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Role of Protective Metabolites and Antioxidants in Mitigating the Harmful Effects of Fluoride Toxicity on Allium cepa L.

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ABSTRACT

Purpose: Presence of fluoride (F) in groundwater and soil above threshold limit is a serious threat, inducing a variety of deleterious impact in plants via overproduction of reactive oxygen species (ROS). The present study was aimed to decipher the impact of F on onion plants.

Methods: Onion bulbs were exposed to 100 and 150 mg L-1 NaF solution. After 25 days, the level of F accumulated in bulb, root and shoot was estimated along with chlorophyll loss, electrolyte leakage and formation of H2O2, and malondialdehyde. The level of protective metabolites like osmolytes (proline and total amino acids), non-enzymatic antioxidants (anthocyanins, flavonoids, glutathione, ascorbate, and cysteine), and enzymatic (SOD, APX, CAT, GPoX, and GST) antioxidants were also determined in bulb, root, and shoot for both the concentration.

Results: The level of F in bulb, root, and shoot of onion plants was elevated linearly with the concentration of NaF applied which suggested unregulated uptake of F- ions via roots. Higher accumulation of F- ions in tissues led to oxidative stress. To combat the detrimental effects of F stress, the level of protective metabolites such as osmolytes and non-enzymatic antioxidants, and activity of enzymatic antioxidants were significantly enhanced in bulb, root, and shoot of onion plants. This rise in the level of protective metabolites and activity of enzymatic antioxidants, though could confer tolerance to some extent, was still not fully sufficient to totally eliminate the deleterious effects of fluoride-induced damage in the tissues.

Conclusions: Based on this study, we concluded that in coming times, appropriate protective measures should be taken to reduce the accumulation of F- ions in onion tissues so as to eventually reduce the toxic effect and thus maintain proper growth and yield of this vital vegetable crop.

Keywords: *Onion; Fluoride; Oxidative damage; Osmolytes; Antioxidants*

INTRODUCTION

In recent times, the whole world is facing an acute problem of environmental contamination. One such pollutant is fluoride (F), whose level has unprecedently risen in the environment in recent decades. F belongs to the halogen group and have unique chemical properties due to its small size and high electronegativity. Natural phenomena such as weathering of mineral rocks, volcanic eruption and marine aerosols or anthropogenic activities like release of untreated water from industries, emission from coal-burning industries, unorganized dumping of household wastes and wash-off from the agricultural field have led to such abrupt rise of F in groundwater and soil.[1] F, being highly soluble in water, gets easily admixed in the surrounding water bodies. Unplanned use of water from such contaminated water sources further leads to the deposition of F in the agricultural field. According to a recent review,[2,3] groundwater in most rural areas of India, used for both drinking and irrigation, is found to be contaminated with fluoride beyond the permissible limit value of 1.0 ppm or 1.5 ppm as per the Bureau of Indian Standards and World Health Organization guidelines, respectively.[4,5] Long-term excessive exposure to fluoride through drinking water and air (industrial fluoride emissions) causes a serious disease called fluorosis in both humans [6,7] and animals, [8,9] causing severe damage to teeth, bones and soft organs,

depending on the concentration of fluoride and the duration of exposure. Long-term exposure of agricultural and horticultural crops to fluoride through any possible source of fluoride also has diverse toxic effects on crop plants.[10,11] Additionally, it was also found that the level of F in soil of such regions was around 69-417 mg kg^{-1} of soil, which directly or indirectly affects around 62 million people including approximately six million children.[12] In a similar study conducted by De et al., abrupt increase in the level of F was found in the soil of Bankura (114 ± 59 mg kg^{-1}) and Purulia (126 ± 65 mg kg^{-1}) districts of West Bengal, India.[13]

Prolonged exposure of crops to contaminated water sources, industrial emissions, and soil leads to higher uptake of F^- ions via roots, ultimately causing its bio-accumulation within tissues. The toxic effects of F on crops like *Vigna* sp., *Cicer arietinum* (chick pea), *Oryza sativa* (rice), *Spinacia oleracea* (spinach) and *Cajanus cajan* (pigeon pea) are widely studied in the country.[13-17] Bio-accumulation of F^- ions in the tissues of the plants above the threshold level leads to deleterious impact on seed germination, nutrient uptake, plant growth, biomass accumulation, and metabolic activities along with lower grain yield.[10,11] In addition, F contamination also inhibits leaf chlorophyll synthesis, resulting in leaf tip burn and chlorosis.[12] Another major event that is triggered due to higher accumulation of F^- ions in the tissues is the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), leading to oxidative stress in plant tissues. F-induced oxidative stress further enhances the activity of lipid peroxidising enzymes such as lipoxygenase (LOX), causing higher membrane damage that in turn leads to higher leakage of electrolytes from the cells and formation of cytotoxic metabolites such as malondialdehyde (MDA).[14,17]

