advanced Crop Improvement, Volume 1 Publisher: Springer Nature Switzerland AG. doi: 10.1007/978-3-031-28146-4_12
2. **Singh A**, Roychoudhury A (2023) Hydrogen peroxide as a signaling molecule in plant abiotic stress. In book: Crop Sustainability and Intellectual Property Rights Publisher: CRC Press (Apple Academic Press). doi: 10.1201/9781003383024-11
3. **Singh A**, Roychoudhury A (2023) Advances in metabolomics research in environmental stress response in plants. In book: Biology and Biotechnology of Environmental Stress Tolerance in Plants, Volume 3: Sustainable Approaches for Enhancing Environmental Stress Tolerance Publisher: Apple Academic Press, Inc. Co-published with CRC Press (Taylor & Francis). doi: 10.1201/9781003346401-17
4. **Singh A**, Roychoudhury A (2023) Exogenous application of trace elements and their uptake by plants to mediate abiotic stress tolerance. In book: Biology and Biotechnology of Environmental Stress Tolerance in Plants, Volume 2: Trace Elements in Environmental Stress Tolerance Publisher: Apple Academic Press, Inc. Co-published with CRC Press (Taylor & Francis). doi: 10.1201/9781003346203-4.

5. **Singh A**, Roychoudhury A (2023) Role of phenolic acids and flavonoids in the mitigation of environmental stress in plants. In book: Biology and Biotechnology of Environmental Stress Tolerance in Plants: Secondary Metabolites in Environmental Stress Tolerance, Volume 1 Publisher: Apple Academic Press, Inc. Co-published with CRC Press (Taylor & Francis). doi: 10.1201/9781003346173-11.
6. **Singh A**, Roychoudhury A (2023) Antioxidants and secondary metabolites of mushrooms as a source of nutraceuticals and functional food. In book: Mushrooms Nutraceuticals and Functional FoodsPublisher: CRC Press, Taylor & Francis group. doi: 10.1201/9781003322238-1.
7. **Singh A**, Roychoudhury A (2022) Mechanism of crosstalk between cytokinin and gibberellin. In book: Auxins, Cytokinins and Gibberellins Signaling in Plants, Signaling and Communication in PlantsPublisher: Springer Nature Switzerland AG. doi: 10.1007/978-3-031-05427-3_4.
8. **Singh A**, Roychoudhury A (2022) Role of nanoparticles in remediation of environmental contaminants. In book: Hazardous and Trace Materials in Soil and PlantsPublisher: Academic Press (Elsevier). doi: 10.1016/B978-0-323-91632-5.00021-5
9. **Singh A**, Roychoudhury A (2022) Protective chemicals and metabolites in stabilizing photosynthesis and respiration machinery during abiotic stresses. In book: Photosynthesis and Respiratory Cycles During Environmental Stress Response in PlantsPublisher: CRC Press (Apple Academic Press). doi: 10.1201/9781003315162-16.
10. **Singh A**, Roychoudhury A (2022) Omics tools to understand abiotic stress response and adaptation in rye, oat and barley. In book: Omics Approach to Manage Abiotic Stress in Cereals Publisher: Springer Nature Singapore. doi: 10.1007/978-981-19-0140-9_21
11. **Singh A,** Roychoudhury A (2021) Role of β-aminobutyric acid in generating stress-tolerant and climate-resilient plants. In book: Plant Perspectives To Global Climate Changes Developing Climate-Resilient Plants Publisher: Academic Press (Elsevier). doi: 10.1016/B978-0-323-85665-2.00005-4.
12. **Singh A,** Roychoudhury A (2021) Hydrogen sulfide and redox homeostasis for alleviation of heavy metal stress. In book: Hydrogen Sulfide and Plant Acclimation to

Abiotic Stresses Publisher: Springer Nature Switzerland AG. doi: 10.1007/978-3-030-73678-1_4.

13. **Singh A,** Roychoudhury A (2021) Salicylic acid and jasmonic acid in generating salt stress-tolerant plants. In book: Jasmonates and Salicylates Signaling in Plants Publisher: Springer Nature Switzerland AG. doi: 10.1007/978-3-030-75805-9_2.
14. **Singh A,** Roychoudhury A (2021) Augmenting the abiotic stress tolerance in plants through microbial association. In book: Microbial Metatranscriptomics Belowground Publisher: Springer Singapore. doi: 10.1007/978-981-15-9758-9_9.
15. **Singh A,** Roychoudhury A (2021) Silicon transporters in plants. In book: Metal and Nutrient Transporters in Abiotic Stress. Publisher: Academic Press (Elsevier). doi: 10.1016/B978-0-12-817955-0.00007-7.
16. **Singh A,** Roychoudhury A (2021) Cytokinin-mediated signalling during environmental stress in plants. In book: Plant Growth Regulators Publisher: Springer Nature Switzerland. doi: 10.1007/978-3-030-61153-8_6.
17. **Singh A,** Chaudhuri B, Roychoudhury A (2020) Influence of night temperature on rice yield and quality. In book: Rice Research for Quality Improvement: Genomics and Genetic Engineering Volume 1 Publisher: Springer Singapore. doi: 10.1007/978-981-15-4120-9_24.
18. **Singh A,** Roychoudhury A (2020) Genetic engineering: A powerful tool to abrogate the effect of metal/metalloid toxicity in rice. In book: Rice Research for Quality Improvement: Genomics and Genetic Engineering Publisher: Springer Singapore. doi: 10.1007/978-981-15-4120-9_15.
19. **Singh A,** Roychoudhury A (2020) Role of γ-aminobutyric acid in the mitigation of abiotic stress in plants. In book: Protective Chemical Agents in the Amelioration of Plant Abiotic Stress Publisher: John Wiley & Sons, Ltd, USA. doi: 10.1002/9781119552154.ch20.
20. Samanta S, **Singh A,** Roychoudhury A (2020) Involvement of sulfur in the regulation of abiotic stress tolerance in plants. In book: Protective Chemical Agents in the Amelioration of Plant Abiotic Stress Publisher: John Wiley & Sons, Ltd, USA. doi: 10.1002/9781119552154.ch22.

Physiology and Molecular Biology of Plants
https://doi.org/10.1007/s12298-023-01355-z

RESEARCH ARTICLE

Deciphering the biochemical regulation of metabolic pathways in abating stress-induced damages during co-exposure of arsenic and fluoride in a non-aromatic and an aromatic rice variety

Ankur Singh[1] · **Aryadeep Roychoudhury**[2]

Received: 25 May 2023 / Revised: 28 July 2023 / Accepted: 28 August 2023

Abstract
The aim of the current work was to decipher the systemic damage and biochemical defense machinery due to combined arsenic (5 mg L^{-1} Na_3AsO_4) and fluoride (50 mg L^{-1} NaF) stress in two rice cultivars viz., IR-64 (non-aromatic) and Gobindobhog (aromatic), grown for 14 days, under 16/8 h light/dark photoperiodic cycle at 32 °C. Higher accumulation of arsenic and fluoride in Gobindobhog generated higher levels of H_2O_2 that caused higher electrolyte leakage, along with malondialdehyde and methylglyoxal formation. Higher oxidative damages severely compromised seed germination and led to chlorophyll loss, inhibition of root and shoot growth and fresh and dry weight of the seedlings. On the contrary, oxidative damage was less pronounced in IR-64, as compared to that of Gobindobhog, which can be attributed to higher accumulation of protective metabolites, i.e., osmolytes and antioxidants. Higher levels of osmolytes (proline, glycine betaine and amino acids) in IR-64 helped in maintaining the osmotic balance of the cells and the integrity of the cell membrane. Additionally, up regulated activity of enzymatic antioxidants (catalase, superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and glutathione S-transferase) along with elevated levels of non-enzymatic antioxidants (flavonoids, phenolics, xanthophylls and carotenoids) played a pivotal role in controlling oxidative damages and strengthening the defense machinery in IR-64, as compared to that of Gobindobhog where lesser enhancement in the level of the above mentioned protective metabolites was noted. The present work illustrated differential phytotoxicity in rice seedlings and elucidated the yet uncharacterized biohazard associated with arsenic and fluoride co-contamination, with better adaptive features of IR-64, compared to Gobindobhog, which appeared as the sensitive variety.

Keywords Fluoride · Arsenic · Reactive oxygen species · Osmolytes · Antioxidants · Rice

Introduction

Due to continuous industrial expansion and ever-growing human population, the discharge of xenobiotic wastes in the environment has risen enormously, making it a matter of great concern. Various anthropogenic activities, viz., rampant release of untreated industrial wastes, unrestricted use of fertilizers and household wastes, several environmental factors like emission of ash during volcanic eruption and natural weathering of minerals have cumulatively contributed toward the rise in the level of toxic chemicals in the environment (Hong et al. 2016). Two such xenobiotics that are highly detrimental to humankind are fluoride (F) and arsenic (As). F and As include the 13th and 20th most abundant element found on the earth's crust, respectively (Chakraborti et al. 2003). The most common contributors of F and As are natural sources such as weathering of mineral rocks or geothermal vents. Along with this, Patel et al. (2019) reported that the synergistic presence of As and F in soil can be due to over-exploitation of mining activities. The deposition of newer sediments and the recharge conditions controlled by the rivers play a significant role in governing the concentration of As and F (Das et al. 2016). Gomez et al. (2013) examined the sediments, rocks and groundwater to analyze As and F co-occurrence. They found that out of 34

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, West Bengal 700016, India

[2] Discipline of Life Sciences, School of Sciences, Indira Gandhi National Open University, Maidan Garhi, New Delhi 110068, India

Published online: 23 September 2023

TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2023) 47: 97-110
© TÜBİTAK
doi:10.55730/1300-008X.2749

Activated charcoal alleviates fluoride stress by restricting fluoride uptake and counteracting oxidative damages in the rice cultivar MTU1010

Ankur SINGH[1], Swarnavo CHAKRABORTY[1], Aryadeep ROYCHOUDHURY[2,*]
[1]Department of Biotechnology, St. Xavier's College (Autonomous), Mother Teresa Sarani, West Bengal, India
[2]Discipline of Life Sciences, School of Sciences, Indira Gandhi National Open University, Maidan Garhi, New Delhi, India

Received: 01.06.2022 • **Accepted/Published Online:** 13.01.2023 • **Final Version:** 23.03.2023

Abstract: This work was aimed to explore the efficacy of activated charcoal (5 mg g^{-1} soil) in abating fluoride (25 mg L^{-1} NaF) stress in rice seedlings, since the protective role of charcoal is widely reported against other forms of abiotic stress. Application of NaF solution reduced germination rate, dry and fresh weight, and shoot and root length of seedlings. Extensive fluoride accumulation lowered the chlorophyll level along with higher electrolyte leakage and formation of H_2O_2, malondialdehyde, and methylglyoxal. Administration of activated charcoal lowered the extent of oxidative damages by inhibiting the uptake of fluoride ions. Exogenous application of activated charcoal also restored the activity of Kreb's cycle enzymes, i.e. PDH, IDH, MDH, and SDH, thereby overcoming the burden of carbon utilization and energy depletion occurring during stress. Additionally, the osmolyte (soluble sugar, proline, glycine-betaine, and amino acid) level was further escalated in presence of activated charcoal. The inhibition in catalase activity in fluoride-stressed seedlings was also restored. The activity of a range of enzymatic antioxidants (guaiacol peroxidase, superoxide dismutase, and ascorbate peroxidase) along with the glyoxalase system associated-enzymes (glyoxalase I and II) was further triggered in stressed seedlings treated with activated charcoal. The enhanced level of carotenoids, ascorbic acid, and total phenolics also enabled efficient scavenging of reactive oxygen species, thereby reducing cellular necrosis in the leaves. Based on the current investigation, it can be concluded that activated charcoal application appears a promising strategy to improve growth and mitigate damages in rice plants, growing in fluoride-polluted soil.

Key words: Activated charcoal, fluoride-induced damage, osmolytes, Kreb's cycle, antioxidants, rice

1. Introduction

Fluorine is the 13th element in terms of its abundance in the globe, which is approximately 0.3 g kg^{-1} of soil present on the earth's surface (Choudhary et al., 2019). This element is placed within the halogen group, and has an important role in the periodic table due to its two extreme characteristics, viz., highest electro-negativity and smallest size. With the progressive rise in various anthropogenic activities, there has been an enhancement in the release of fluoride (F) into the environment. Contamination of the surface water with F salts via industrial discharges, agricultural run-offs, sewage, and other household wastes has been commonly observed in modern times (Hong et al., 2016; Singh et al., 2021a). Moreover, the depletion in the level of ground water has further led to the contamination of the water bodies used for household supply as well as for irrigation in the fields (Banerjee and Roychoudhury, 2019a; Mondal, 2017). Subsequently, the usage of this contaminated water in the agricultural field elicits the accumulation of F salts in the soil that severely declines the growth and yield of crops, ultimately posing a genuine warning to worldwide food safety. The extent of F pollution in different locations of the Indian subcontinent has been attributed to a higher value of F like 69-417 mg kg^{-1} in soil (Bhattacharya and Samal, 2018).

Rice plants require substantial quantities of water for their optimal growth and yield, and have been demonstrated to uptake considerable amounts of F via chloride channels (Banerjee and Roychoudhury, 2019b). Excess deposition of F salts within the tissues of plant leads to chlorosis, leaf tip burn, reduced germination percentage, lower seedling biomass, enhanced generation of toxic reactive oxygen species (ROS), i.e. superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) which in turn disintegrate the lipid membrane, ultimately resulting in higher leakage of electrolytes along with up-regulating the cytotoxic metabolite production [methylglyoxal (MG) and malondialdehyde (MDA)] and ultimately cell death (Singh et al., 2020). Additionally, higher uptake of F salts from soil eventually leads to their accumulation in rice grains that is

* Correspondence: aryadeep.rc@gmail.com

Environmental Science and Pollution Research (2023) 30:25024–25036
https://doi.org/10.1007/s11356-021-14624-9

CHALLENGES IN MANAGING AND RISK ASSESSMENT OF EMERGING ENVIRONMENTAL POLLUTANTS

Salicylic acid–mediated alleviation of fluoride toxicity in rice by restricting fluoride bioaccumulation and strengthening the osmolyte, antioxidant and glyoxalase systems

Ankur Singh[1] · Aryadeep Roychoudhury[1]

Received: 30 March 2021 / Accepted: 25 May 2021 / Published online: 1 June 2021

Abstract

The aim of the manuscript was to demonstrate the efficacy of salicylic acid (SA) in abrogating the fluoride-induced oxidative damages in the susceptible rice cultivar, MTU1010. Prolonged exposure of seedlings to sodium fluoride (25 mg L^{-1}) severely impaired growth and overall physiological parameters like germination percentage, biomass and root and shoot length and incited the formation of hydrogen peroxide that enhanced electrolyte leakage, formation of cytotoxic products like malondialdehyde and methylglyoxal and lipoxygenase activity. Exogenous application of SA (0.5 mM) enhanced the endogenous level of SA that restored the chlorophyll content and catalase activity and further escalated the activity of other enzymatic antioxidants (superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, glutathione peroxidase and glutathione S-transferase), formation of non-enzymatic antioxidants (anthocyanins, carotenoids, flavonoids, phenolics, ascorbate and reduced glutathione) and osmolytes (proline, amino acids and glycine betaine) that cumulatively maintained the integrity of membrane structure and homeostatic balance of the cells by scavenging the accumulated hydrogen peroxide. SA-mediated formation of proline and flavonoids was linked with the enhanced activity of Δ^1-pyrroline-5-carboxylate synthetase and phenylalanine ammonia lyase. Fluoride stress enhanced the activity of enzymes like glyoxalase I and glyoxalase II which were further aggravated in the seedlings upon treatment with SA, effectively detoxifying the methylglyoxal formed during stress. Overall, the manuscript depicts the pivotal role played by exogenous SA in ameliorating the effects of fluoride-induced damages in the seedlings and proves its potentiality as a protective chemical against fluoride stress when applied exogenously in rice.

