RESPONSES OF PLANTS TO FLUORIDE: AN OVERVIEW OF OXIDATIVE STRESS AND DEFENSE MECHANISMS

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ABSTRACT: The fluoride ion (F) is widespread in the environment and toxic for all living organisms. Prolonged exposure to F leads to physiological, biochemical, and molecular modifications in plants. F is known as a potential metabolic inhibitor, interfering with the overall responses of plants including seed germination, growth and productivity, biomass accumulation, photosynthesis, enzyme activities, protein synthesis, gene expression patterns, and reactive oxygen species (ROS) production. It has also been shown to amend the functionalities of various antioxidants leading to oxidative stress inside plants. However, in recent years, a few attempts have been made to mitigate the ill effects of F on plants and their productivity responses. Modulation of cellular thiol molecules for protection against ROS-induced injury has been considered to be one of the effective strategies against F toxicity. In addition, the accumulation of proline and exogenous supplementation with salicylic acid have been shown to be potential measures for protecting the plants from F toxicosis. The present review covers the information available so far on the mechanism(s) of F uptake by plants, the F-induced physiological, biochemical, and molecular amendments in plants, and the potential for mitigation of F-stress in plants by using various molecules. The authors have also attempted to highlight the gaps in the existing knowledge for F toxicity in plants and the future research prospects.

Keywords: Antioxidants; Fluoride toxicity; Growth responses; Oxidative stress; Salicylic acid.

INTRODUCTION

Contamination by the fluoride ion (F) of ground water and soils is a great concern in several countries when it is present at levels above the permitted guidelines.[1] The World Health Organization set in 1984, and reaffirmed in 1993, a guideline for a “desirable” upper limit of F in drinking water of 1.5 ppm (mg/L). The country standards are lower in India (1 ppm) and Senegal, West Africa, (0.6 ppm) with a rider to the Indian limit noting “lesser the fluoride the better, as fluoride is injurious to health.” F is not an essential trace element for plants, animals, or humans although some consider it to have a beneficial effect in reducing dental caries when applied topically to the teeth. Chronic exposure to F induces an array of deleterious impacts in domestic[2-8] and experimental[9] animals, humans,[10-13] and a variety of plants.[14] Some plants accumulate F and are able to grow even at high F concentrations (4000 µg/g) without showing any signs of injury while several other plants sustain damage if exposed to even low F concentrations (<20 µg/g).[15] Exposure to F for a prolonged period primarily leads to chlorosis and then necrosis in the leaves of plants.[16] It was long been known as a potential metabolic inhibitor, interfering with the overall responses of plants including seed germination, growth and productivity, biomass accumulation,

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photosynthesis, activities of enzymes, protein synthesis and secretion, patterns of gene expression, and production of reactive oxygen species (ROS).[17,18] Changes in the activities of enzymes and intermediary metabolism, induced by F, may result in altered growth, development, and reproduction.[19]

To combat abiotic stress, like F-promoted ROS, plant cells are armed with a set of antioxidants, which incorporate both enzymatic and non-enzymatic members, which can protect the stressed cells under unfavorable circumstances.[20] Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POX), glutathione reductase (GR), monodehydroascorbate reductase, and glutahione-S-transferase are a few of the enzymic antioxidants. Non-enzymic antioxidants include ascorbic acid (AA), glutathione, proline (Pro), α-tocopherol, and flavonoids.[21] In several instances, stressed cells are shown to accumulate compatible solutes or osmolytes like glycinebetaine, Pro, mannitol, sugars, etc., to counteract with water imbalance conditions.[22] It has been recognized that the accumulation of osmolytes is an important attribute for the protection and survival of plants under stress.[22] Recently, salicylic acid (SA) was shown to ameliorate F-imposed injuries in plants.[23] Being a phenolic compound, SA plays a crucial role in the signaling of several physio-biochemical responses of plants.[24] In this review, our emphasis is on collating the findings on the various aspects of plant metabolism in relation to F-toxicity, and information of the mechanisms for the management of F-toxicity.