To survive under such harsh environmental conditions, plants have developed diverse protective machineries which comprise of osmolyte production and activation of enzymatic and non-enzymatic antioxidants. Osmolytes such as amino acids and proline are electrically neutral, small non-toxic metabolites that scavenge free ROS generated due to abiotic stressors, eventually maintaining the integrity of lipid membrane and regulating the cellular osmotic balance.[18] In addition, they also protect and maintain proteins in their active conformation. The enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (GPoX), and glutathione S-transferase (GST) synergistically play a crucial role in detoxifying the cellular ROS. Superoxide radicals formed in the cells due to higher accumulation of F^- ions are degraded by SOD to H_2O_2 which is further detoxified by the activity of other enzymatic antioxidants like APX, CAT, GST, and GPoX.[18] Apart from enzymatic antioxidants, the participation of non-enzymatic antioxidants like anthocyanins, flavonoids, glutathione, ascorbate and cysteine in stabilizing the lipid membrane by reducing the damaging effects of ROS is also widely reported. The protective role of osmolytes and antioxidants (enzymatic and non-enzymatic) was previously reported in several crops like *Cajanus cajan* (pigeon pea), *Oryza sativa* (rice), *Triticum aestivum* (wheat), *Carthamus tinctorius (safflower),* and *Camellia sinensis (tea)*.[17,20-23]

Onion (*Allium cepa* L.) is a vegetable crop of high economic importance worldwide. It is high in nutrients, minerals, vitamins, and antioxidant compounds while being low in calories.[24] Additionally, onion bulbs are a rich source of flavonoids and other bioactive polyphenols that are important for human diet in protecting from cardiovascular diseases and cancer.[25] India ranks first in area and second in production of onion next to China, contributing to 22% of global production.[26] In spite of higher crop area, the productivity of onion to that of crop area is quite less in India, as compared to that of other countries across the globe.[27] One of the major reasons for its lower productivity is F contamination in soil and ground water bodies. Onion thrives best at slightly lower pH of around 6.0, which also favours the higher dissociation and uptake of F^- ions from the soil via roots. Earlier, Ruan et al. showed higher accumulation of F^- ions in the leaf, root, and stem of tea plants at pH 5.5 which could be attributed to the higher dissociation of NaF and HF molecules, that eventually induce F^- ions to get concentrated in the soil.[28] Thus, hindered production of onion in India might be due to higher presence of F^- ions that are easily taken up via roots at lower pH. In spite of substantial economic importance, no detailed reports are available till date, demonstrating the negative impact of F toxicity on onion. Thus, the main aim of this study was to showcase the deleterious impact of F on the growth of onion, exposed to two different concentrations, viz., 100 and 150 mg L^{-1} NaF. These two concentration of NaF was selected, based on the earlier work of Bhattacharya and Samal, where they showed that the level of F in water in some of the major onion growing states of India such as Maharashtra, Karnataka, Rajasthan, Gujarat, and Madhya Pradesh ranges between 50 and 200 mg kg^{-1} of soil.[3] After 25 days, F content in bulb, root, and shoot was measured along with other damage-associated parameters such as chlorophyll loss, leakage of electrolyte, and formation of H_2O_2 and MDA in cells. The fate of protective metabolites like osmolytes and enzymatic and non-enzymatic antioxidants was also analysed to check their efficacy in lowering the effects of F toxicity.