Keywords Antioxidants · Fluoride toxicity · *Oryza sativa* · Osmolytes · Oxidative stress · Salicylic acid

Introduction

Fluoride (F) belongs to the halide group and demands attention due to its high electronegativity and small size that accounts for its unique chemical and biological properties. The main source of F in nature is parent rocks, but in recent times, various anthropogenic activities have also enhanced the discharge of F in the environment. In certain regions of India, the level of F is around 69–417 mg kg^{-1} of soil (Bhattacharya and Samal 2018) which affects around 62 million people including approximately six million children (Choudhary et al. 2019). Excess accumulation of F salts in soil also contaminate the water bodies, which if used for irrigation in the agricultural fields cause physiological and molecular abnormalities in plants (Banerjee and Roychoudhury 2019).

The toxic effect of F on crops like *Oryza sativa*, *Cicer arietinum*, *Vigna* sp. and *Cajanus cajan* is widely reported (Singh et al. 2020a; Dey et al. 2012; Singh et al. 2020b; Yadu et al. 2017). Prolonged exposure of roots to high F level results in higher transportation of F^- ions within tissues through the chloride channels. Accumulation of F^- ions above the threshold level causes deleterious effects on nutrient uptake, seed germination, plant growth, metabolic activities and biomass accumulation, along with reduced yield. Excess

Responsible Editor: Gangrong Shi

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, West Bengal 700016, India

Plant Cell Reports (2022) 41:2261–2278
https://doi.org/10.1007/s00299-022-02919-1

ORIGINAL ARTICLE

Extensive cross-talk among stress-regulated protective metabolites, biogenic-amines and phytohormone-signalling, co-ordinated by dopamine-mediated seed-priming, governs tolerance against fluoride stress in rice

Swarnavo Chakraborty[1] · Ankur Singh[1] · Aryadeep Roychoudhury[1]

Received: 23 June 2022 / Accepted: 9 August 2022 / Published online: 30 August 2022

Abstract

Key message **Seed priming with dopamine reduced fluoride bioaccumulation, induced endogenous dopamine level, thereby orchestrating phytohormone homeostasis and biogenic amine metabolism, and modulating osmolyte and antioxidant machinery to enhance fluoride tolerance in rice.**

Abstract The aim of this study was to decipher the efficacy of seed priming with dopamine in curtailing the adverse impacts of fluoride toxicity in rice seedlings. Fluoride-stressed seedlings exhibited severe growth retardation, high fluoride bioaccumulation, electrolyte leakage and marked cellular injuries. Dopamine priming stimulated the overall physiological growth parameters during stress, via reduced formation of H_2O_2, malondialdehyde and methylglyoxal, due to lesser fluoride-accumulation. Fluoride stress-induced endogenous dopamine level was further induced upon dopamine priming, marked by the up regulated *DOPA decarboxylase* expression. Additionally, dopamine treatment led to escalated activity of catalase, superoxide dismutase and glutathione peroxidase in the stressed seedlings, concomitant with altered *CAT, SOD* and *GPX* expression. The higher accumulation of protective osmolytes (proline and total amino acids) and non-enzymatic antioxidants (phenolics, flavonoids, anthocyanins, glutathione and carotenoids), upon dopamine priming, during fluoride stress, could be linked with the altered expression pattern of the respective genes. Dopamine promoted active utilization of the biogenic amine (polyamines and ϒ-amino butyric acid) pools for toxicity mitigation, correlated with the modulation of the concerned enzyme activity and gene expression. Dopamine stimulated the accumulation of phytohormones like gibberellin and salicylic acid, via inducing the biosynthetic genes like *gibberellin-3-oxidase (GA3ox)* and *isochorismate synthase (ICS)*, respectively, while depreciating the abscisic acid and melatonin level during fluoride stress. To our knowledge, this is the first documented report for the remedial role of dopamine priming against fluoride stress in any plant species. This study will open new arenas in sustainable agriculture for the exploitation of this pulsating biomolecule against fluoride stress.

Keywords Dopamine · Seed priming · Fluoride stress · Rice · Osmolytes · Antioxidants · Phytohormones · Biogenic amine

Introduction

Due to progressive human population explosion and industrialization, xenobiotic discharge into the environment has risen enormously, making it a matter of great global concern. Fluoride (F), being one of such potent xenobiotics, exhibits unique biological and chemical properties, owing to its very small size and high electronegativity. Being a member of the halide group, F has been designated as the thirteenth element in terms of its abundance across the globe (Armienta and Segovia, 2008). F is highly water-soluble and tends to pollute both surface and ground water. Across India, the F

Communicated by Prakash P. Kumar.

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata 700016, West Bengal, India

Journal of Plant Growth Regulation
https://doi.org/10.1007/s00344-022-10780-5

Fluoride Toxicity Imposes Differential Reprogramming of the Representative Intermediates and Enzymes Belonging to Nitrogen Metabolism in Two *indica* Rice Varieties, Varying in their Pattern of Fluoride Stress Response

Ankur Singh[1] · **Aryadeep Roychoudhury**[1]

Received: 15 April 2022 / Accepted: 23 August 2022

Abstract
The main aim of this manuscript was to demonstrate the reprogramming of nitrogen and GABA metabolism in the two rice cultivars (Khitish and MTU1010) in response to fluoride stress (25 mg L^{-1}) for 10 and 20 days. The seedlings were grown under 16/8 h light/dark photoperiodic cycle in a plant growth chamber. Fluoride stress significantly hampered growth and biomass of Khitish and MTU1010 after 10 days and 20 days of treatment, respectively. Fluoride stress reduced the level of total and soluble nitrogen, nitrate and nitrite, concomitant with inhibited activity of NR and NiR, along with corresponding gene expression level. In response to fluoride stress, the total amino acids were significantly enhanced in the seedlings, contributing towards better growth. Level of ammonium ions was also enhanced in the stressed seedlings, along with inhibited activity of GS and GOGAT, implying that the incorporation of ammonium ions into glutamine and glutamate via GS and GOGAT was hindered during fluoride stress. Expression level of *GS* and *GOGAT* was accordingly regulated in the seedlings of both the varieties. The endogenous GABA content was enhanced in both the cultivars along with the induced catalytic activity of GDH and GAD and their corresponding gene expression level. Our work clearly demonstrated that differential reprogramming of the metabolites and enzymes of nitrogen assimilation pathways was largely responsible for the differential pattern of adaptive strategies against fluoride stress in the two varieties, viz., Khitish and MTU1010, the former exhibiting resilience against long-term stress, whilst the latter showing greater susceptibility.

Keywords Nitrogen metabolism · GABA · Fluoride stress · Rice · Amino acids

Introduction

With the explosion of human population, the release of xenobiotic substances in the environment has increased enormously to a point of grave concern. Fluoride (F) is one of such pollutants whose continuous release in the environment has raised a serious cause of concern amongst various researchers and environmentalist groups. Along with several anthropogenic activities (release of untreated water from industries, excess application of fertilisers in agricultural fields and various household activities), various environmental factors (natural weathering of minerals, emissions from volcanic ash and marine aerosols) have also contributed towards such abrupt rise of F in the environment (Hong et al. 2016). F being highly soluble in water is easily admixed in the surrounding water bodies and pollutes both ground and surface water. According to a recent study conducted by Bhattacharya and Samal (2018), the level of F in drinking water in some parts of India is found to be as high as 0.8–1.3 mg L^{-1} (Bankura and Purulia districts of West Bengal) and 0.3–9.8 mg L^{-1} (Newai Tehsil, Rajasthan). India is an agriculture-based country where the farmers heavily rely on such water bodies for irrigating agricultural fields. Unplanned irrigation of agricultural land further deposits F salts, which are readily absorbed by the plants. In a recent study, De et al. (2021) reported

Handling Editor: Sudhir K. Sopory.

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata 700016, West Bengal, India

Published online: 09 September 2022

Protoplasma (2022) 259:1331–1350
https://doi.org/10.1007/s00709-022-01740-7

ORIGINAL ARTICLE

Fluoride tolerance in rice is negatively regulated by the 'stress-phytohormone' abscisic acid (ABA), but promoted by ABA-antagonist growth regulators, melatonin, and gibberellic acid

Ankur Singh[1] · Aditya Banerjee[1] · Aryadeep Roychoudhury[1]

Received: 2 November 2021 / Accepted: 17 January 2022 / Published online: 27 January 2022

Abstract
The present manuscript aimed at investigating whether abscisic acid (ABA) promotes fluoride tolerance, similar to inciting salt adaptation in rice. Seeds of three salt-tolerant rice genotypes were maintained at 32 °C under 16/8 h light/dark photoperiodic cycle with 700 µmol photons m^{-2} s^{-1} intensity and 50% relative humidity in a plant growth chamber for 20 days. Suppressed ABA biosynthesis, and downregulated expression of ABA-inducible genes like *Rab16A*, *Osem*, and *TRAB1* triggered NaCl-induced growth inhibition and physiological injuries like chlorophyll degradation, electrolyte leakage, formation of H_2O_2, malondialdehyde, and methylglyoxal in Matla. Reduced ABA accumulation increased the levels of melatonin and gibberellic acid in NaF (50 mg L^{-1})-stressed Nonabokra and Matla, which altogether promoted fluoride tolerance. Higher ABA content in NaF-stressed Jarava stimulated fluoride uptake via chloride channels, thus exhibiting severe fluoride susceptibility, in spite of higher production of ABA-associated osmolytes like proline, glycine-betaine and polyamines via the concerted action of genes like *PDH*, *ADC*, *ODC*, *SAMDC*, *SPDS*, *SPMS*, *DAO*, and *PAO*. Increased accumulation of compatible solutes in presence of high endogenous ABA promoted salt tolerance in Jarava; the same was insufficient to ameliorate fluoride-induced injuries in this cultivar. Treatment with ABA biosynthetic inhibitor, Na_2WO_4 promoted fluoride tolerance in Jarava, whereas further supplementation with exogenous ABA resulted in reversion back to fluoride-susceptible phenotype. Our work clearly established that ABA cannot always be considered as a 'universal' stress hormone as known in literature, since it acts as a negative regulator of fluoride tolerance which is more tightly regulated in rice via melatonin- and gibberellic acid-dependent pathways in ABA-independent manner.

Keywords Abscisic acid (ABA) · Fluoride stress · Salt stress · ABA inhibitor · ABA-melatonin-gibberellic acid interaction · ABA-dependent pathway · Salt-tolerant rice · Varietal difference

Introduction

Increasing environmental contamination with toxic xenobiotic substances is a major global problem in the present times. Release of fluoride (F) in the environment due to various anthropogenic activities from industries, mines, agricultural fields, and various household activities is gradually turning out to be a menace to the environmental safety (Hong et al. 2016). According to a recent study conducted by Bhattacharya and Samal (2018), the level of F in soil in some parts of India such as Bankura and Purulia (West Bengal) and Newai Tehsil (Rajasthan) ranges between 55–399 and 50 – 180 mg kg^{-1} of soil, respectively, whereas the level of F in water ranges between 0.8–1.3 and 0.3–9.8 mg L^{-1} which is significantly higher than that of 1.5 mg L^{-1}, as recommended by the World Health Organization (WHO). The use of contaminated water for crop irrigation leads to the widespread accumulation of F^- ions in plant biomass. Earlier, Gautam et al. (2010) reported that irrigation of crops with water having 7.4–14 mg L^{-1} F led to the accumulation of F in plants like spinach (26 mg kg^{-1} fresh weight) and fenugreek (16 mg kg^{-1} fresh weight). Water-intensive crops like rice uptake F^- ions from the surrounding environment through the chloride channels, viz., CLC1 and CLC2

Communicated by Handling Editor: Bhumi Nath Tripathi.

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata 700016, West Bengal, India

Journal of Plant Growth Regulation (2021) 40:2454–2464
https://doi.org/10.1007/s00344-021-10328-z

Seedling Priming with Sodium Nitroprusside Rescues *Vigna radiata* from Salinity Stress-Induced Oxidative Damages

Aryadeep Roychoudhury[1] · Ankur Singh[1] · Tariq Aftab[2] · Poulomi Ghosal[3] · Nilanjana Banik[3]

Received: 29 October 2020 / Accepted: 26 January 2021 / Published online: 26 February 2021

Abstract

The main focus of this manuscript was to highlight the efficacy of seedling priming with 0.06 mM sodium nitroprusside (SNP), a potent NO donor in the alleviation of salt-induced oxidative and ionic stress in *Vigna radiata*. Treatment of seedlings with 100 mM NaCl reduced their overall growth and severely affected the basic growth and physiological parameters such as fresh weight, dry weight, root, shoot and leaf length, and relative water content and led to chlorophyll loss, accumulation of H_2O_2 and malondialdehyde (MDA) and induced the ascorbic acid oxidase (AAO) activity. Priming with SNP enhanced the level of protective metabolites such as proline, reducing sugars, total amino acids and total phenolics, lowered the AAO activity, and modulated the activity of antioxidative enzymes such as catalase, ascorbate peroxidase, superoxide dismutase and guaiacol peroxidase that efficiently scavenged the H_2O_2 formed, lowered the MDA level and chlorophyll loss, and improved the overall physiological traits. The activity of polyphenol oxidase, nitrate reductase and α-amylase was also induced in presence of SNP during salt stress that ensured better utilization of polyphenolic compounds for defence, and improved the nitrogen assimilation efficiency with better mobilization of stored starch, so as to facilitate germination and enhance seedling tolerance in response to salinity. Our communication clearly established the prospects of seedling priming with SNP as a cost-effective strategy to develop salt-tolerant *V. radiata* seedlings with better survival capacity.

Keywords *Vigna radiata* · Sodium nitroprusside · Salt stress · Osmolytes · Antioxidants

Introduction

Around 6% of the total land area of the Earth which comprise almost 800 million hectares of cultivable land is affected due to salinity that hampers the growth, development and yield of various crops (Yang and Guo 2017). Accumulation of sodium (Na^+) and chloride (Cl^-) ions are responsible for salt toxicity in plants (Ismail et al. 2014). Ionic stress occurs due to accumulation of Na^+ and Cl^- ions in the tissues that are absorbed by the roots, translocated to the shoots and finally accumulated in the leaves (Munns and Tester 2008). Higher accumulation of Na^+ ions also hampers the K^+/Na^+ homeostasis which creates osmotic disbalance and inhibits the growth and development of plants (Roychoudhury et al. 2008). Excess soluble salts in the soil also reduce water availability, i.e. water potential of the root system that leads to osmotic stress (Hasegawa et al. 2000). Ionic and osmotic stress due to excess soluble salt leads to secondary stress that includes formation of reactive oxygen species (ROS), i.e. H_2O_2 which in turn produce cytotoxic metabolites such as malondialdehyde (MDA) (Roychoudhury et al. 2011). In addition, salt stress also reduces the chlorophyll content that might be due to higher activity of chlorophyllase enzyme, or breakdown of chlorophyll due to deposition of ions or disruption of ultrastructure of chloroplasts (Saha et al. 2010). Garchery et al. (2013) reported that osmotic stress in tomato seedlings induced the activity of ascorbic acid oxidase (AAO; EC 1.10.3.3) that ultimately lowered the ascorbic acid content of the apoplast which negatively affected the tolerance level of the seedlings.

Handling Editor: Rhonda Peavy.