UPTAKE AND ACCUMULATION OF F BY PLANTS

The gradual accumulation of F into the plant sub-cellular compartments results later in toxicity responses in the plants. F enters into plants mainly through two pathways. Initially, F deposited over leaves enters *via* stomata, and secondly, through the soil and water into the roots by a passive diffusion process.[25] However, studies showed that the uptake of F from the atmospheric air is more significant than from the soil.[20] Subsequently, F is transported via xylem tissues through the apoplastic and symplastic pathways into the shoots.[26] A part of the F is also transported through biological membranes via non-ionic diffusion of hydrogen fluoride (HF). The small neutral molecule of HF crosses the cell membranes seven times faster than the fluoride ion (F). However, at the present time, the precise mechanisms of F uptake into the cells has not been fully explored. The bioaccumulation of F in the different plant parts varies depending upon its transfer from the soil to the roots, and then into the shoots. Its accumulation was found to be highest in the roots with progressively lower levels in the leaves, seeds, and shoots.[20]

PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF F TOXICITY

High internal F concentrations disturb almost all the physiological and metabolic processes of plants and their parts.[20]

Growth attributes: The seed germination and early seedling growth stages are physiologically complex processes and are severely affected by high concentrations of F. With an excess of F, plants exhibit inhibited seed germination

responses, vigor, stress tolerance index, root-shoot length, number of seeds formed (due to an imbalanced nutrient uptake), and biomass accumulation (Figure).[20]

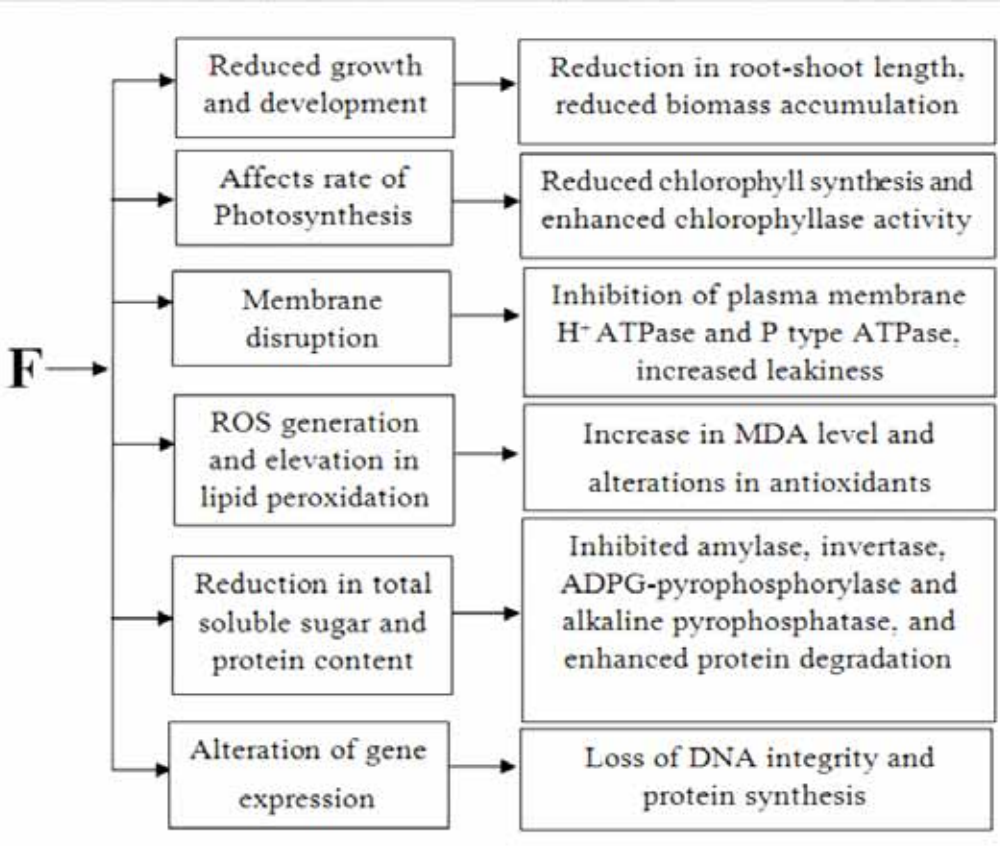

Figure. F-induced physiological, biochemical and molecular modifications in plants.