MATERIAL AND METHODS

Plant growth and stress treatment

In this study, onion (*A. cepa* L.) was used as the experimental plant sample. Initially, onion bulbs were treated with “Kay Bee Fungo Raze” fungicide (Kay Bee

Bio Organics Pvt Ltd) before planting to avoid the growth of fungus on bulb. Individual bulb was planted in clean sand, mixed with 0.1 % (v/w) fungicide and placed in growth chamber. The temperature of the incubator was maintained at 25 ± 2°C with relative humidity of 50% and 16/8 h photoperiod light/dark cycle. For stress imposition, the bulbs were maintained in either 100 mg L^{-1} or 150 mg L^{-1} NaF solution for 25 days. Water-treated bulbs served as experimental control. Thus, the experimental sets which were maintained in triplicate were as follows:

Set 1: Onion bulb maintained in water only
Set 2: Onion bulb maintained with 100 mg L^{-1} NaF solution
Set 3: Onion bulb maintained with 150 mg L^{-1} NaF solution
After completion of 25 days, onion bulbs were collected and stored at −20°C for experimental analysis.

Determination of F levels

For determination of F level in bulb, root, and shoot (0.5 g each), the respective tissues were homogenized in 3.0 mL TISAB (pH 5.2). F present in the homogenate [mg F present kg^{-1} fresh weight (FW)] was estimated using Oakton® PC 2700 Benchtop pH/conductivity meter (Cole-Palmer, USA), having F^- ion-sensitive electrode (Oaktonion-selective electrode, F, Cole-Palmer, USA).[29]

F-induced oxidative damage parameters

Chlorophyll level was determined by homogenizing 0.5 g of healthy green shoot tissue in 80% (v/v) acetone and was expressed as µg chlorophyll present g^{-1} tissue.[18] MDA and H_2O_2 level, present in root, shoot, and bulb, was estimated, following the earlier work of Yadu et al.[17] 0.3 g of tissue was homogenized in 0.5% (w/v) 2-thiobarbituric acid (TBA), dissolved in 20% (w/v) trichloroacetic acid (TCA) for MDA estimation, while homogenized in 0.1% (w/v) TCA for H_2O_2 estimation. MDA and H_2O_2 content of tissues was represented as µM MDA and nM H_2O_2 present g^{-1} FW, respectively. The leakage of cellular electrolytes was estimated following the work of Campos et al. and represented as percent of loss of electrolytes from the cells.[30]

Level of amino acids and proline in tissues

For the determination of amino acids and proline, the earlier work of Roychoudhury et al. was followed.[31] Amino acid and proline content of the tissues was represented as µg amino acid or µg proline present g^{-1} FW.

Activity of SOD, APX, CAT, GPoX and GST

SOD activity was determined following the earlier work of Singh et al. and was represented as unit of enzyme required to inhibit 50% reduction of p-nitroblue tetrazolium.[29] APX and CAT activity was measured using the previous protocol reported by Campos et al.[32] The activity of APX was represented as µM ascorbate oxidized min^{-1} mg^{-1} total protein, while CAT activity was represented as µM H_2O_2 decomposed min^{-1} mg^{-1} total protein. The activity of GPoX was determined, following the work of Srinivas et al. and was measured as µM tetraguaiacol formed min^{-1} mg^{-1} total protein.[33] For the determination of GST activity, the earlier work of Banerjee and Roychoudhury was followed; GST activity was expressed as µM CDNB (1-chloro-2, 4-dinitrobenzene) utilized min^{-1} mg^{-1} total protein.[19]

Estimation of anthocyanins, flavonoids, glutathione, ascorbate and cysteine

The level of anthocyanins and flavonoids accumulated in tissues was measured following the earlier work of Singh and Roychoudhury and was expressed as µM anthocyanins present g^{-1} FW and µg flavonoids present g^{-1} FW tissues, respectively.[20] Glutathione content was determined, following the work of Banerjee and Roychoudhury and expressed as mM glutathione present g^{-1} FW tissues.[19] The concentration of ascorbate in plant tissues was estimated using the earlier work of Sayed and Soliman.[34] Ascorbate level in tissues was expressed as µg ascorbate present g^{-1} FW. Level of cysteine was measured using the procedure described by Gaitonde and was represented as mM cysteine present g^{-1} FW tissues.[35]

Estimation of total protein

Protein level in plant tissues was measured, using the protocol developed by Bradford with bovine serum albumin as the standard.[36] Equal amount of protein was used for the estimation of enzyme activity for each treatment.

Statistical analysis

Each treatment set, i.e., water-treated onion bulb, NaF (100 mg L^{-1})-treated onion bulb and NaF (150 mg L^{-1})-treated onion bulb was maintained in triplicate (n = 3) in completely randomized design. The data was represented as mean ± standard error and statistical significance was determined at $p \leq 0.05$ using analysis of variance (ANOVA).