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata 700016, West Bengal, India

[2] Department of Botany, Aligarh Muslim University, Aligarh 202002, India

[3] Department of Botany, Scottish Church College, 1&3 Urquhart Square, Kolkata 700006, West Bengal, India

Journal of Plant Growth Regulation (2021) 40:2342–2357
https://doi.org/10.1007/s00344-020-10285-z

Differential Responses of *Vigna radiata* and *Vigna mungo* to Fluoride-Induced Oxidative Stress and Amelioration via Exogenous Application of Sodium Nitroprusside

Ankur Singh[1] · Aditya Banerjee[1] · Aryadeep Roychoudhury[1]

Received: 4 October 2020 / Accepted: 25 November 2020 / Published online: 1 January 2021

Abstract

The manuscript aimed to explore the differential responses of *Vigna radiata* and *Vigna mungo* to fluoride (25 mg L^{-1} NaF) stress and validate the protective roles of sodium nitroprusside (SNP) (50 µM). *V. radiata* showed 53.5% higher bioaccumulation of fluoride compared to *V. mungo*. The overall growth in *V. radiata* was more affected (32.0%) compared with *V. mungo* (6.3%), which on the contrary displayed regulated fluoride entry and fluoride-adaptive phenotype. Fluoride bioaccumulation in *V. radiata* significantly triggered H_2O_2 and methylglyoxal accumulation, electrolyte leakage, chlorophyll degradation and lipid peroxidation, which accounted for its fluoride sensitivity. Exogenous SNP alleviated fluoride toxicity in both the species by synthesizing osmolytes (proline, glycine-betaine and total amino acids), non-enzymatic antioxidants (anthocyanins, flavonoids, total phenolics, carotenoids, ascorbate and glutathione) and activating antioxidative enzymes (APX, SOD, GPX, GST, GPOX and GR). Fluoride toxicity inhibited the CAT activity to a higher degree in *V. radiata* (51.0%) than *V. mungo* (31.3%). However, SNP supplementation rescued the CAT activity, lowered the fluoride uptake and membrane damage, and also alleviated oxidative stress in both the species, along with accelerated growth recovery and chlorophyll conservation. Cytotoxic methylglyoxal was efficiently detoxified in presence of SNP due to enhanced activity of the glyoxalases. Exogenous SNP also triggered endogenous NO formation along with up regulating the activity of P5CS and PAL, which stimulated proline and flavonoid biosynthesis during stress. Statistical analyses revealed that the ameliorating potential of SNP was more prominent in the susceptible species. Overall, our investigation established *V. radiata* and *V. mungo* as fluoride-sensitive and fluoride-tolerant species, respectively, and also illustrated the efficacy of SNP in abating stress-induced damages in *Vigna* by augmenting the defence machinery.

Keywords Antioxidative machineries · Fluoride · Glyoxalase · Osmolytes · Oxidative damages · Sodium nitroprusside · *Vigna radiata* · *Vigna mungo*

Introduction

In recent times, reckless human activities have enhanced the releases of fluoride (F) salts from the factories, agricultural fields and domestic wastes that get admixed with the surrounding water bodies (Hong et al. 2016). The irrigation of agricultural fields from water previously contaminated with F salts leads to the deposition of F in the soil that ultimately reduces the productivity. The F level in agricultural soil in some regions of India such as Newai Tehsil, Rajasthan and Bankura and Purulia, West Bengal is recorded to be as high as 50–180 and 55–399 mg F kg^{-1} of soil, respectively (Bhattacharya and Samal 2018). Accumulated F in the soil is readily taken up by the plants through their chloride channels and accumulated in the plants cells (Banerjee and Roychoudhury 2019a). Deposition of F in the plant tissues causes reduced growth and hampers the overall development along with visible symptoms of damages such as leaf tip burn, formation of secondary roots, higher degradation of chlorophyll due to chlorosis, and reduced biomass and height of plants. Various studies have previously shown the toxic effects of F in crops like *Vigna radiata*, *Oryza sativa*, *Cicer arietinum* and

Handling Editor: Tariq Aftab.

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, West Bengal 700016, India

Journal of Plant Growth Regulation (2021) 40:1579–1593
https://doi.org/10.1007/s00344-020-10202-4

Fluoride Stress-Mediated Regulation of Tricarboxylic Acid Cycle and Sugar Metabolism in Rice Seedlings in Absence and Presence of Exogenous Calcium

Ankur Singh[1] · Aryadeep Roychoudhury[1] · Santanu Samanta[1] · Aditya Banerjee[1]

Received: 12 December 2019 / Accepted: 14 August 2020 / Published online: 7 September 2020

Abstract
The aim of the current study was to investigate the regulation of tricarboxylic acid (TCA) cycle and sugar metabolism in the seedlings of the rice variety, Khitish, during fluoride stress, either in absence or presence of exogenous calcium compounds, viz., calcium hydroxide and calcium nitrate. The inhibitory effect of fluoride stress on energy production was reflected by lowered levels of pyruvic acid, citric acid and malic acid, substantial interference with the respiratory enzymes such as PDH, CS, IDH and SDH, and inhibiting the expression of the concerned genes. Seed priming with both calcium compounds elevated the organic acid levels, restored the activity of the above enzymes and expression of the corresponding genes. On the contrary, the enhanced fumarase and MDH activity and *MDH* gene expression, during fluoride stress, was lowered by calcium pre-treatment. The sucrose level, together with SPS and SS activity and expression of the corresponding genes were restored to higher levels, concomitant with lowered INV activity and gene expression, in presence of calcium compounds, suggesting lesser utilization of sucrose to combat fluoride stress. The efficacy of calcium compounds in conserving starch as the major food reserve, without provoking its catabolic breakdown, was established on the basis of enhanced starch content with lowered α-amylase and starch phosphorylase activity and corresponding gene expression. The present communication for the first time documented the detrimental impact of fluoride stress on TCA cycle metabolites and majority of the enzymes, and endogenous sucrose and starch accumulation, as well as highlighted the reversal of the negative effects with the exogenous application of calcium compounds.

Keywords Tricarboxylic acid cycle · Rice · Fluoride stress · Calcium compounds · Sugar metabolism

Introduction

Fluorine occupies 0.06–0.09% of the total surface area of earth and is the 13th most abundant element to be found on earth surface (Armienta and Segovia 2008). It exerts toxic effects when exceeds the safe limit of 1.5 mg L^{-1} as suggested by the World Health Organization (WHO) (Banerjee and Roychoudhury 2019a). In India, the fluoride content recorded in some regions is as high as 48 mg L^{-1} (Susheela 1999). Groundwater contamination with fluoride is also an acute menace in recent times. Rice plants requiring enormous volume of water for completion of their life cycle uptake fluoride from both soil and groundwater through chloride channels (Banerjee and Roychoudhury 2019b). Bharti et al. (2017) reported that for each 100 ppm rise in the fluoride level in the soil up to 2200 ppm, the internal fluoride content of the plants rises by 3 ppm. Bhattacharya et al. (2017) observed that the fluoride concentration in some parts of West Bengal in India ranges between 55 and 399 mg kg^{-1} of soil which leads to fluoride accumulation, in crops and vegetables like radish, carrot, onion bulb, brinjal, potato tuber, cauliflower, cabbage, coriander and pigeon pea, to as high as 13–63 mg kg^{-1} of dry weight. In another study, Bhat et al. (2015) reported fluoride concentration to be varying between 0.69 and 0.71 ppm in crops like onion, cabbage and tomato, grown within 1 km range of a zinc smelter in Debari, Udaipur, Rajasthan in India where soil fluoride concentration was reported to be as high as 179–189 ppm. The fluoride level in countries like China and Croatia is reported

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, West Bengal 700016, India

Environmental Science and Pollution Research (2021) 28:40220–40232
https://doi.org/10.1007/s11356-020-10604-7

EMERGING TRENDS IN BIOTECHNOLOGY FOR SUSTAINABLE DEVELOPMENT AND POLLUTION PREVENTION

Fluoride toxicity variably affects overall physiology and grain development in three contrasting rice genotypes, representing a potential biohazard

Aditya Banerjee[1] · Ankur Singh[1] · Aryadeep Roychoudhury[1]

Received: 10 June 2020 / Accepted: 23 August 2020 / Published online: 15 September 2020

Abstract

Ingestion of fluoride through consumption of contaminated food grains has been regarded to be hazardous for consumer health. The current study indicated the possible occurrence of such biohazard due to fluoride bioaccumulation in rice grains and straw (cattle feed). The effects of fluoride toxicity at three stages of grain development in three rice genotypes, viz., IR-64, Gobindobhog (aromatic), and Khitish, were also studied. Irrigation with fluoride-infested water inhibited grain formation in IR-64 and reduced grain yield in Gobindobhog. Fluoride toxicity promoted seed sterility in IR-64 by triggering reactive oxygen species (ROS) production and cellular necrosis, suppressing genes like *GIF1*, *DEP1*, and *SPL14* (positively controlling seed formation) and inducing *GW2* (negatively mediating grain development). Gobindobhog showed intermediate fluoride sensitivity and accumulated high levels of proline, anthocyanins, flavonoids, and phenolics due to the induction of genes like *P5CS*, *ANS*, and *PAL* in developing grains. The agronomic attributes in Khitish were unaffected by fluoride stress due to regulated fluoride uptake and high expression of *GIF1*, *DEP1*, and *SPL14* along with an increased synthesis of anthocyanins, flavonoids, and phenolics. Khitish also accumulated low ROS as a result of which lowest *lipoxygenase* expression (among selected cultivars) was observed in developing grains. Fluoride entry was accelerated in the straw of Khitish, possibly due to the absence of regulated uptake mechanism in dead seedlings. Furthermore, the ecological concerns regarding fluoride bioaccumulation and reduced grain yield at the varietal level were also established, based on statistical modelling.

Keywords Fluoride · Rice grain development · Reactive oxygen species · Biohazard · Varietal response · Statistical modelling

Introduction

Environmental pollution due to fluoride is a rapidly growing concern, especially in the Southeast Asian countries, since fluoride acts as a potent xenobiotic (Zhou et al. 2019). Unplanned exploitation of groundwater has drastically reduced the depth of the presiding water table. As a result, water extracted from deep-bored pipes is contaminated with fluorides that leach out from the mineral-bed (Banerjee and Roychoudhury 2019a). Apart from being used for drinking and other household purposes, a large proportion of groundwater is adequately used to irrigate the agricultural fields that are extensively distributed in South East Asia (Mondal 2017). Rice is one of the most widely cultivated food crops in this area, with China, India, Bangladesh, and Pakistan being some of the largest producers of rice grains (Peng et al. 2009). The states of West Bengal (rice bowl of India), Odisha, Bihar, and Chhattisgarh are largely infested with endemic fluorosis, and as high as 48 mg L^{-1} of fluoride has been detected across the Indian states (Susheela 1999). Fluoride has been regarded as a dreaded groundwater toxin in Guizhou Province, Yuncheng Basin, and Northwest China (Sivasankar et al. 2016). Alarming levels of fluoride have been detected in the Gangetic delta of Bangladesh (exclusively used for rice cultivation) and the Lahore, Quetta, and Tehsil Mailsi provinces of Pakistan (Rasool et al. 2018). Enhanced mobility of fluoride in Indian groundwater (popularly used for irrigation) was due to the abundance of zeolite-rich sediments which accelerated overall ion-exchange (Mondal and Kumar 2017). Ingestion of

Responsible Editor: Gangrong Shi

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, West Bengal 700016, India

Chemosphere 262 (2021) 127826

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Silicon nanoparticle-pulsing mitigates fluoride stress in rice by fine-tuning the ionomic and metabolomic balance and refining agronomic traits

Aditya Banerjee [a], Ankur Singh [a], M. Sudarshan [b], Aryadeep Roychoudhury [a, *]

[a] Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, 700016, West Bengal, India
[b] UGC-DAE Consortium for Scientific Research, Kolkata Centre, 3/LB Bidhannagar, Kolkata, 700098, India

HIGHLIGHTS

- Nano-silicon strongly stimulated overall rice growth during fluoride toxicity.
- Fluoride-mediated inhibition in grain hardening and lower yield were reversed.
- Nano-silicon enhanced macro and micronutrient uptake during stress.
- ROS was scavenged by the antioxidants and methylglyoxal detoxified by glyoxalases.
- Nano-silicon promoted nanozymatic effect.

ARTICLE INFO

Article history:
Received 13 June 2020
Received in revised form
14 July 2020
Accepted 24 July 2020
Available online 6 August 2020

Handling Editor: T Cutright

Keywords:
Silicon nanoparticles
Fluoride toxicity
Rice
Ionomics
Antioxidants and glyoxalase machinery
Amelioration

ABSTRACT

The present manuscript investigates the roles of silicon nanoparticles (SiNPs) in ameliorating fluoride toxicity in the susceptible rice cultivar, IR-64. Fluoride toxicity reduced overall growth and yield by suppressing grain development. Fluoride stress alarmingly increased the accumulation of cobalt, which together with fluoride triggered electrolyte leakage, malondialdehyde, methylglyoxal and hydrogen peroxide accumulation and NADPH oxidase activity. The overall photosynthesis was compromised due to chlorosis and inhibited Hill activity. Nano-Si-priming efficiently ameliorated molecular injuries and restored yield by reducing fluoride bioaccumulation particularly in the grains. The level of non-enzymatic antioxidants like anthocyanins, flavonoids, phenolics and glutathione was stimulated upon SiNP-priming. Nano-Si-pulsing removed fluoride-mediated inhibition of glutathione synthesis by activating glutathione reductase. Glutathione was utilized to activate glyoxalases and associated enzymes like glutathione-S-transferase and glutathione peroxidase. Uptake of nutrients like silicon, potassium, zinc, copper, iron, nickel, manganese, selenium and vanadium improved seedling health even during prolonged fluoride stress. Nano-Si-pulsing produced a nanozymatic effect, since high level of crucial co-factors like copper, zinc and iron stimulated the activity of superoxide dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase, which synergistically with other enzymatic and non-enzymatic antioxidants scavenged reactive oxygen species and promoted fluoride tolerance. Overall, the study supported by statistical modelling using principal component analysis, t-distributed stochastic neighbour embedding and multidimensional scaling, established the potential of SiNP to promote safe rice cultivation and precision farming even in fluoride-infested environments.

1. Introduction

Rising level of fluoride in groundwater is one of the major causes of persistent environmental pollution, owing to its high biomagnifying potential (Banerjee and Roychoudhury, 2019a). The crucial factors for leaching of fluorides from mineral beds are: (i) anthropogenic water extraction from underground aquifers, and (ii) increased rates of weathering (due to climate change) and ion-exchange reactions (Mukherjee and Singh, 2018). As a result, endemic fluorosis has become a rising concern in extensive areas

* Corresponding author.
E-mail address: aryadeep.rc@gmail.com (A. Roychoudhury).

https://doi.org/10.1016/j.chemosphere.2020.127826
0045-6535/

Journal of Biotechnology 324 (2020) 220–232

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Exogenous supplementation of melatonin alters representative organic acids and enzymes of respiratory cycle as well as sugar metabolism during arsenic stress in two contrasting indica rice cultivars

Santanu Samanta, Ankur Singh, Aditya Banerjee, Aryadeep Roychoudhury *

Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, 700016, West Bengal, India

ARTICLE INFO

Keywords:
Arsenic stress
Melatonin
Respiratory cycle
Organic acids
Sugar metabolism
Rice

ABSTRACT

The objective of the present investigation was to understand the impact of exogenously applied melatonin on mitochondrial respiration and sugar metabolism in two contrasting rice cultivars, viz., Khitish (arsenic-susceptible) and Muktashri (arsenic-tolerant) under arsenic-stress. Melatonin effectively restored the level of organic acids like pyruvic acid, malic acid and more particularly citric acid by 33 % in Khitish which were lowered during arsenic-stress, whereas their levels were further elevated in Muktashri to provide energy for defence against arsenic-induced injury. Arsenic-exposure led to a significant inhibition in enzyme activities as well as corresponding transcript level of key respiratory enzymes, viz., pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase and malate dehydrogenase, intriguingly more prominently in case of Khitish. Conversely, melatonin supplementation, irrespective of cultivars, considerably improved the activity of the above enzymes and corresponding gene expressions during stress, indicating acceleration in the rate of Krebs cycle. Melatonin supplementation also stimulated the accumulation of total soluble sugars by 62 % and 25 %, reducing sugars by 50 % and 44 % and non-reducing sugars by 75 % and 14 % in Khitish and Muktashri respectively, concomitant with higher activities of acid invertase, sucrose synthase and sucrose phosphate synthase enzymes, along with the expression of corresponding genes. Enhanced starch accumulation via regulation of alpha amylase and starch phosphorylase activities and gene expression, by melatonin also contributed towards better stress tolerance. Overall, this work illustrated the efficacy of melatonin in the regulation of representative organic acids and enzymes of respiratory cycle along with starch and sugar metabolism in rice cultivars under arsenic toxicity.