The failure of germination may be the consequence of reduced water uptake, inhibited cell division and enlargement in the embryo, and/or an overall fall in the metabolic activity associated with these processes. Matching observations were recorded for the F-treated seeds/seedlings of *Vigna radiata* (mung),[22] *Cicer arietinum* (Bengal gram),[27] *Raphanus sativus* (radish),[28] and *Triticum aestivum* (wheat).[29] In addition, F-imposed prevention of the dephosphorylation of phytin compounds through inhibited phytase enzyme, mineral nutrition, and amylase activity, were held responsible for the reduced rates of seed germination.[23, 30] A close connection between a deficiency of key nutrients and growth responses was revealed in *Citrus aurantium* (citrus) seedlings.[31] F has also been shown to lower DNA synthesis which leads to decreased RNA and protein synthesis, resulting in reduced cell division and elongation, and consequently inhibited growth.[30] Leaves and green stems can accumulate a larger proportion of atmospheric F due to their wide surface area and the presence of stomata. Likewise, F also accumulates in the tips and margins of monocotyledonous and dicotyledonous leaves, respectively,

which may result in chlorosis and necrosis, the first visible symptoms of F injury.[30] In several instances, shoot growth was more affected than the roots, which was probably due to an improper nutrient uptake by the affected seedlings.

Photosynthesis: F affects the rate of photosynthesis chiefly by reducing chlorophyll synthesis or by degradation of the ultra-structure of the chloroplasts and an inhibition of the Hill reaction.[25] Amongst other parts, the chloroplasts are shown to be the chief accumulator of F.[32] Many investigations have highlighted that F caused deleterious impacts on photosynthetic pigments such as chlorophylls-a and b, carotenoids, and anthocyanins.[32] High F was found to reduce the availability of Fe^{2+} ions which are essential for chlorophyll synthesis, and to increase the activity of chlorophyll degrading enzymes, like chlorophyllase, with consequent affects on the process of photosynthesis (Figure). Moreover, F instigated inhibition in the electron transport rate, particularly at the photosystem-II, is also one of the mechanisms of F-injury.[25] It has also been demonstrated that oxygen is continuously produced during the photolysis of water. Substitution of Cl^- ions of the photosystem-II, by the F, inhibits the photo-oxidation of water and causes the generation of new free radicals in the proteins of this system, which is incapable of compensating by the process of photolysis.[33] It has been proposed that the inhibited photosynthesis may possibly be the result of the loss of sub-cellular organization, granulation of the chloroplasts, and low stomatal conductance linked a limited CO_2 uptake.[32] In addition, in the chloroplasts, F is also shown to affect the activities of RUBISCO (ribulose 1,5-biphosphate carboxylase), sucrose synthase, sucrose synthetase, and the enzymes associated with CO_2 fixation.[20,32]

ATPase: An excessive availability of F has been demonstrated to inhibit the ATP synthase enzyme, thus affecting energy metabolism in the higher plants (Figure).[34] The plasma membrane is quite sensitive to F and hence is considered to be a critical site for attack.[20] Moreover, the membranes of the tonoplasts were shown to be most susceptible to F.[20] In F affected *Beta vulgaris* (sweet potato), the plasma membrane ATPase was adversely affected by F ions.[34] It was further suggested that the degree of inhibition in the plasma membrane ATPase increased drastically with increasing concentrations of free Mg^{2+} in the reaction medium.[20] This indicates that the F-induced suppression in the plasma membrane H^+ ATPase and the P type ATPase is at the active site of the enzymes, and is mediated by a magnesium-fluoro complex.[20]