RESULTS

F bioaccumulation in onion tissues

F treatment significantly hampered the shoot length of the onion plants (Fig. 1).

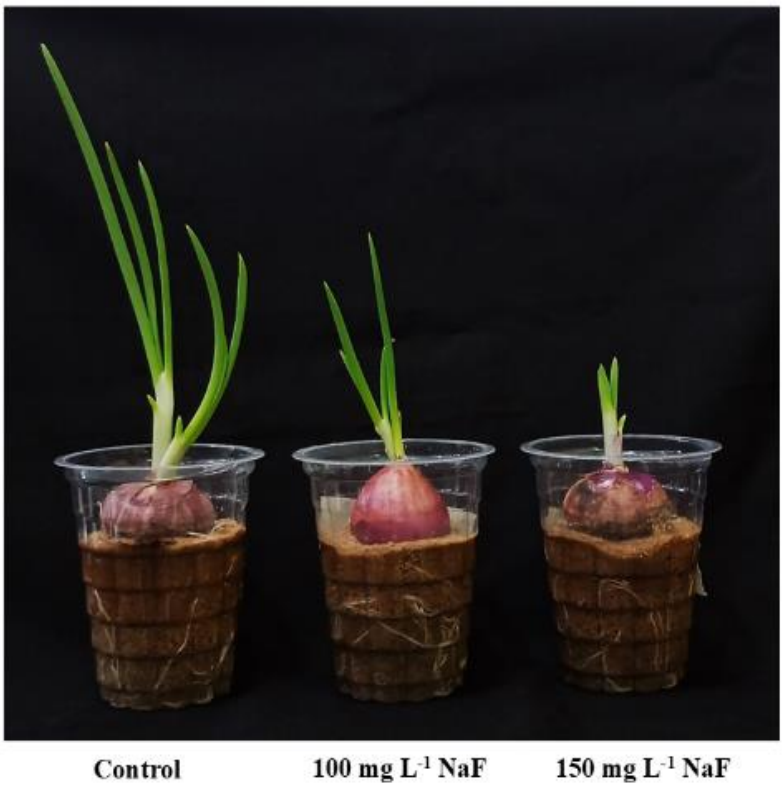

Figure 1. Effect of application of 100 and 150 mg L^{-1} NaF on growth performance of onion plants, monitored for 25 days; untreated (non-stressed) plant served as experimental control.

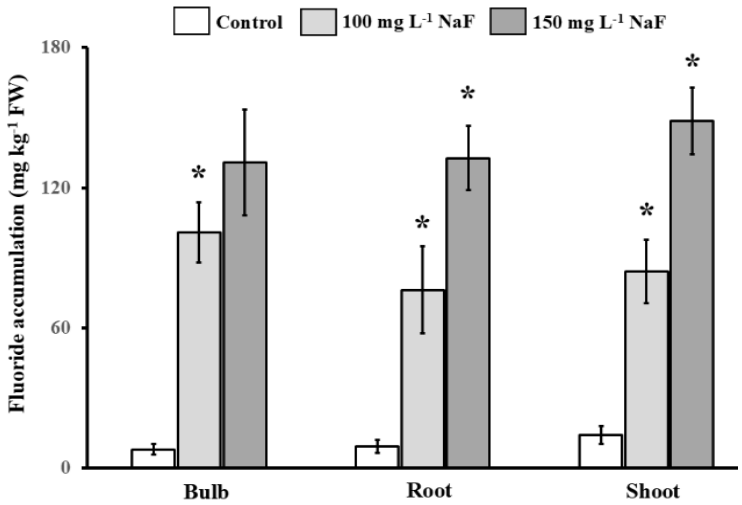

Figure 2. F content in onion tissues, grown in presence of 100 or 150 mg L^{-1} NaF. Data represented here are the mean value (n = 3) ± standard error; '*' represents data with significant differences at $P \leq 0.05$, compared against respective control tissues (grown in water only).