1. Introduction

Arsenic (As) ranks as the 20th most abundant element in the Earth's crust and 12th in the human body (Singh et al., 2015). Vast stretches of Vietnam, Bangladesh, along with West Bengal and other states of India depend on As-contaminated groundwater for irrigation of staple food crops such as rice (Kumar and Puri, 2012). Although the provisional guideline for As in drinking water is 0.01 mg L^{-1} (WHO, 2011), nearly 50 million people, mainly from Bengal Deltaic Plains, are at risk of being exposed to $>$ 0.05 mg L^{-1} of As, which is the maximum limit of As-concentration in drinking water in most of the Asian countries (Shrivastava et al., 2015; Rahaman et al., 2013). Arsenic has prominent adverse effects on plant growth and agronomic grain yield. In plants, As causes physio-biochemical alterations, interferes with the metabolic processes and induces generation of reactive oxygen species (ROS), which triggers oxidative stress in plants (Samanta et al., 2020; Abbas et al., 2018; Khan and Gupta, 2018). Due to similar chemical properties, [$As^{(V)}$] inhibits phosphate-dependent reactions and forms unstable arsenate-ADP [$As^{(V)}$-ADP] by competing with phosphate and uncouples ATP synthesis from electron transport (Finnegan and Chen, 2012). This leads to detrimental changes of energy bypass in mitochondrial respiration, including electron transport chain (ETC), making mitochondria a strong target of As-stress (Cozzolino et al., 2010).

The respiratory cycle acts as a central hub of metabolism in all living cells. Pyruvic acid, the end product of glycolysis, is oxidized to acetyl-CoA by the enzyme, pyruvate dehydrogenase complex (PDH) (Lin et al., 2003). Initiation of Krebs cycle occurs by condensation of acetyl-CoA with oxaloacetate by the enzyme, citrate synthase (CS)

* Corresponding author.
E-mail address: aryadeep.rc@gmail.com (A. Roychoudhury).

https://doi.org/10.1016/j.jbiotec.2020.10.013
Received 26 June 2020; Received in revised form 8 October 2020; Accepted 11 October 2020
Available online 14 October 2020

Environmental Pollution 267 (2020) 115378

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

De novo RNA-Seq analysis in sensitive rice cultivar and comparative transcript profiling in contrasting genotypes reveal genetic biomarkers for fluoride-stress response☆

Aditya Banerjee, Ankur Singh, Aryadeep Roychoudhury*

Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, 700016, West Bengal, India

ARTICLE INFO

Article history:
Received 26 May 2020
Received in revised form
13 July 2020
Accepted 4 August 2020
Available online 17 August 2020

Keywords:
RNA-Seq
de novo assembly
Fluoride toxicity
Differentially expressed genes
IR-64 and Khitish
Statistical modelling

ABSTRACT

The fluoride-sensitive indica rice cultivar, IR-64 was subjected to NaF-treatment for 25 days, following which RNA-Seq analysis identified significant up and down regulation of 1,303 and 93 transcripts respectively. Gene ontology (GO) enrichment analysis classified transcripts into groups related to 'cellular part', 'membrane', 'catalytic activity', 'transporter activity', 'binding', 'metabolic processes' and 'cellular processes'. Analysis of differentially expressed genes (DEGs) revealed fluoride-mediated suppression of abscisic acid (ABA) biosynthesis and signaling. Instead, the gibberellin-dependent pathway and signaling via ABA-independent transcription factors (TFs) was activated. Comparative profiling of selected DEGs in IR-64 and fluoride-tolerant variety, Khitish revealed significant cytoskeletal and nucleosomal remodelling, accompanied with escalated levels of autophagy in stressed IR-64 (unlike that in stressed Khitish). Genes associated with ion, solute and xenobiotic transport were strongly up regulated in stressed IR-64, indicating potential fluoride entry through these channels. On the contrary, genes associated with xenobiotic mobility were suppressed in the tolerant cultivar, which restricted bioaccumulation and translocation of fluoride. Pairwise expression profile analysis between stressed IR-64 and Khitish, supported by extensive statistical modelling predicted that fluoride susceptibility was associated with high expression of genes like *amino acid transporter*, *ABC transporter2*, *CLCd*, *MFS monosaccharide transporter*, *SulfT2.1* and *PotT2* while fluoride tolerance with high expression of *Sweet11*.

1. Introduction

Fluorine is the 13th most abundant element found in Earth's crust and acts as a toxic pollutant in the ecological cycle (Choubisa, 2013). Fluorine is usually detected in the form of fluorides. Increase in fluoride contamination in groundwater is a rising concern for overall health of consumers, including animals and even humans. Ingestion of fluoride beyond the safe limit of 1.5 mg L^{-1} (as prescribed by the World Health Organization) is known to cause severe fluorosis along with irreversible skeletal and neurological deformities (Yadu et al., 2018). Internationally acclaimed and recognized pollution-controlling agencies like the Occupational Safety and Health Administration (OSHA), Agency for Toxic Substances and Disease Registry (ATSDR), American Conference of Governmental Industrial Hygenists (ACGIH), Directly Observed Treatment (DOT), National Institute for Occupational Safety and Health, Washington D. C (NIOSH), The International Agency for Research on Cancer (IARC), National Fire Protection Association (NFPA) and Environmental Protection Agency (EPA) have recognized fluoride as a severely hazardous element.

Uncontrolled anthropogenic activity and unplanned extraction of groundwater have led to drastic reduction in the water-table, leading to unprecedented leaching of fluorides from low-lying mineral rocks (Mondal, 2017). Currently, groundwater fluoride pollution is a rising problem in South-East Asian countries largely involved in rice production (Banerjee and Roychoudhury, 2019a). Rice is the staple food crop in a large number of countries and is water-intensive in nature. Unfortunately, the groundwater reserves of the largest rice-producing countries like China, India, Bangladesh and Pakistan are drastically affected by endemic fluorosis (Sivasankar et al., 2016). Highest fluoride level has been detected to be as much as 48 mg L^{-1} across Indian states (Susheela, 1999).

In our previous communications, we showed that the use of

☆ This paper has been recommended for acceptance by Jörg Rinklebe.

* Corresponding author.
E-mail address: aryadeep.rc@gmail.com (A. Roychoudhury).

https://doi.org/10.1016/j.envpol.2020.115378
0269-7491/

DOI: https://doi.org/10.36094/sc.v86.2020.Alleviation_of_Floride-mediated.Singh.317

Research Communication

Sci. and Cult. 86 (9–10) 320-324 (2020)

Alleviation of Fluoride-Mediated Toxicity via Seed Priming with Calcium Oxide in *Oryza sativa* L. cv. Khitish

Abstract **: The effect of seed priming with calcium oxide (CaO) in the mitigation of fluoride-mediated stress is not yet explored. The present manuscript highlights the amelioration of fluoride toxicity in an indica rice cultivar, viz., Khitish, by initial priming of seeds for 24 h with two different concentrations of CaO (0.3 mM and 0.5 mM). Fluoride toxicity brought about considerable decrease in the seedling emergence, affecting the overall physiology of seedlings. Chlorophyll degeneration, enhanced endogenous malondialdehyde and H_2O_2, and electrolyte leakage due to higher bioaccumulation of fluoride were observed. While the catalase activity was decreased, the other antioxidants like guaiacol peroxidase, proline and total amino acids were elevated. Seed priming with CaO largely improved plant performance under fluoride stress by enhancing germination efficiency, with better physiology of plants, reducing fluoride bioaccumulation and overall oxidative damages. The protective effect of CaO was also evident from restoration of the catalase activity, and lowering the level of guaiacol peroxidase, proline and total amino acids, even under stressed condition. The decrement in the level of carotenoids and total phenolics as a consequence of stress was also overcome via CaO priming. The lower (0.3 mM) concentration appeared to be more potential in stress mitigation. Thus, CaO priming offers a reliable strategy in amelioration of fluoride-mediated injuries in Khitish by inhibiting fluoride uptake and improving the overall plant growth.**

Keywords: **Fluoride stress, calcium oxide, seed priming, antioxidants, osmolytes, rice**

Fluoride contamination is globally recognized as a severe threat to biotic components of the environment, hindering water and mineral transport. In plants, fluoride toxicity affects the overall physiology, causing chlorophyll damage which ultimately leads to chlorosis, leaf margin burn, growth retardation and necrosis of leaf tip. It interferes with the activity of ferric ion-containing enzymes such as catalase, cytochrome oxidase and peroxidase, hinders phosphorylation of proteins and rate of photosynthesis, and generates reactive oxygen species (ROS) which interfere with other metabolic processes[1,2]. Bioaccumulation of fluoride in edible parts can occur through translocation of fluoride ions via root-shoot system, thereby enhancing the endogenous fluoride level[1].

In order to counteract oxidative stress, plants recruit an elaborate defence mechanism involving compatible solutes like proline and amino acids, as well as non-enzymatic and enzymatic antioxidants like phenolics, carotenoids, peroxidase, catalase, etc[3]. In addition to these protective machineries, presence of various divalent elements in the soil like calcium and magnesium has been reported to bind with fluoride ion which results in lower uptake of fluoride from soil. The mitigation of fluoride toxicity by aluminium has been emphasized, but in recent times, several studies have particularly reported the protective role of calcium against fluoride stress. Calcium chelates the fluoride ions forming a CaF_2 complex, ultimately reducing fluoride uptake by plant tissues and reducing the overall damages. 0.6 g L^{-1} of CaO nanoparticles have been used successfully to adsorb 100 mg L^{-1} fluoride ions when present in aqueous solution[4]. The liming of soil with CaO has been shown to decrease the level of fluoride in *Camellia sinensis* L. leaves by 10.5% in comparison with the plants, where Ca was not applied exogenously[5].

Seed priming is an effective and practical technique which accelerates rapid and uniform seedling emergence, high seedling vigor and better yield, and normal metabolic processes under unfavourable environmental conditions. Higher and synchronized germination of primed seeds primarily occurs due to reduction in the lag time of imbibition, build-up of germination-enhancing metabolites, enzyme activation, metabolic repair during imbibition and osmotic adjustment[6]. Although the role of CaO in overcoming fluoride stress has been advocated, there is no available work on the protective action of seed priming

Earlier reports have shown that seed priming with $CaCl_2$ mitigated the adverse effects of drought stress in sunflower seedlings by increasing the different growth criteria like SL, leaf area, fresh and dry weight of shoot, increasing the antioxidant enzymes and photosynthetic pigments as well as decreasing the level of H_2O_2, MDA, soluble phenolic and flavonoid contents, as compared to drought-stressed seedlings[20]. Overall, the present study contributes to our mechanistic understanding of Ca priming-mediated attenuation of fluoride accumulation in rice and provides useful information regarding improvement of the adaptability of rice to fluoride stress by seed priming with CaO. The results evidently highlight that CaO priming can be an economic strategy for the farmers engaged in cultivation of rice to maintain usual growth and yield during fluoride stress under field conditions.

Acknowledgments

Financial assistance from Science and Engineering Research Board, Government of India through the grant [EMR/2016/004799] and Department of Higher Education, Science and Technology and Biotechnology, Government of West Bengal, through the grant [264(Sanc.)/ST/P/S&T/1G-80/2017] to Dr. Aryadeep Roychoudhury is gratefully acknowledged. ❐

ANKUR SINGH,
ADITYA BANERJEE,
ARYADEEP ROYCHOUDHURY*

Post Graduate Department of Biotechnology,
St. Xavier's College (Autonomous),
30, Mother Teresa Sarani, Kolkata - 700016,
West Bengal, India

* E-mail: aryadeep.rc@gmail.com

Received : 19 April, 2020

References

1. A. Banerjee, A. Roychoudhury and P Ghosh, *Plant Physiol. Biochem.* **142**, 143-150 (2019).
2. K.A. Kumar, P. Varaprasad and A.V.B Rao, *Res. J. Earth Sci.* **1**, 69-73 (2009).
3. A. Banerjee and A. Roychoudhury, *Plant Cell Rep.* **38**, 1217-1233 (2019).
4. G. Patel, U. Pal and S. Menon, *Sep. Sci. Technol.* **44**, 2806-2826 (2009).
5. J. Ruan , L. Ma , Y. Shi and W. Han, *Ann. Bot.* **93**, 97-105 (2004).
6. A. Banerjee and A. Roychoudhury, In: Advances in seed priming (Rakshit A & Singh HB, Eds) Springer, Singapore, pp 81 (2018).
7. S. Hussain, F. Khan, H.A. Hussain and L. Nie, *Front. Plant Sci.* **7**, 1-14 (2016).
8. A. Roychoudhury, S. Basu and D.N. Sengupta, *Acta Physiol. Plant.* **34**, 835-847 (2011).
9. O. Lowry, N. Rosebrough, A. Farr and R. Randall, *J. Biol. Chem.* **193**, 265-275 (1951).
10. Addinsoft XLSTAT v. 2015.1.01: data analysis and statistics software for Microsoft Excel. Addinsoft, Paris. (2017).
11. U. Dey, N.K. Mondal, K. Das and J.K. Datta, *Fluoride.* **45**, 389-393 (2012).
12. B.R. Gadi, P. Verma and A. Ram A, *J. Chem. Biol. Phys. Sci.* **2**, 1371-1378 (2012).
13. Y. Tak and B. Asthir, *Fluoride,* **50**, 324-333 (2017).
14. A. Roychoudhury, A. Banerjee and V. Lahiri, *Turk. J. Bot.* **39**, 887-910 (2015).
15. N. Misra, A.K. Gupta and U.N. Dwivedi, *J. Plant Sci.* **1**, 56-66 (2006).
16. S. Chakrabarti and P.K. Patra, *Fluoride.* **48**, 56-61 (2015).
17. A. Rezazadeh, A. Ghasemnezhad, M. Barani and T. Telmadarrehei, *Res. J. Med. Plants.* **6**, 245-252 (2012).
18. G. Yuan, X. Wang, R. Guo and Q. Wang, *Food Chem.* **121**, 1014-1019 (2010).
19. V. Chandrakar, A. Dubey and S. Keshavkant, *J. Soil Sci. Plant Nutr.* **16**, 662-676 (2016).
20. M. Madany and R. Khalil, *Egypt. J. Exp. Biol. (Bot.).* **13**, 119-133 (2017).