Reactive oxygen species (ROS): The ROS are a group of free radicals such as singlet oxygen: $^1O_2$, superoxide anion: $O_2^{\bullet -}$, hydrogen peroxide: H_2O_2, and hydroxyl radical: $OH^{\bullet}$, derived from O_2.[35,36] In plants, the production of ROS is the unavoidable consequence of different metabolic pathways operating in the cellular compartments like the mitochondria, chloroplasts, peroxisomes, etc. These ROS are strong oxidants that can readily react with almost all the components of the cell and cause severe damage to lipids, proteins, and nucleic acids.[35,36] Under abiotic stresses including F-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.[21] This triggers a cascade of reactions generating oxygen radicals very aggressively.[37] Moreover, $O_2^{\bullet-}$ is also produced by two distinct pathways in the peroxisomes; (i) in the matrix where xanthine oxidase catalyzes the oxidation of xanthine and hypoxanthine into the uric acid, and (ii) in the membranes where a small electron transport chain (ETC) is operative.[21]

Although over production of ROS can lead to phytotoxicity, at low levels ROSs can serve as signaling molecules. Thus, the effects and/or functioning of ROS depend upon their concentration, which tends to increase significantly under stressful conditions including F-availability.[37,38] A prevalence of $O_2^{\bullet-}$ was detected at high F concentrations, while at low levels there was a dominance of $OH^{\bullet}$, produced through the Haber-Weiss reaction.[39] Similar rises in ROS were also documented by several other researchers in F-stressed plants.[25,30,38,40]

FLUORIDE-INDUCED ROS VS BIOMOLECULES

Carbohydrates: Increased glucose was shown to be linked directly with ROS quenching and NADPH production, which serves as source of energy for the growing seeds/seedlings.[40] Low F was found to raise the sugar content in *Citrullus lanatus* (watermelon) while at high dose it resulted in reduced sugar accumulation in *Oryza sativa* (rice) seedlings.[23,40] The actions of F in inhibiting photosynthesis, reducing the activities of invertase and amylase, and increasing oxidative stress may possibly be responsible for this reduced sugar accumulation.[33] The activities of more than 300 enzymes are shown to be inhibited severely by F *via* removal of a cofactor Mg^{2+}.[33] Moreover, the partitioning of the photo assimilates for sucrose and starch synthesis, is directed by F-mediated pyrophosphate (PPi) accumulation.[41] The synthesis of PPi is regulated by the ADPG-pyrophosphorylase and alkaline pyrophosphatase enzymes. ADPG-pyrophosphorylase accelerates the conversion of glucose-1-phosphate to ADP-glucose, a precursor of starch, leading to PPi synthesis, and alkaline pyrophosphatase hydrolyzes PPi.[41] ADPG-pyrophosphorylase is completely inhibited by traces of both PPi and F while alkaline pyrophosphatase is suppressed by F, allowing the accumulation of PPi and thereby reducing starch synthesis.[41]

Lipids: The most damaging and key process known to occur prominently in the plasma membranes is the oxidation of the poly unsaturated fatty acid (PUFA) fractions of lipids.[21] The peroxidation reaction of this macromolecule includes three important steps viz., initiation, progression, and termination.[42] Peroxidation of membrane lipids is initiated when a $OH^{\bullet}$ radical abstracts one hydrogen atom from a PUFA.[21] Under aerobic conditions, oxygen combines with the carbon-centered lipid radical of the fatty acid and forms the $ROO^{\bullet}$ radical. This $ROO^{\bullet}$ radical abstracts one hydrogen from an adjacent PUFA and in this way the peroxidation reaction is progressed.[21,43] The PUFAs after the $^1O_2$ and $OH^{\bullet}$ attack releases lipid hydroperoxide as a by-product.[43] Increased peroxidation of phospholipids (a type of PUFA), results in decreased membrane fluidity and a rise

in leakiness.[43] Moreover, the lipid peroxidation reaction also exacerbates the oxidative damage through the production of lipid-derived secondary free radicals that themselves can react with and damage both proteins and DNA.[44] Additionally, the PUFAs get oxidized from ROS, and generate a number of cytotoxic by-products namely malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), and hydroxyl and keto fatty acids (Figure).[43] A significant change in the pace of the lipid peroxidation reaction with increasing F and time of exposure was observed in *Helianthus annuus* (sunflower).[33]