NaF exposure led to 12.7- and 16.4-fold higher F accumulation in the bulb, exposed to 100 and 150 mg L^{-1} NaF solution, respectively, as compared to that of control onion bulb. Similarly, 8.2- and 5.9-fold higher level of F^- ions was noted in root and shoot of onion, exposed to 100 mg L^{-1} NaF solution, respectively, while 14.3- and 10.5-fold higher F^- ions was noted in root and shoot of onion, respectively, exposed to 150 mg L^{-1} NaF (Fig. 2).

Extent of oxidative damages in onion tissues

Almost 1.6- and 3.0-fold lower chlorophyll content was noted in onion shoot, exposed to 100 and 150 mg L^{-1} NaF, respectively. Higher F accumulation also induced the leakage of electrolytes by 2.2-, 2.5-, and 2.4- fold from the bulb, root and shoot, respectively of onion, treated with 100 mg L^{-1} NaF solution, while 4.0-, 4.4-, and 6.5- fold from the bulb, root and shoot,

respectively, during treatment with 150 mg L^{-1} NaF. Similarly, the level of H_2O_2 and MDA was enhanced by 1.5- and 2.4- fold, respectively, in bulb, 1.4- and 5.1-fold, respectively in root, and 1.7- and 3.1- fold, respectively, in shoot tissue during 100 mg L^{-1} NaF treatment. Treatment of seedlings with 150 mg L^{-1} NaF induced H_2O_2 formation by 1.7-, 2.1-, and 2.2- fold in bulb, root and shoot, respectively, as compared to the respective control tissues. Higher dose of F (150 mg L^{-1} NaF) also induced the formation of MDA by 3.5-, 6.9-, and 4.4- fold in bulb, root and shoot tissues of onion seedlings (Table 1).

Table 1. Chlorophyll content, electrolyte leakage, H_2O_2, and MDA content of onion tissues, grown in presence of 100 or 150 mg L^{-1} NaF

	Treatment	Chlorophyll (µg g^{-1} tissue)	Electrolyte leakage (%)	H_2O_2 (nM g^{-1} FW)	MDA (µM g^{-1} FW)
Bulb	Control	-	5.73 ± 0.23	168.21 ± 12.89	1.24 ± 0.22
	100 mg L^{-1} NaF	-	13.05 ± 0.69*	260.26 ± 9.23*	2.95 ± 0.33*
	150 mg L^{-1} NaF	-	23.26 ± 1.33*	295.90 ± 12.52	4.34 ± 0.52*
Root	Control	-	6.38 ± 0.42	154.62 ± 9.04	0.98 ± 0.26
	100 mg L^{-1} NaF	-	16.15 ± 1.11*	223.72 ± 8.27*	5.05 ± 0.40*
	150 mg L^{-1} NaF	-	28.20 ± 1.28*	333.08 ± 12.85*	6.81 ± 0.33
Shoot	Control	34.56 ± 1.06	4.42 ± 0.51	167.18 ± 10.83	1.76 ± 0.26
	100 mg L^{-1} NaF	21.11 ± 2.30*	10.55 ± 1.33	279.62 ± 8.33*	5.56 ± 0.37*
	150 mg L^{-1} NaF	11.49 ± 0.92	28.73 ± 0.81*	367.95 ± 11.16*	7.79 ± 0.23*

Data represented here are the mean value (n = 3) ± standard error; '*' represents data with significant differences at *P* ≤ 0.05, compared against respective control tissues (grown in water only). H_2O_2: hydrogen peroxide, MDA: malondialdehyde.

Level of major osmolytes

The level of total amino acids and proline was found to be induced by 2.1- and 3.0- fold, respectively, in bulb, 4.0- and 2.8- fold, respectively, in root and 2.1- and 2.4-fold, respectively, in shoot tissues of onion seedlings in presence of 100 mg L^{-1} NaF. Similarly, treatment with 150 mg L^{-1} NaF further aggravated the level of total amino acids by 2.6-, 6.2-, and 3.1- fold and the level of proline by 4.5-, 3.7-, and 4.3- fold in bulb, root, and shoot tissues, respectively, compared to that of their respective control counterparts (Fig. 3).