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: http://ees.elsevier.com

Research article

Silicon-regulated antioxidant and osmolyte defense and methylglyoxal detoxification functions co-ordinately in attenuating fluoride toxicity and conferring protection to rice seedlings

Ankur Singh, Aryadeep Roychoudhury *

Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, 700016, West Bengal, India

ARTICLE INFO

Keywords
Fluoride
Silicon
Rice
Damages
Osmolytes
Antioxidants
Methylglyoxal

ABSTRACT

The objective of this manuscript was to demonstrate the efficacy of silicon supplementation in relieving the fluoride-induced damages in rice cultivar, Khitish. The exposure of seedlings to two different concentrations of fluoride, viz., 25 and 50 mg L^{-1} NaF caused increase in fluoride accumulation, as a result of which the seedlings suffered severe oxidative stress, as evident from growth inhibition, reduction in seed germination, tissue biomass, root and shoot length, decline in chlorophyll content, increases in electrolyte leakage, H_2O_2 content, lipid peroxidation (malondialdehyde content and lipoxygenase activity), protein carbonylation and protease activity. The extent of damage was more at higher fluoride concentration. Silicon amendment, irrespective of fluoride concentrations, led to large build-up of endogenous silicon level and brought considerable improvement in all the parameters examined with respect to fluoride stress. The fluoride-mediated enhancement in methylglyoxal level was lowered by silicon, because of the prominent activation of glyoxalase I and glyoxalase II. While the stress-mediated induction in antioxidative enzymes like GPOX, APX, SOD, GPX and GR was lowered by silicon, the inhibition in CAT activity was relieved. The antioxidative defense mechanism was also boosted up via enhanced content of total phenolics and carotenoids. However, the fluoride-mediated increase in anthocyanins, flavonoids, xanthophyll, ascorbate and reduced glutathione, and osmolytes like total amino acids, proline and glycine-betaine, were all lowered in presence of silicon, together with reduced PAL and P5CS activity. Overall, silicon reduced oxidative damages to develop fluoride-tolerant rice plants through augmentation of different antioxidant and osmolyte defense and methylglyoxal detoxification system.

1. Introduction

Fluorine belongs to the halogen family and is labelled as the 13th most profuse non-metal found on the outer layer of the Earth's crust (Armienta and Segovia, 2008). Due to various anthropogenic activities, the range of fluoride (F) pollution in some parts of India is as high as 69–417 mg kg^{-1} of F in soil (Bhattacharya and Samal, 2018). The World Health Organization suggested that consumption of F above 1.5 mg L^{-1} causes toxic effects in human population (Banerjee and Roychoudhury, 2019a). Owing to the presence of F-salts in soil, groundwater also gets polluted due to leaching so that crops like rice take up adequate amounts of F^- ions continuously from both groundwater and soil through their chloride channels (Banerjee and Roychoudhury, 2019b) and deposit them within their cells at a level much above the threshold limit. Endogenous F^- accumulation hampers the overall growth of the plants. The F-toxicity causes leaf tip burn, chlorosis due to breakdown of chlorophyll, secondary root formation and reduction of biomass of rice seedlings (Banerjee and Roychoudhury, 2019c). Recent reports have shown the effect of F-toxicity on various plants such as *Oryza sativa*, *Cajanus cajan* and *Cicer arietinum* (Banerjee et al., 2019a, 2019b; Dey et al., 2012; Singh et al., 2020; Yadu et al., 2017). Rice seedlings subjected to 25 mg L^{-1} NaF treatment underwent considerable tissue damages and stress injuries along with massive accumulation of F^- far above the permissible limit (Banerjee and Roychoudhury, 2019c).

Over accumulation of F^- in plant tissues leads to oxidative stress which enhances the formation of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2). Higher level of ROS enhances lipoxygenase activity (LOX; EC 1.13.11) which triggers the peroxidation of lipid moieties of cell membrane, thereby up regulating the formation of by-products like malondialdehyde (MDA) and increasing the leakage of electrolytes from cells (Banerjee and Roychoudhury, 2019b). Oxidative burst in plants leads to the accumulation of other cytotoxic compounds such as methylglyoxal (MG) (Banerjee and Roychoudhury, 2018) and induces the carbonylation of certain amino acids present in proteins which distort their integrated structure, making them susceptible to the activity of protease (EC 3.4.21). The higher uptake of F^- within the tissues leads to the ultimate accumulation of F^- in the grains of the agricultural crops (Banerjee and Roychoudhury, 2019c).

To counteract the toxic effect of F^- and the F^--induced oxidative burst in the cells, plants have various protective machineries in the form of enzymatic and non-enzymatic antioxidants and osmolytes. The major enzymatic antioxidants involve glutathione reductase (GR; EC 1.6.4.2), guaiacol peroxidase (GPOX; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), ascorbate peroxidase (APX; EC 1.11.1.11) and superox-

* Corresponding author.
E-mail address: aryadeep.rc@gmail.com (A. Roychoudhury)

Journal of Hazardous Materials 390 (2020) 121978

Contents lists available at ScienceDirect

Journal of Hazardous Materials

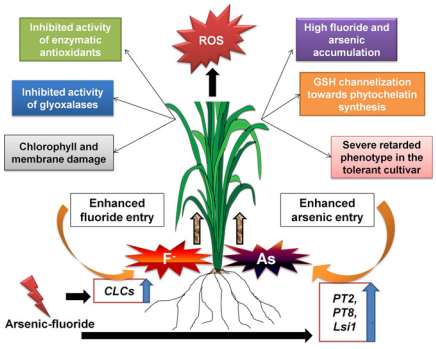
journal homepage: www.elsevier.com/locate/jhazmat

Deciphering the molecular mechanism behind stimulated co-uptake of arsenic and fluoride from soil, associated toxicity, defence and glyoxalase machineries in arsenic-tolerant rice

Aditya Banerjee, Santanu Samanta, Ankur Singh, Aryadeep Roychoudhury*

Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, 700016, West Bengal, India

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: L. Eder

Keywords:
Arsenic-fluoride co-contamination
Rice
Synergistic uptake mechanism
Bioaccumulation
Antioxidants

ABSTRACT

The current study elucidates the uncharacterized biohazard associated with rice growth in arsenic and fluoride co-contaminated sites. Analysis of the arsenic-tolerant rice cultivar, Muktashri (known to restrict arsenic uptake) revealed that fluoride largely stimulated arsenic bioaccumulation in the stressed tissues and vice versa. Gene expression studies revealed that high arsenic uptake was facilitated by the fluoride-dependent up regulation of *phosphate transporter2 (PT2)*, *PT8* and *low silicon rice1* (*Lsi1*), and elevated fluoride accumulation was stimulated by the arsenic-mediated induction of *chloride channels* (*CLCs*). The endogenous accumulation of fluoride and arsenic increased reactive oxygen species (ROS), O_2^-, membrane peroxidation and arsenic localization within tissues. This inhibited plant growth by triggering chlorosis, electrolyte leakage, malondialdehyde production (due to high lipoxygenase activity), protein carbonylation, protease activity and methylglyoxal accumulation due to inhibited glyoxylase activity. Metabolic analysis showed inhibited proline biosynthesis along with increased channelization of glutathione towards *phytochelatin synthase* and *glutathione-S-tranferase*-dependent pathways. Inhibition of the antioxidant enzymes like catalase, ascorbate peroxidase and guaiacol peroxidase validated the inefficient scavenging of H_2O_2 during combined stress. *In silico* analyses predicted the ecotoxicological risks of arsenic-fluoride complex formed during joint stress. Overall, our investigation illustrated the underlying mechanism of arsenic-fluoride co-uptake which resulted in complete suppression of the 'tolerant'-phenotype in Muktashri seedlings.

* Corresponding author.
E-mail address: aryadeep.rc@gmail.com (A. Roychoudhury).

https://doi.org/10.1016/j.jhazmat.2019.121978
Received 16 October 2019; Received in revised form 19 December 2019; Accepted 24 December 2019
Available online 25 December 2019

Protoplasma (2020) 257:767–782
https://doi.org/10.1007/s00709-019-01460-5

ORIGINAL ARTICLE

Seed priming with calcium compounds abrogate fluoride-induced oxidative stress by upregulating defence pathways in an indica rice variety

Ankur Singh[1] · Aditya Banerjee[1] · Aryadeep Roychoudhury[1]

Received: 3 September 2019 / Accepted: 18 November 2019 / Published online: 18 December 2019

Abstract

The aim of this manuscript was to investigate the role of calcium compounds, viz., $Ca(OH)_2$, $Ca(NO_3)_2$, and $CaCl_2$ (each used at 0.3 mM and 0.5 mM concentration) as seed priming agents to ameliorate fluoride toxicity in rice. The stressed seedlings exhibited high fluoride bioaccumulation, severe growth retardation, and cellular damages. Calcium compounds improved plant performance by increasing seed germination, seedling biomass, and root and shoot length, avoiding chlorophyll degeneration and leakage of electrolytes, along with lowering the levels of malondialdehdye, H_2O_2, and endogenous fluoride. Calcium-regulated defence was mediated by proline synthesised due to increased *Δ¹-pyrroline 5-carboxylate synthetase* (*P5CS*) and lowered *proline dehydrogenase* (*PDH*) expression, and glycine betaine synthesised due to *betaine aldehyde dehydrogenase 1* (*BADH1*) expression. While the stress-mediated lowering of carotenoids and total phenolics was relieved by calcium priming, stress-enhanced flavonoids and ascorbic acid content was restored to the normal condition, along with releasing the fluoride-induced inhibition of ascorbic acid oxidase (AAO) activity. The activities of antioxidant enzymes like catalase, guaiacol peroxidase, and superoxide dismutase, and the expression of *catalase* and *superoxide dismutase* genes were also affected by calcium priming. The elevated endogenous calcium level, brought about by priming, enhanced the expression of genes related to calcium signalling pathway, particularly the *calcineurin-B-like 10* (*CBL10*) gene. $Ca(OH)_2$ (0.3 mM) appeared to be the most efficient of all the three priming agents. Overall, the present work highlighted the efficacy of calcium compounds as priming agents in abrogating fluoride toxicity in rice.

Keywords Calcium compounds · Fluoride stress · Oxidative damage · Rice · Seed priming

Introduction

Fluorine is ranked as the 13th most abundant element in nature, covering 0.06–0.09% of total earth surface (Armienta and Segovia 2008). Rapid industrialisation has led to the higher release of fluorides in the atmosphere which ultimately increases the fluoride level above the safety limits. The endogenous fluoride level in plants is estimated to increase by 3 ppm for each 100 ppm increase in soil fluoride up to the 2200-ppm level (Bharti et al. 2017). The overall physiology of the plants is affected due to higher level of fluoride, thereby developing symptoms like chlorosis, leaf tip necrosis, and reduced growth (Banerjee and Roychoudhury 2019a). In addition, the biochemical processes like enzyme activity, synthesis of pigments, and proteins are severely impaired under fluoride stress. The redox homeostatic conditions of the cell are disrupted due to over production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). Increased level of ROS results in membrane perturbation and lipid peroxidation which increases the level of malondialdehyde (MDA) (Yadu et al. 2016) and leakage of electrolytes (Kumar and Singh 2015). Transportation of fluoride through root-shoot system leads to the bioaccumulation of fluoride in the edible parts of the plants (Banerjee and Roychoudhury

Key message Seed priming with calcium compounds mitigate fluoride stress in rice seedlings by lowering fluoride accumulation and oxidative damages, and triggering the endogenous calcium level which stimulate the osmolytes, antioxidants, and calcium-dependent genes.

Handling Editor: Néstor Carrillo

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, West Bengal 700016, India

Environmental Science and Pollution Research (2019) 26:36598–36614
https://doi.org/10.1007/s11356-019-06711-9

RESEARCH ARTICLE

Spermidine application reduces fluoride uptake and ameliorates physiological injuries in a susceptible rice cultivar by activating diverse regulators of the defense machinery

Aditya Banerjee[1] · Ankur Singh[1] · Aryadeep Roychoudhury[1]

Received: 15 June 2019 / Accepted: 7 October 2019 / Published online: 16 November 2019

Abstract

The manuscript illustrates the ameliorative effects of exogenously applied higher polyamine (PA), spermidine (Spd) in the susceptible indica rice cultivar IR-64 subjected to prolonged fluoride stress. The Spd treatment drastically reduced fluoride bioaccumulation by restricting entry of the anions through chloride channels and enabled better maintenance of the proton gradient via accumulation of P-H^+/ATPase, thereby improving the root and shoot lengths, fresh and dry weights, RWC, chlorophyll content and activities of pyruvate dehydrogenase (PyrDH), α-amylase, and nitrate reductase (NR) in the Spd-treated, stressed plants. Expression of *RuBisCo*, *PyrDH*, *α-amylase*, and *NR* was stimulated. Spd supplementation reduced the molecular damage indices like malondialdehyde, lipoxygenase, protease activity, electrolyte leakage, protein carbonylation, H_2O_2, and methylglyoxal (detoxified by glyoxalase II). Mitigation of oxidative damage was facilitated by the accumulation and utilization of proline, glycine-betaine, total amino acids, higher PAs, anthocyanin, flavonoids, β-carotene, xanthophyll, and phenolics as verified from the expression of genes like *P5CS*, *BADH1*, *SAMDC*, *SPDS*, *SPMS*, *DAO*, *PAO*, and *PAL*. Spd treatment activated the ascorbate-glutathione cycle in the stressed seedlings. Expression and activities of enzymatic antioxidants showed that GPOX, APX, GPX, and GST were the chief ROS scavengers. Exogenous Spd promoted ABA accumulation by upregulating *NCED3* and suppressing *ABA8ox1* expression. ABA-dependent osmotic stress-responsive genes like *Osem*, *WRKY71*, and *TRAB1* as well as ABA-independent transcription factor encoding gene *DREB2A* were induced by Spd. Thus, Spd treatment ameliorated fluoride-mediated injuries in IR-64 by restricting fluoride uptake, refining the defense machinery and activating the ABA-dependent as well as ABA-independent stress-responsive genes.

Keywords Fluoride stress · Exogenous spermidine · Rice physiology · Osmolytes · Antioxidants · Abscisic acid · Gene expression

Introduction

Fluorine is a toxic xenobiotic with about 950 mg L^{-1} stored as fluorides within the earth's crust. Biomagnification of fluoride in the ecological food chain is a potential biohazard since the World Health Organization (WHO) has prohibited the ingestion of fluoride beyond 1.5 mg L^{-1}. Doses above this safe limit lead to irreversible fluorosis and neurological diseases in humans and animals (Banerjee and Roychoudhury 2019a, b). Susheela (1999) recorded the extent of fluoride contamination to be as high as 48 mg L^{-1} in the groundwater across Indian states. Stretches of cultivable lands in West Bengal, Bihar, Bangladesh, and Pakistan face acute endemic fluorosis (Banerjee and Roychoudhury 2019c). Large parts of such fluoride-infested areas are dedicated towards the cultivation of rice, the staple food crop. Being a water-intensive crop, irrigation with fluoride-contaminated groundwater leads to fluoride bioaccumulation within rice tissues (Banerjee and Roychoudhury 2019c; Banerjee et al. 2019a). Limited studies are available on the detailed mechanism of fluoride-mediated injuries in rice. Fluoride acts as an accumulative poison that promotes oxidative stress in plants (Ghassemi-Golezani and Farhangi-Abriz 2019). It is absorbed by the roots via the

Responsible editor: Gangrong Shi

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11356-019-06711-9) contains supplementary material, which is available to authorized users.

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, West Bengal 700016, India

Plant Cell Reports (2023) 42:961–974
https://doi.org/10.1007/s00299-023-03013-w

REVIEW

Abscisic acid in plants under abiotic stress: crosstalk with major phytohormones

Ankur Singh[1] · **Aryadeep Roychoudhury**[2]

Received: 4 February 2023 / Accepted: 28 March 2023 / Published online: 20 April 2023

Abstract

Key message **Extensive crosstalk exists among ABA and different phytohormones that modulate plant tolerance against different abiotic stress.**

Being sessile, plants are exposed to a wide range of abiotic stress (drought, heat, cold, salinity and metal toxicity) that exert unwarranted threat to plant life and drastically affect growth, development, metabolism, and yield of crops. To cope with such harsh conditions, plants have developed a wide range of protective phytohormones of which abscisic acid plays a pivotal role. It controls various physiological processes of plants such as leaf senescence, seed dormancy, stomatal closure, fruit ripening, and other stress-related functions. Under challenging situations, physiological responses of ABA manifested in the form of morphological, cytological, and anatomical alterations arise as a result of synergistic or antagonistic interaction with multiple phytohormones. This review provides new insight into ABA homeostasis and its perception and signaling crosstalk with other phytohormones at both molecular and physiological level under critical conditions including drought, salinity, heavy metal toxicity, and extreme temperature. The review also reveals the role of ABA in the regulation of various physiological processes via its positive or negative crosstalk with phytohormones, viz., gibberellin, melatonin, cytokinin, auxin, salicylic acid, jasmonic acid, ethylene, brassinosteroids, and strigolactone in response to alteration of environmental conditions. This review forms a basis for designing of plants that will have an enhanced tolerance capability against different abiotic stress.