Proteins: The addition of F reduced the protein content in both a dose and time dependent manner in a variety of seedlings.[22,27] Reduced synthesis, enhanced degradation, and/or usage for energy production are held to be responsible for this lowering of protein in stressed seedlings.[25] F was also found to amend the pace of RNA synthesis and nitrogen metabolism, and to enhance ribonuclease activity, with a consequently lower protein turnover.[33] Proteins also make conjugates with the by-products of fatty acid oxidation.[44] Additionally, like lipids, proteins are also prone to ROS attack, which may cause deleterious modifications via nitrosylation, carbonylation, formation of disulphide bonds, and glutathionylation.[44] ROS oxidizes Arg, His, Lys, Pro, Thr, and Trp of proteins into γ-glutamyl semialdehyde, aminoketobutyrate, and amino adipic semialdehydes, thereby making them sensitive to proteases.[37,44] Proteins with S-containing amino acids and thiol-groups, such as Cys and Met, are quite sensitive to $^1O_2$ and $OH^{\bullet}$.[44] Based on the existing literature until the present time, only a few reports have been published pertaining to F-instigated carbonyl formation in the plants but this has been more extensively discussed in relation to several other stresses.[37,45] Moreover, F has also been shown to be involved in the synthesis of misfolded proteins in the endoplasmic reticulum and consequent ROS generation.[38] The expressions of genes associated with stress response factors (e.g., heat shock proteins-70), signal transduction components, and apoptosis related proteins were demonstrated to be up-regulated by exogenous F application.[38]

DNA: F-toxicity revealed reduced synthesis of DNA which was possibly linked to insufficient DNA polymerase activity.[30] This reduced DNA is also related to decreased RNA and protein synthesis, and reduced rates of cell division and elongation.[30] Fluoride has also been shown to instigate genotoxic responses. Additionally, ROS are proven to induce strand breaks, point/deletion mutations, crosslinks, chromosomal aberrations, and the production of 8-oxoguanosine (8-OHdG) in stressed tissues.[37,46] Among these, 8-OHdG is a highly mutagenic miscoding lesion that causes the G:C to T:A transversion mutation.[46] Moreover, reduced telomere length and inhibited DNA repair processes are possible reasons for the genotoxic responses in the affected tissues.[47] RAPD analyses of stressed plants revealed that the disappearance of normal bands may probably be associated with events of DNA damage.[48] Additionally, electrophoresis and qRT-PCR studies demonstrated that the rate of DNA damage and the expression of the caspase-3 and -9 genes respectively, were significantly enhanced with exogenous F application.[39,49] However, up until the present time, only a few reports have

been published pertaining to the genotoxic responses of F, particularly in plants and their parts.

ROS SCAVENGING SYSTEM

To maintain homeostasis, cells are armed with an integrated ROS scavenging system network that comprises enzymic (SOD, CAT, POX, APX, and GR) and non-enzymic (AA, α-tocopherol, flavonoids, Pro, and glutathione) candidates.[36] Among the enzymatic components, SOD, a family of metalloenzymes, catalyzes the disproportionation of $O_2^{\bullet-}$ into H_2O_2 and O_2, and is present in almost all aerobic organisms.[42] Three isoforms of it namely; Mn-SOD (mitochondria), Fe-SOD (chloroplasts), and Cu/Zn-SOD (cytosol, chloroplasts, peroxisomes, and mitochondria) are shown to exist.[37,42] CAT, a heme-containing enzyme, catalyzes the dismutation of H_2O_2 into H_2O, and O_2.[37,42] POX, a heme containing protein, has four conserved disulfide bridges and takes part in lignin biosynthesis and organogenesis via the decomposition of auxin or the biosynthesis of ethylene.[37,42] Additionally, APX, a central component of the ascorbate-glutathione cycle, uses two moles of ascorbate to reduce H_2O_2 into water, and generates two moles of monodehydroascorbate.[37,42] Another scavenger GR, a flavo-protein oxidoreductase, maintains a reduced state of glutathione inside the cells.[21]