Keywords ABA perception and signaling · Abiotic stress · Antioxidants · Stress amelioration · Gene interactions

Introduction

Unlike animals, plants are sessile organism and are, thus, constantly challenged by unfavorable environment, such as drought, salinity, fluctuant temperature, and metal toxicity that significantly hamper their survival capability and longevity. The negative effects of abiotic stress impact the plants at all phase of their life cycle which are evident at both molecular and physiological level. According to the report of dos Reis et al. (2012), around 90% of the cultivable land is affected by one or more abiotic stress (drought, high salinity, cold, and heat) which accounts for about 70% yield loss of the major crops (Mantri et al. 2012). Qadir et al. (2014) reported that during last two decades (1990–2013), salinity of the arable land has been increased by 37%. Additionally, enhanced evapotranspiration due to change in global precipitation patterns and global warming has increased the severity and frequency of drought stress (Dai 2011). Recently, in a meta-analysis study, Raftery et al. (2017) predicted that the average temperature of the earth is likely to be increased by 2.0 to 4.9 °C. Anthropogenic activities can further worsen the condition through discharge of the contaminated water and solid wastes in cropland that eventually leads to higher accumulation of heavy metals. Accumulation of such toxic elements above the safety level not only limits the productivity of crops, but also causes serious risk to human health (Rehman et al. 2018). Estimates based on the integration of climate change and crop yield model further suggest that in coming days, the severity of the damage due to abrupt

Communicated by Wusheng Liu.

✉ Aryadeep Roychoudhury
aryadeep.rc@gmail.com

[1] Department of Biotechnology, St. Xavier's College (Autonomous), 30 Mother Teresa Sarani, Kolkata 700016, West Bengal, India

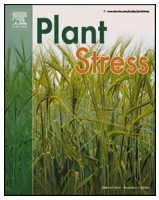
[2] Discipline of Life Sciences, School of Sciences, Indira Gandhi National Open University, Maidan Garhi, New Delhi 110068, India

Plant Stress 6 (2022) 100117

Contents lists available at ScienceDirect

Plant Stress

journal homepage: www.sciencedirect.com/journal/plant-stress



Biogenic nanoparticles and generation of abiotic stress-resilient plants: A new approach for sustainable agriculture

Swarnavo Chakraborty [1], Ankur Singh [1], Aryadeep Roychoudhury [*]

Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30 Mother Teresa Sarani, Kolkata, West Bengal 700016, India

A B S T R A C T

Agricultural crop production is getting a significant setback due to global warming and climate change in the modern era. These two factors have significantly induced the severity of abiotic stresses such as drought, salinity, extreme temperature and heavy metal toxicity. Upon being exposed to harsh environmental conditions, cytotoxic metabolites such as reactive oxygen species are generated which in turn disturb the homeostatic balance of cells along with inciting membrane damage. In order to overcome the negative effects of abiotic stresses, plant and agrarian scientific reports have shown significant progresses in promoting the efficient and eco-friendly approaches for the synthesis of plant- and microorganism-based synthesis of nanoparticles. Owing to their nanoscale size, nanoparticles possess a large surface/volume ratio that significantly alters their property and characteristic as compared to that of their normal counterparts. Nanoparticles can be physically, chemically and biologically synthesised. While physical and chemical methods have been successfully applied for the generation of nanoparticles of high purity and desired size, these processes are costly and require toxic chemicals. Thus, in the modern era, several investigators have focused their interest on biological methods of nanoparticle synthesis, since such methods are fast, cost effective and eco-friendly. In this review, we attempt to deliver information about plant- and microorganism-mediated synthesis of nanoparticles, based on recent scientific works. We also demonstrate the significance of these nanoparticles in the mitigation of negative impact of abiotic stresses on crop plants which might come handy in near future to the agricultural community.

Biogenic nanoparticle; Microorganisms; Plants; Abiotic stressors; Phytohormones

1. Introduction

Rapidly expanding population and global climate change evokes serious global agricultural food crisis. Abiotic stress is one such dreaded environmental issue that has been threatening the ecological stability at an alarming rate. Salinity, drought, extremes of temperature and heavy metal toxicity are some of the most common abiotic stressors, hampering global agricultural production (He et al., 2018; Khan et al., 2021). Osmotic stress forms the hall mark of all forms of abiotic stresses, leading to altered plant metabolism and ionic allocation (Imran et al., 2021). Owing to the rise in anthropogenic activities, random climatic change and poor techniques of agriculture, acres of agricultural lands suffer from stress-induced deterioration of soil quality, causing widespread reduction in the productivity of food crops globally (Mantri et al., 2011; Wahid et al., 2020). Abiotic stressors trigger the generation of harmful reactive oxygen species (ROS), which alter plant cellular metabolism at varying levels, including photosynthesis, carbon assimilation, biochemical cascades and permeability of membranes (Jaspers and Kangasjärvi, 2010; Khan and Khan, 2017). Hence, a series of efficient and eco-friendly techniques have been exploited to mitigate the ill effects associated with various abiotic stressors and to promote plant stress adaptive capability. Nanotechnology is one such promising platform, ensuring enhanced stress adaptability and sustainable agriculture worldwide (Saxena et al., 2016; Das and Das, 2018). Nanoparticles have gained extensive importance in the field of molecular biology, owing to their unique physical and chemical properties, viz., tiny size (ranging from 1-100 nm); remarkable cellular stability, enormous surface area and reactive power (Nejatzadeh, 2021). Nanoparticles can have both positive and negative impacts upon the plant system. At very high doses, several nanoparticles can impose oxidative injuries to biomolecules, leading to plant cellular damages and even cell death. However, at optimal levels (low nanomolar concentrations), nanoparticles behave as crucial regulators of plant development and growth (Tariq et al., 2021). Nanoparticle application enhances plant stress adaptive capability via alteration of the crucial metabolic cascades and boosting the anti-oxidative defence machinery and free radical detoxification mechanisms (Jalil and Ansari, 2019). Specific plant parts, viz., seeds, flowers, fruits, stem, bark, leaves, peels and roots have been widely exploited for the biosynthesis of an array of nanoparticles, including iron, gold,

* Corresponding author.
E-mail address: aryadeep.rc@gmail.com (A. Roychoudhury).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.stress.2022.100117
Received 30 July 2022; Received in revised form 25 September 2022; Accepted 1 October 2022
Available online 2 October 2022

Received: 1 May 2021 | Revised: 24 June 2021 | Accepted: 14 July 2021

DOI: 10.1111/ppl.13502

SPECIAL ISSUE ARTICLE

Physiologia Plantarum

Gene regulation at transcriptional and post-transcriptional levels to combat salt stress in plants

Ankur Singh | Aryadeep Roychoudhury

Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, India

Correspondence
Aryadeep Roychoudhury, Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata, West Bengal 700016, India.
Email: aryadeep.rc@gmail.com

Funding information
Science and Engineering Research Board, Government of India, Grant/Award Number: EMR/2016/004799; Department of Higher Education, Science and Technology and Biotechnology, Government of West Bengal, Grant/Award Number: 264(Sanc.)/ST/P/S&T/1G-80/2017

Edited by V. Kumar

Abstract

Soil salinity is a major challenge that will be faced more and more by human population in the near future. Higher salt concentrations in the soil limit the growth and production of crops, which poses serious threats to global food production. Various plant breeding approaches have been followed in the past which are reported to reduce the effect of salt stress by inducing the level of protective metabolites like osmolytes and antioxidants. Conventional breeding approaches are time-consuming and not cost-effective. In recent times, genetic engineering has been largely followed to confer salt tolerance through introgressions of single transgenes or stacking multiple transgenes. However, most of such works are limited only at the laboratory level and field trials are still awaited to prove the long-term efficacy of such transgenics. In this review, we attempt to present a broad overview of the current strategies undertaken to develop halophytic and salt-tolerant crops. The salt-induced damages in the plants are highlighted, followed by representing the novel traits, associated with salt stress, which can be used for engineering salt tolerance in glycophytic crops. Additionally, the role of transcriptional and epigenetic regulation in plants for amelioration of salt-induced damages has been reviewed. The role of post-transcriptional mechanisms such as microRNA regulation, genome editing and alternative splicing, during salt stress, and their implications in the development of salt-tolerant crops are also discussed. Finally, we present a short overview about the role of ion transporters and rhizobacteria in the engineering of salt tolerance in crop species.

1 | INTRODUCTION

To supply food for the growing population of the world, it is necessary to increase the area of the cultivable land for crop production and introduce new intensive and ever-growing productive crops. According to the reports of the Food and Agricultural Organization (FAO, 2011), the world population is estimated to cross a mark of 9.1 billion by 2050. To feed this enormous population, crop production should be raised by 70% (FAO, 2011). Salinization of land inflicts a serious threat and appears as a major challenge to meet this demand by hampering the growth and productivity of crops.

According to Flowers et al. (2010), salt contamination of around 800 million hectares of land is causing a loss of about 12 trillion dollars per year to the global agricultural economy. Thus, approximately one third of the total agricultural land that covers around 6% of the total land area is highly affected by salt (Mian et al., 2009). The regions that receive inadequate amount of rainfall or have a high evapotranspiration rate, that is, arid and semi-arid regions, are more prone to salt stress (Singh et al., 2015). Plants cultivated on saline soil accumulate sodium (Na^+) and chloride (Cl^-) ions in their tissues that lead to ionic and osmotic stress. Ionic stress hampers the K^+/Na^+ homeostasis, ultimately inhibiting plant growth and development (Roychoudhury et al., 2008). High Na^+ levels also interfere with enzyme activities and destabilize protein structures by interfering with their surface charges. Osmotic stress due to salt toxicity causes the formation of reactive oxygen species (ROS) such as superoxide

RNA Interference (RNAi) Technology: An Effective Tool in Plant Breeding

Ankur Singh and Aryadeep Roychoudhury

Abstract Traditional plant breeding is widely practised and is very successful in producing transgenic plants with desired traits, but it is very time-consuming, not cost effective and is only limited to the species which are very closely related. RNA interference (RNAi) technology has provided the breeders a new tool by inserting a small piece of non-coding RNA which has the ability to decrease the expression of a specific gene. Insertion of non-coding RNA suppresses the expression of the gene which provides an opportunity to acquire a new trait by accumulating or eliminating specific traits which do not exist in wild type plants. RNAi is an evolutionary mechanism acquired by the plants as a defence mechanism against foreign genes, but in recent times, it has been used by the breeders as a tool for generating better traits in organisms. RNAi is a reliable and fast method for studying gene function by silencing a gene, based on its sequence specificity. Application of RNAi in plants has led to the generation of plants which are resistant against stress condition, less susceptible to infection, have better nutritional value along with higher yield. This chapter deals with RNAi mechanism, major players involved in RNAi and methods which are used for insertion of RNAi in plants. In addition, we focus on the application of RNAi in plants to acquire new characters which are difficult to obtain by traditional breeding.

Keywords RNA interference · Micro RNA · Plant breeding · Gene silencing · Quality traits · Stress

A. Singh
Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

A. Roychoudhury (✉)
Discipline of Life Sciences, School of Sciences, Indira Gandhi National Open University, Maidan Garhi, New Delhi, India

A. Raina et al. (eds.), *Advanced Crop Improvement, Volume 1*,
https://doi.org/10.1007/978-3-031-28146-4_12

CHAPTER 9

Hydrogen Peroxide as a Signaling Molecule in Plant Abiotic Stress

ANKUR SINGH and ARYADEEP ROYCHOUDHURY

Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30 Mother Teresa Sarani, Kolkata, West Bengal, India

ABSTRACT

Hydrogen peroxide (H_2O_2) is widely generated as a reactive oxygen species (ROS) during abiotic stress in all plant systems. H_2O_2 is formed in an uncontrolled way during mitochondrial respiration and photosynthesis via electron transport process. In addition, activity of various enzymes like amine oxidases, oxalate, and flavin-containing enzymes also contribute toward tight regulation of H_2O_2 formation and its level in plants via both enzymatic and nonenzymatic H_2O_2 scavengers. Salinity, drought, light, and extreme temperature like external stress can lead to excess formation of H_2O_2, which acts as cytotoxic metabolite in cells. However, in recent time, it is well established that optimal concentration of H_2O_2 acts as a signaling molecule regulating a wide range of biochemical, physiological, and molecular response in plants during stressed environment. Various reports have shown that H_2O_2 plays a major role in plant development to control physiological processes like senescence, seed germination, regulation of stomatal aperture, shoot and root development, and programmed cell death. In addition, the crosstalk of H_2O_2 with other signaling molecules likes nitric oxide, ethylene, jasmonic acid, salicylic acid, abscisic acid, and calcium plays a key role to abrogate

Crop Sustainability and Intellectual Property Rights. Soumya Mukherjee, Piyali Mukherjee & Tariq Aftab (Eds)

CHAPTER 14

Advances in Metabolomics Research in Environmental Stress Response in Plants

ANKUR SINGH and ARYADEEP ROYCHOUDHURY*

Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata – 700016, West Bengal, India

**Corresponding author. E-mail: aryadeep.rc@gmail.com*

ABSTRACT

Abiotic stress is one of the major limiting factors that significantly hampers the development of plants and also negatively affects the quality and quantity of crop yield. To overcome the negative effects of abiotic stresses, plants reprogram their metabolic pathway which in turn enhances the level of primary and secondary metabolites. Metabolomics is an important technique for deciphering the unknown metabolites or quantification of known metabolites in plants formed on being exposed to such limiting factors. Metabolome refers to the total metabolite constituent of any plant species that can be analyzed to further explore the changes that take place in plants on being exposed to environmental stresses. Metabolomics is a promising tool that helps in the proper identification of metabolic networks linked with abiotic stress that might help in the identification of stress-specific marker in plants. Advanced metabolomics tools such as non-destructive nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GS-MS), liquid chromatography-mass spectrometry (LC-MS) and direct flow injection mass spectrometry have enhanced the rate of metabolome profiling of plants. Presently, integration of metabolomics with other omics

Biology and Biotechnology of Environmental Stress Tolerance in Plants, Volume 3: Sustainable Approaches for Enhancing Environmental Stress Tolerance. Aryadeep Roychoudhury (Ed.)

CHAPTER 3

Exogenous Application of Trace Elements and Their Uptake by Plants to Mediate Abiotic Stress Tolerance

ANKUR SINGH and ARYADEEP ROYCHOUDHURY*

Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata – 700016, West Bengal, India

**Corresponding author. E-mail: aryadeep.rc@gmail.com*

ABSTRACT

With the rise in global population, the demand for oil, fibers, foods, and crop yields is increasing day by day but in contrast to this unfavorable environmental condition hinders the productivity of plants. To attain global food demand, it is necessary to improve the yields of the plants by reducing the negative effects of abiotic stresses. Abiotic stresses hamper the normal growth of plants by inhibiting seed germination, reduced photosynthetic rate, water, and nutritional imbalance, higher formation of cytotoxic metabolites and lower accumulation of dry mass which ultimately reduces the yield and quality of plants. Trace elements (TEs) are a secondary requirement of plants for their normal growth, but various reports have demonstrated their importance in ameliorating the negative effects of abiotic stresses. At the primarily level, TEs are important constituents of several metalloproteins, cofactors for various enzymes, cell membrane and organelles and at secondary level, they regulated the key metabolic pathways involved in gene expressions, production of phytohormones and formation of lipids, carbohydrates, proteins, osmolytes, and antioxidants that protects the plants from stress-induced

Biology and Biotechnology of Environmental Stress Tolerance in Plants: Trace Elements in Environmental Stress Tolerance, Volume 2. Aryadeep Roychoudhury (Ed.)

CHAPTER 9

Role of Phenolic Acids and Flavonoids in the Mitigation of Environmental Stress in Plants

ANKUR SINGH and ARYADEEP ROYCHOUDHURY

Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata – 700016, West Bengal, India

**Corresponding author. E-mail: aryadeep.rc@gmail.com*

ABSTRACT

Phenolic compounds are an important class of secondary metabolites that are produced in plants in optimal and suboptimal conditions and play a pivotal role in their life cycle. This diverse group of metabolites contains various structures, from simple forms consisting of one aromatic ring to more complex ones consisting of a large number of polymerized molecules. Based on their structures, different polyphenolic compounds show different functions that range from protection against abiotic stress to plant growth and reproduction. To cope with abiotic stresses, plants enhance the production of secondary metabolites such as phenolic acid and flavonoids that detoxify the cytotoxic metabolites and help in improving the growth, development, and yield of plants. Polyphenolic compounds are synthesized in plants via. shikimate/phenylpropanoid pathway. The major enzymes of this pathway are phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), cinnamate 4-hydroxylase (C4H), and 4-coumarate: CoA ligase (4CL). Phenylpropanoid pathway is activated under abiotic stress conditions (drought, heavy metal, salinity, high/low

Biology and Biotechnology of Environmental Stress Tolerance in Plants: Secondary Metabolites in Environmental Stress Tolerance, Volume 1. Aryadeep Roychoudhury (Ed.)

CHAPTER 1

Antioxidants and Secondary Metabolites of Mushrooms as a Source of Nutraceuticals and Functional Food

Ankur Singh and *Aryadeep Roychoudhury**

1. Introduction

Since origin, man has mostly lived in a hunter-gatherer society, depending on the biodiversity of their surroundings for their sustenance. Mushrooms have probably been eaten for as long as people have walked on the surface of the Earth. The history of mushrooms dates back to 300 BC when Theophrastus first mentioned it as 'truffles'. Romans regarded mushrooms as 'food of the Gods' whereas Greek warriors used to consume them during battles to acquire strength. Basidiomycete fungi, during sexual reproduction, produce fruiting bodies that are commonly known as 'mushrooms'. Spores released from the fruiting bodies germinate to form mycelia which in the presence of suitable substrate (dead branches, fallen leaves, wood, etc.) further develop into primoridia. Finally, primoridia gives rise to the fruiting bodies, i.e., the mushrooms. According to Royse et al. (2017), the production of mushrooms was 30 million tons at a cost of about $63 billion in 2013 which shows a significant rise in the production as compared to that of 1978 when only one million ton of mushrooms was produced worldwide. Of all the cultivated mushrooms, around 54% (~ $34 billion) was edible. Most of the cultivated edible mushrooms consist of five genera: *Flammulina* (11%), *Agaricus* (15%), *Auricularia* (17%), *Pleurotus* (19%) and *Lentinula* (22%) (Raut, 2019). For centuries, mushrooms have been widely used as traditional medicines and foods. However, in spite of wide use, the pharmacological and nutritional property of mushrooms has been recently recognised worldwide. In recent times, various scientific reports have described the pharmacological properties (antifungal,

Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30 Mother Teresa Sarani, Kolkata – 700016, West Bengal, India.
* Corresponding author: aryadeep.rc@gmail.com

Mechanism of Crosstalk Between Cytokinin and Gibberellin

Ankur Singh and Aryadeep Roychoudhury

Abstract Phytohormones play an integral role in various plant biological processes and regulates the signaling pathways that helps to maintain the growth and development of plants in unheralded environmental conditions. Cytokinin and gibberellin are two such major phytohormones that regulate the growth of the plants; however, various studies have demonstrated that an antagonistic interaction occurs between cytokinin and gibberellin during several physiological processes such as shoot and root elongation, shoot regeneration in culture, cell differentiation and meristem activity. This delicate balance between cytokinin and gibberellin in plants is maintained by various proteins such as KNOX, SPY and SEC. KNOX proteins enhance the expression of cytokinin-biosynthesis gene *Isopentenyl Transferase 7* that accumulates cytokinin in meristems, whereas SPY and SEC are two Serine and Threonine O-linked N-acetylglucosamine (O-GlcNAc) transferase that inhibits the gibberellin response and enhances cytokinin signaling in plants. The development of plants requires a dynamic balance between these two hormones. Thus, the main objective of this book chapter is to present all the recent works that was done focusing the crosstalk between cytokinin and gibberellin. We also tried to explain the role of major components (SPY, SEC and KNOX) involved in this complex network and effects of their mutation in plants.

1 Introduction

Plants have extraordinary capability of being potentially 'immortal'. Some plants survived several years and their death mostly occurred due to external factors such as environmental stress, pathogen infection and other diseases. This long life expectancy of the plants is mainly due to presence of long lasting stem cells that continuously produces new organs and tissues. According to Heidstra and Sabatini (2014), this

A. Singh · A. Roychoudhury (✉)
Department of Biotechnology, St. Xavier's College (Autonomous), 30 Mother Teresa Sarani, Kolkata 700016, West Bengal, India
e-mail: aryadeep.rc@gmail.com

T. Aftab (ed.), *Auxins, Cytokinins and Gibberellins Signaling in Plants*,
Signaling and Communication in Plants,
https://doi.org/10.1007/978-3-031-05427-3_4

Chapter 23

Role of nanoparticles in remediation of environmental contaminants

Ankur Singh and Aryadeep Roychoudhury
Post-Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

23.1 Introduction

The industrial revolution brought about economic prosperity, along with the release of various kinds of pollutants in the environment which is becoming a serious cause of concern for the growing human population. Environmental pollutants mostly consist of pesticides, fertilizers, heavy metals, herbicides, sewage from households, toxic gases, industrial effluents, oil splits, and particulate matter. Several new techniques have been initiated to remove these contaminants from soil, air, and water. Since these pollutants are a mixture of various kinds of substances of different nature, the removal of these pollutants is quite complex. Thus, to get rid of this problem, recent studies have adopted the use of nanotechnology for eliminating toxic pollutants from the environment (Tratnyek & Johnson, 2006).

In recent times, the nanoparticle has gained a lot of popularity due to its distinct size that accounts for its unique properties. Nanoparticles are stated as particles having sizes lower than 100 nm and higher than 1 nm in two or three dimensions. Due to their greater surface-to-volume ratio, nanoparticles have higher reactivity as compared to their bulky counterparts (Guerra, Smith, Alexis, & Whitehead, 2017). Surface functionalization or grafting of nanoparticles with various functional groups helps in inducing its binding capacity with desired molecules like pollutants (Guerra, Campbell, Whitehead, & Alexis, 2017). In addition, various morphological properties like porosity, size, and chemical composition of nanoparticles can also be modified which directly affects the nature of nanoparticles which can be further utilized for enhancing their properties. Thus, surface grafting and morphological changes of nanoparticles as desired offer significant advantages for the elimination of unwanted pollutions from the environment over conventional methods (Campbell, Guerra, Dhulekar, Alexis, & Whitehead, 2015). Using biodegradable materials provides an alternative safer and greener method for removing pollutants from the environment. Thus, various groups have focused on nanotechnology for the efficient removal of wastes from the environment because nanoparticles can be easily engineered to make them more effective and target-specific (Pandey & Fulekar, 2012). Despite such a significant advantage over conventional methods, nanoparticles are often unstable under normal conditions. Recent advancement in nanoscience has paved the way for the use of nanoparticles for efficient removal of environmental contaminants by increasing their stability, preventing their agglomeration, and enhancing their monodispersity. To develop a good nanomaterial, a keen understanding of their process of fabrication, the material platforms, and optimized performance are required. Thus, this chapter will aim to provide an outline of some recent development in the field of nanotechnology that will be effective in abrogating toxicity emanating from the environmental pollutants.

23.2 Interaction between nanoparticles and biotic and abiotic factors

According to Avio, Gorbi, and Regoli (2017), about 10 million tons of toxic chemicals are discharged by the industries into the environment, which in reaction with other compounds, release more toxic by-products like polychlorinated dibenzofurans or polychlorinated dibenzo-p-dioxins. In addition to their complex physical and chemical properties, their interactions with abiotic and biotic environmental factors like animals, plants, water, soil, microorganism, and air have further complicated the process of their elimination from the environment.

Hazardous and Trace Materials in Soil and Plants. DOI: https://doi.org/10.1016/B978-0-323-91632-5.00021-5

CHAPTER 16

Protective Chemicals and Metabolites in Stabilizing Photosynthesis and Respiration Machinery during Abiotic Stresses

ANKUR SINGH and ARYADEEP ROYCHOUDHURY*

Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30, Mother Teresa Sarani, Kolkata 700016, West Bengal, India

**Corresponding author. E-mail: aryadeep.rc@gmail.com*

ABSTRACT

Unfavorable environmental conditions suspend the development and growth of plants and also limit their production that ultimately threatens the food security of the world. Various abiotic factors such as drought, salinity, temperature, heavy metals, and excessive light hamper the photosynthetic and respiratory process in plants. These factors either directly inhibit the activity of enzyme involved in the above-mentioned process or indirectly distort the structure of thylakoids, chloroplast and degrade photosynthetic pigments via the formation of reactive oxygen species that again inhibits the above-mentioned processes and directly lowers the yield of the plants. However, to survive in these hostile environment, plants have developed an extensive network of protective metabolites comprising of osmolytes and secondary metabolites. Additionally, various reports have also demonstrated that exogenous application of certain chemicals such as salicylic acid, nitric

Photosynthesis and Respiratory Cycles during Environmental Stress Response in Plants. Aryadeep Roychoudhury (Ed.)

Omics Tools to Understand Abiotic Stress Response and Adaptation in Rye, Oat and Barley

21

Ankur Singh and Aryadeep Roychoudhury

Abstract

Rye, oat and barley are one of the most highly consumed cereals in the world after wheat and rice. Their demand in the food industries has enormously increased; however, despite high productivity, continuous supply as per the market demand is hard to achieve, mostly because of periodic crop losses occurring due to abiotic stresses such as drought, salinity, heavy metal toxicity and non-uniform temperature. Generation of abiotic stress-tolerant crops is one of the most serious challenges that need to be addressed by the scientific communities. In this regard, efforts are being made to understand the stress tolerance mechanism, gene discovery and interaction of genetic and environmental factors. Several omics tools and approaches have recently been used for the development of stress-tolerant crops having better grain quality. Modern sequencing technologies have greatly accelerated the genomics and transcriptomics studies in the above-mentioned species. In contrast, limited efforts have been made in other omics branches like proteomics and metabolomics. Extensive cataloguing of omics resources has highlighted the need for integration of omics approaches for efficient utilization of resources and a better understanding of the molecular mechanism. The information provided in this chapter will be helpful to understand the plant responses and genetic regulatory networks involved in abiotic stress tolerance and efficient utilization of omics resources for improvement in performances of rye, oat and barley.

Keywords

Omics · *Secale cereale* · *Avena sativa* · *Hordeum vulgare* · Microarray · Mass spectroscopy · Abiotic stresses

A. Singh · A. Roychoudhury (✉)
Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

A. Roychoudhury et al. (eds.), *Omics Approach to Manage Abiotic Stress in Cereals*,
https://doi.org/10.1007/978-981-19-0140-9_21

CHAPTER

21

Role of β-aminobutyric acid in generating stress-tolerant and climate-resilient plants

Ankur Singh and Aryadeep Roychoudhury

Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

1 Introduction

Beta-aminobutyric acid (BABA) is a nonproteinaceous rare amino acid found in plants (Jisha and Puthur, 2015). It is an isomer of γ-aminobutyric acid (GABA) and α-aminobutyric acid (AABA) with a common molecular formula $C_4H_9NO_2$. Due to its lower abundance in nature, BABA was earlier considered as xenobiotics, but Gamliel and Katan (1992) showed that root exudate of tomato plants grown in solarised soil contains BABA. Baccelli et al. (2017) demonstrated the presence of BABA in plants and stated that during abiotic stress, the endogenous level of BABA was enhanced in plants. BABA is a well-known neurotransmitter in animals that is perceived by the target cells on being released from the neurones and acts by inducing a change in the functional properties of the cells (Deutch, 2013). Due to its ability to induce tolerance in plants against stressed environment and its dynamic nature, Baccelli and Mauch-Mani (2017) considered BABA as a novel priming hormone for plants. Exogenous application of BABA plays a pivotal role in inducing the tolerance level of the plants against both biotic and abiotic stress like salinity, water deficit, and heat shock (Jakab et al., 2001; Cohen, 2001; Zimmerli et al., 2008). The protective role of BABA in plants against harsh environmental condition is mainly explained by its interaction with several stress regulating hormones in plants such as abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and ethylene (Ton et al., 2005; Jakab et al., 2005). Luna et al. (2014) identified *impaired in BABA-induced immunity 1* (*IBI1*) gene that encodes for aspartyl-tRNA synthetase in *Arabidopsis*. On priming the *Arabidopsis* mutants with BABA against the biotrophic oomycete *Hyaloperonospora arabidopsidis*, it was found that both SA-dependent and SA-independent pathways were blocked which led to the conclusion that perception of BABA in plants is mediated by an

https://doi.org/10.1016/B978-0-323-85665-2.00005-4

Chapter 4
Hydrogen Sulfide and Redox Homeostasis for Alleviation of Heavy Metal Stress

Ankur Singh and Aryadeep Roychoudhury

Abstract The industrial development has led to the release of heavy metals (HMs) in the environment beyond the safe limit. The effluents of industries carrying HMs like lead, mercury, cadmium, aluminium, zinc, copper, arsenic, etc. are admixed with the surrounding water bodies. Agricultural fields when irrigated with water from these polluted sources also get contaminated which ultimately has led to the toxicity in plants due to accumulation of metals in their tissues above the threshold limit. Metal toxicity reduces plant growth and affects crop yield. In addition, metal toxicity also induces the formation of reactive oxygen species (ROS) which disturb the redox homeostasis of the cells. ROS, when present above a threshold level in cells, damage the lipid membranes and other macromolecules. However, plants have some protective machinery such as osmolytes, and enzymatic and non-enzymatic antioxidants which protect them from metal stress and helps in the detoxification of ROS. As a potent endogenous gasotransmitter, hydrogen sulfide (H_2S) can enhance the function of these protective machineries of plants when exogenously applied. H_2S also protects proteins which are sensitive to damage by ROS through persulfidation of cysteine residues present in the protein. During metal stress, H_2S can mediate the signalling pathways of calcium and nitric oxide (NO). This chapter mainly deals with the toxic effect of different HMs in plants, metabolism of H_2S and its protective role during metal toxicity in plants.