Exposure to F was shown to reduce SOD activity in *H. annuus*.[33] In contrast, an increase in it was recorded in F-treated seedlings of *O. sativa*.[40] This fluctuating behavior of SOD may probably be related to altered metabolic status or its biosynthesis, in F-affected tissues.[40] Moreover, a remarkable fall in the ATPase and phosphatase levels in response to exogenous F can be attributed to the binding of F to Mg^{2+} and Ca^{2+}, thus reducing their availability for the functioning of various enzymes.[33] However, the enhanced activity of antioxidants may be an adaptive strategy which can be considered as a positive feedback mechanism against oxidative stress.[40] Like SOD, CAT was also reduced with F-stress in *O. sativa*.[40] The findings suggested that with F-toxicity, the $OH^{\bullet}$ attached to the Fe^{2+} atoms of CAT are replaced by low molecular weight anions thereby inhibiting enzyme activity.[40] In contrast, enhanced POD and APX levels were measured in *C. arietinum* and *O. sativa*.[27,40] Moreover, a dose dependent fall in GR was discernible in F treated *H. annuus*.[33] Like the enzymic candidates, the content of AA fell initially in response to F, but then rose drastically with increased F exposure and F concentration, in *O. sativa*.[40] The binding of F with ascorbic acid oxidase allowed an increased accumulation of AA with low F. At higher F doses, the bond(s) between F and ascorbic acid oxidase may broken down allowing low levels of AA. In addition, with increasing F, a rise in Pro, an inhibitor of programmed cell death and scavenger of ROS, was evident in several species due to the reduced activities of Pro catabolic enzymes, up-regulation in its *de-novo* synthesis and biosynthetic enzymes, and/or breakdown of Pro rich proteins.[22,27] The enhanced accumulation of amino acids during abiotic stresses suggests that these molecules may be serving as sinks for excess nitrogen, transient sources of both carbon and nitrogen, and regulators of cellular osmoticum.[33]

AMELIORATION OF F TOXICITY IN PLANTS

In practice, plants are able to minimize the stress exerted by metabolic disruptions up to a certain extent by involving their defense system. In several instances, the candidates of defense system become hyperactive in order to provide better control over the ROS, the key facilitators of injury symptoms.[37,42] To make this system proactive against ROS during F-toxicity, exogenous salicylic acid (SA) was found to be quite effective.[23] It is a phenolic compound that plays a signaling role in the plants.[24] Exogenous SA increased seed germination, and shoot-root growth in F-treated *C. lanatus*.[23] SA was also shown to stimulate seed germination and growth traits via enhanced synthesis of gibberellic acid and the prevention of auxin oxidation respectively.[23] Exogenous SA was also implicated in enhancing photosynthesis through cytokinin mediated chlorophyll biosynthesis and the inhibition of chlorophyllase. Moreover, SA was proven to enhance the soluble sugar and phenolic content in F affected seedlings, thereby indicating SA to be a potential ROS quencher.[23]

CONCLUSIONS AND FUTURE PERSPECTIVES

The published literature indicated that excess F adversely affects the overall physiological and biochemical processes of plants, right from seed germination to the expression of metabolically important genes. In addition, members of integrated defense system are found to be markedly altered in the presence of high F, thereby making the plants sensitive towards it. Although, attempts has been made in the past to assess the deleterious effects of F on plants, this has been only up to a limited extent. Studies have yet to be performed to generate information on (i) the membrane channels/proteins involved in the uptake, translocation, and accumulation of F, (ii) the alterations in DNA, RNA, and their metabolism and repair systems, (iii) the amendments in the expressions of antioxidants and stress responsive genes, and (iv) the efficient management of F-toxicity using potential molecules.

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