Keyword Hydrogen sulphide · Metal stress · Redox homeostasis · Reactive oxygen species · Osmolytes · Antioxidants

A. Singh · A. Roychoudhury (✉)
Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India
e-mail: aryadeep.rc@gmail.com

M. N. Khan et al. (eds.), *Hydrogen Sulfide and Plant Acclimation to Abiotic Stresses*, Plant in Challenging Environments 1, https://doi.org/10.1007/978-3-030-73678-1_4

Salicylic Acid and Jasmonic Acid in Generating Salt Stress-Tolerant Plants

Ankur Singh and Aryadeep Roychoudhury

Abstract Salt toxicity is a severe environmental constraint which limits the growth and development of the plants. To overcome this unfavourable environmental situation, plants possess endogenous protective metabolites which reduce the toxicity of salt stress. Such metabolites cumulatively reduce salt-induced toxicity by reducing the accumulation of Na^+ ions via up regulating the endogenous protective mechanisms of plants. Two such protective compounds are salicylic and jasmonic acids, also characterized as well-known stress related phytohormones. The precursor of salicylic acid is phenylalanine and chorismate, whereas that of jasmonic acid is α-linolenic acid. After being synthesised in the tissues, they are further processed to give methylated or glycosylated products which are utilized by plants for the induction of systemic acquired resistance against various abiotic stresses. These two acids do not only play an independent role during abiotic stress, but also work in a complex signal network with other plant hormones which help to abrogate the toxicity caused due to excess accumulation of Na^+ ions and lower the level of other cytotoxic metabolites and reactive oxygen species during salt stress. This chapter focuses on the harmful effects of salt stress in various crops followed by metabolism and transportation of salicylic and jasmonic acids. The protective role of salicylic and jasmonic acid has been discussed along with their crosstalk with other plant hormones that synergistically help to ameliorate the salt-induced toxicity in plants.

1 Introduction

Production of agricultural crops around the world is severely hampered by abiotic stresses like drought, salinity, extreme temperature, metal and xenobiotic toxicity along with several biotic stresses like insects and other diseases which restrict the growth and development of plants. Unfavourable environmental conditions reduce the yield of the plants and are a major cause of concern for human food safety. The

A. Singh · A. Roychoudhury (✉)
Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), 30 Mother Teresa Sarani, Kolkata, West Bengal 700016, India

T. Aftab and M. Yusuf (eds.), *Jasmonates and Salicylates Signaling in Plants*, Signaling and Communication in Plants,
https://doi.org/10.1007/978-3-030-75805-9_2

Augmenting the Abiotic Stress Tolerance in Plants Through Microbial Association

9

Ankur Singh and Aryadeep Roychoudhury

Abstract

Recurring climate change due to irresponsible behaviour of human leads to unfavourable climatic condition such as drought, salt stress, extreme temperature and metal toxicity, which significantly decrease the quality and yield of various plants. Several physiological, biochemical and molecular parameters are hampered due to abiotic stress conditions. To enhance the fertility of soil, chemical fertilizers are used which causes soil pollution. Hence, it is necessary to develop a method which is safe and can increase the productivity of the plants by inducing their tolerance capability against abiotic stress. Application of soil dwelling microorganisms is a promising method which can be effectively applied in the field as a bio-fertilizer to induce the crop yield and overcome other symptoms of abiotic stress. Microbes enhance the tolerance mechanism in plants by increasing the mobilization of major elements present in soil, thus facilitating their uptake by plants. In addition, they also induce the formation of hormones, siderophores, osmolytes and antioxidants, which can combat the effect of unfavourable conditions. The interaction between plants and microbes is essential as it is a biological process and in the near future, it can replace the conventional methods of farming which decrease the fertility of lands. In this chapter, our aim is to review various mechanisms adopted by the soil microbes to abrogate the negative effects of abiotic stresses in plants for their better growth and productivity.

Keywords

Abiotic stress · Microbes · Siderophores · Rhizobacteria · Exopolysaccharides · Plant–microbe interaction

A. Singh · A. Roychoudhury (✉)
Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

M. Nath et al. (eds.), *Microbial Metatranscriptomics Belowground*,
https://doi.org/10.1007/978-981-15-9758-9_9

7

Silicon transporters in plants

Ankur Singh and Aryadeep Roychoudhury

POST GRADUATE DEPARTMENT OF BIOTECHNOLOGY, ST. XAVIER'S COLLEGE (AUTONOMOUS), KOLKATA, WEST BENGAL, INDIA

1 Introduction

Silicon (Si) is placed in the 14th group of the periodic table and is the most abundant element known to be found on the Earth's surface after oxygen. It is a tetravalent element that is mostly found as a silicate, aluminosilicates, or as various forms of SiO_2. In earlier days, Si was assumed to be a nonessential element for plant growth and development, but in recent years, several studies have shown that the presence of Si in plants is necessary for their resistance against biotic stress, like fungal and bacterial infection, and abiotic stress, like drought, salt, heavy metals, etc. (Luyckx et al., 2017), which makes it a quasiessential element for plants (Epstein and Bloom, 2005). Plants that can accumulate 1.5% or more Si of their dry weight are considered as high Si-accumulators, whereas plants accumulating 0.2% Si of their dry weight in leaves are termed as low Si-accumulators (Tubana and Heckman, 2015). Graminaceous plants like rice, wheat, barley, sugarcane, etc. mostly uptake and accumulate more Si compared with other plants like tomato and beans, which cannot absorb Si from the soil (Nikolic et al., 2006). Graminaceous plants absorb Si as monosilicic acid or orthosilicic acid by their roots and then deposit it to their epidermal tissue where, in the presence of calcium and pectin ions, Si forms a cellulose membrane-Si, which provides protection to the plants (Rodrigues et al., 2003; Bélanger et al., 2003). However, this passive role of Si is too simplistic to explain the better growth of plants supplemented with Si under stress conditions. Si priming is also effective in providing protection to the plants against abiotic stress conditions. The priming of nonaccumulator plants with Si also provides protection against stress, such as priming of tomato seeds with Si upregulates the expression of genes involved in ethylene, jasmonic acid signaling, and other stress-related genes when plants are infected with *Ralstonia solanacearum*, an aerobic nonspore-forming plant pathogen (Ghareeb et al., 2011). Si also provides protection to the plants externally by decreasing the pH of the soil and ultimately decreases the bioavailability of heavy metal to the plants. Si generates gelatinous metasilicic acid that retains the heavy metals in the soil, thus providing protection to the plants against heavy metals (Gu et al., 2011).

Plants absorb soluble Si from the soil and form phytolith, which is an important form of biogenic silica. Total phytolith fixed by the terrestrial vegetation (60–200 Tmol-Si $year^{-1}$) is comparable to that of total biogenic silica produced by marine vegetation (240 Tmol-Si

Metal and Nutrient Transporters in Abiotic Stress. https://doi.org/10.1016/B978-0-12-817955-0.00007-7

Chapter 6
Cytokinin-Mediated Signalling During Environmental Stress in Plants

Ankur Singh and Aryadeep Roychoudhury

6.1 Introduction

Plants are sessile living organisms which cannot escape unfavourable environment by shifting their positions. Harsh environmental conditions are negative factors which adversely affect the growth, biomass and yield of plants. Stress faced by plants may be due to the invading pathogens or infestations of insects collectively known as biotic stress and abiotic stress which occurs due to hostile environmental conditions like drought, salinity, extreme temperature or heavy metal toxicity (Roychoudhury et al. 2013). Harsh conditions lead to oxidative burst in plant tissues which ultimately enhances the formation of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide ($\cdot O_2^-$) and hydroxyl radicals ($\cdot OH$). These ROS lead to cellular membrane damages and formation of toxic products like malondialdehyde (MDA) and methylglyoxal (Banerjee and Roychoudhury 2018). Plants have several mechanisms which help them adapt and survive under unfavourable conditions. Production of various endogenous signalling molecules helps to coordinate and enhance the functions of the internal defensive pathways which ultimately induce their survival capability under severe environmental conditions.

In addition to the action of other stress hormones such as abscisic acid (ABA), jasmonate and salicylic acid, cytokinin (CK) also plays an important role in regulating the action of protective machineries of plants (Ha et al. 2012). CKs were initially believed to be only involved in cell division and in the regulation of the cell cycle (Schaller et al. 2014), but later it was found that CKs also govern various other functions like inhibition of root growth, maintenance of apical dominance, formation of shoot meristem, growth of lateral buds, expansion and senescence of leaves and nitrogen signalling pathways (Frebort et al. 2011; Giulini et al. 2004; Miyawaki

A. Singh · A. Roychoudhury (✉)
Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

T. Aftab, K. R. Hakeem (eds.), *Plant Growth Regulators*,
https://doi.org/10.1007/978-3-030-61153-8_6

Influence of Night Temperature on Rice Yield and Quality

Ankur Singh, Bodhisatwa Chaudhuri, and Aryadeep Roychoudhury

Abstract

Rice is an important food crop for a substantial proportion of the human population and due to the risks posed by global warming and climate change, there is significant interest in analysing the effects of environmental factors such as temperature on rice yield and quality. Compared to studies on the influence of day temperatures on rice yield and quality, there are fewer studies on the influence of night temperature on these parameters. Any change in ambient temperature is known to activate stress-associated signalling pathways in plants. Night temperature is a crucial environmental factor that affects metabolic processes such as respiration and translocation of nutrients. An increase in night temperature reduces rice yield by reducing the translocation of non-structural carbohydrate and nitrogen which ultimately reduces grain-filling rate, grain weight and quality. Other parameters such as the percentage of chalky rice kernels are also adversely affected by an increase in night temperature. High night temperature (HNT) increases spikelet sterility, thereby decreasing pollination. At the cellular level, high night temperature decreases membrane thermal stability and increases the rate of respiration. Treatment with exogenous effectors such as salicylic acid reduces the effects of high night temperature by increasing the total anti-oxidant capacity and membrane thermal stability. An increase in Ca signalling proteins and heat shock proteins (HSPs) also enhances tolerance to high night temperature. This chapter therefore focuses on the overall influence of night temperature on rice physiology, grain yield and the endogenous signalling events.

A. Singh · A. Roychoudhury (✉)
Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

B. Chaudhuri
Indian Institute of Science, Bangalore, India

A. Roychoudhury (ed.), *Rice Research for Quality Improvement: Genomics and Genetic Engineering*, https://doi.org/10.1007/978-981-15-4120-9_24

Genetic Engineering: A Powerful Tool to Abrogate the Effect of Metal/Metalloid Toxicity in Rice

Ankur Singh and Aryadeep Roychoudhury

Abstract

Environmental pollution is a matter of growing concern all over the world. The release of elements from industries pollutes the surface water, which when used for irrigation deposit heavy metals in the field. Crops like rice, which is a major food crop of the world, require large amounts of water during their life span. They accumulate toxic elements in their grains when irrigated with contaminated water due to their higher translocation factor. Metal toxicity can be of two types: deficiency of metals, which is the most common problem for metals like iron, and excess of metal present in the surrounding, which is commonly seen for mercury, arsenic, cadmium, etc. Both types of toxicity hamper plant growth and reduce the yield and nutritional content of the grains. To overcome the problem of metal toxicity, several methods have been used. One such method is genetic engineering. In this approach, a gene is introduced in the plants, which enhances the tolerance level of the plants against the metal stress without changing the agronomic trait. Till date, several transgenic rice plants have been introduced, which confer tolerance against higher concentration of heavy metals. This chapter mainly deals with the toxic effects of such metals in rice plants and the genes introduced in the plants, which help them overcome the toxic effects and enhance their survival under stress condition.

Keywords

Heavy metals · Metalloids · Rice · Transgenic approach · Phytoremediation · *Schizosaccharomyces pombe* · *merAB*

A. Singh · A. Roychoudhury (✉)
Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

A. Roychoudhury (ed.), *Rice Research for Quality Improvement: Genomics and Genetic Engineering*, https://doi.org/10.1007/978-981-15-4120-9_15

20

Role of γ-Aminobutyric Acid in the Mitigation of Abiotic Stress in Plants

Ankur Singh and Aryadeep Roychoudhury

Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

20.1 Introduction

Gamma-aminobutyric acid (GABA) was first reported to be naturally present in the tuber of *Solanum tuberosum* (Steward et al. 1949). GABA is a short, four-carbon amino acid that is nonprotein in nature and plays a significant role in the protection of plants against several environmental stresses. GABA is a well-known neurotransmitter in animals; however, its role in plants was less understood and it was only considered to be a metabolite in plants. In recent times, several studies have shown that GABA is not just a metabolite but also plays a major role in abiotic stress amelioration in plants. Under different biotic and abiotic stresses, around 1000-fold increase occurs in the concentration of GABA depending on the level of stress (Bown and Shelp 1997). In *Capsicum annuum*, photosynthesis and antioxidative enzyme activity were positively increased upon exogenous application of GABA (Li et al. 2017). Some other functions such as regulation of citric acid cycle metabolism and proline synthesis (Signorelli et al. 2015), metabolism of carbon and nitrogen during stress conditions in plants (Barbosa et al. 2010), and amelioration of oxidative damage (Shelp et al. 2012) are also controlled by GABA. In some cases, GABA acts as an inducer molecule for gene expression (Renault et al. 2011) In addition to its protective functions, GABA also plays a role in plant signal transduction pathways (Beuve et al. 2004). Reduction of root length occurs when the level of GABA rises in plants (Renault et al. 2013), and it also ensures the successful fertilization in plants by guiding the pollen tubes to the ovary (Palanivelu et al. 2003). The signal transducer of GABA in plants is aluminum-activated malate transporter (ALMT). GABA can also act as a signal for growth in plants by altering the cellular signal, which is an important event in tissue growth (Ramesh et al. 2015).

Biological stress may be defined as any condition that alters the initial homeostasis of the organisms; stress in plants can be described as any change in surrounding environmental condition that alters the optimal homeostasis state. Plant stresses can be classified into two types. Biotic stress is caused due to insects and any type of disease or infection, and abiotic stress mainly occurs due to physical imbalance of environmental factors such as light,

Protective Chemical Agents in the Amelioration of Plant Abiotic Stress: Biochemical and Molecular Perspectives, First Edition. Edited by Aryadeep Roychoudhury and Durgesh Kumar Tripathi.

22

Involvement of Sulfur in the Regulation of Abiotic Stress Tolerance in Plants

Santanu Samanta[#], Ankur Singh[#], and Aryadeep Roychoudhury

Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India
[#] Equally contributed

22.1 Introduction

Abiotic stresses are negative impact of nonliving factors that harmfully affect survival, biomass production, accumulation, and grain yield of most crops. There are different types of abiotic stressors such as dehydration, intense sunlight, extreme temperature, or others that reduce the crop yield below the optimum level. Plants are frequently challenged by many types of abiotic stresses because of constant fluctuations of climate. When these stresses are severe, both quality and quantity losses are incurred on a wider scale. Abiotic stress is a major global problem, which is limiting the productivity of crops. Different types of abiotic stresses are a serious problem limiting the yield of the traditional cultivars (Roychoudhury et al. 2013).

Plant responses to all abiotic stresses are dynamic and complex. Acute responses cause cell death and sub-acute responses lead to changes at biochemical and gene expression levels (Toivonen 2005). When plants are exposed to many types of abiotic stresses, they actively produce reactive oxygen species (ROS), which are highly reactive and cause damage to many important biomolecules such as proteins, lipids, carbohydrates, as well as DNA. On the other hand, cells may use these toxic molecules as biological stimuli to trigger changes at gene expression level. Generally, abiotic stressors stimulate perturbation in the crop cellular homeostasis, leading to enhanced production of ROS. The ability of the cell to suppress the oxidative damages largely depends upon the endogenous free-radical-scavenging activity (Das and Roychoudhury 2014).

For improvement of crop quality, plant breeding may also be used. However, this method takes a long time to release a new variety resistant to a particular stress. Transgenic technology has emerged as an expanding component of agricultural biotechnology over the past decades, since it allows the development of plants with qualities in a much shorter period of time as compared to conventional breeding. However, there are several ethical and biosafety issues of transgenic technology due to which it is restricted till now in terms of commercial release.

Protective Chemical Agents in the Amelioration of Plant Abiotic Stress: Biochemical and Molecular Perspectives, First Edition. Edited by Aryadeep Roychoudhury and Durgesh Kumar Tripathi.