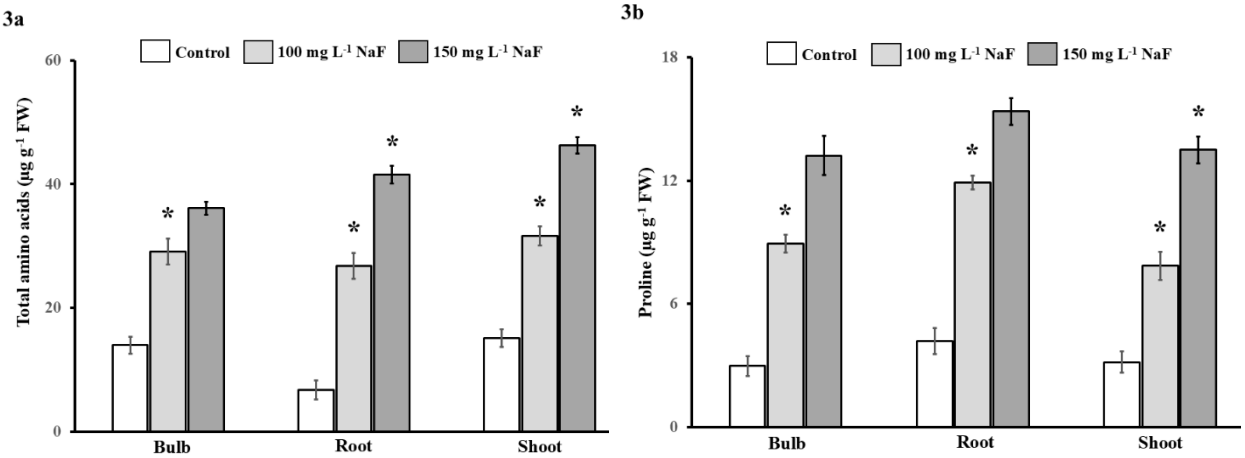

Figure 3. Total amino acids (a) and proline (b) content of onion tissues, grown in presence of 100 or 150 mg L^{-1} NaF. Data represented are the mean value (n = 3) ± standard error; '*' represents data with significant differences at P ≤ 0.05, compared against respective control tissues (grown in water only).

Table 2: Activity of SOD, CAT, APX, GPoX and GST in onion tissues, grown in presence of 100 or 150 mg L^{-1} NaF

	Treatment	SOD (Unit mg^{-1} total protein)	CAT (µM H_2O_2 decomposed min^{-1} mg^{-1} total protein)	APX (µM ascorbate oxidized min^{-1} mg^{-1} total protein)	GPoX (µM tetraguaiacol formed min^{-1} mg^{-1} total protein)	GST (µM CDNB (utilized min^{-1} mg^{-1} total protein)
Bulb	Control	5.50 ± 0.79	14.07 ± 2.27	147.80 ± 17.21	9.30 ± 1.40	35.91 ± 3.29
	100 mg L^{-1} NaF	10.48 ± 1.21*	16.82 ± 1.91	252.88 ± 9.97*	25.43 ± 2.37*	64.94 ± 4.53*
	150 mg L^{-1} NaF	12.51 ± 0.40	25.29 ± 1.72*	431.01 ± 19.54*	38.13 ± 3.08*	106.64 ± 3.13*
Root	Control	2.71 ± 0.61	17.72 ± 1.44	109.95 ± 21.14	10.87 ± 2.10	52.23 ± 6.59
	100 mg L^{-1} NaF	8.94 ± 1.35*	20.96 ± 2.98	227.87 ± 37.23*	30.82 ± 2.27*	105.60 ± 16.53*
	150 mg L^{-1} NaF	13.33 ± 0.57*	22.14 ± 2.05	366.95 ± 32.21*	43.90 ± 3.74*	120.07 ± 4.96
Shoot	Control	6.80 ± 0.65	7.88 ± 2.09	162.95 ± 17.31	8.85 ± 2.71	31.77 ± 10.58
	100 mg L^{-1} NaF	9.59 ± 0.22*	15.62 ± 2.70*	310.38 ± 23.10*	21.52 ± 2.47*	66.91 ± 3.50*
	150 mg L^{-1} NaF	15.24 ± 1.73*	20.00 ± 2.73	411.95 ± 47.16	36.90 ± 4.36*	119.23 ± 7.84*

Data represented here are the mean value (n = 3) ± standard error; '*' represents data with significant differences at $P \leq 0.05$, compared against respective control tissues (grown in water only). SOD: superoxide dismutase, CAT: catalase, APX: ascorbate peroxidase, GPoX: guaiacol peroxidase, GST: glutathione S-transferase.

Activity of enzymatic antioxidants

The activity of SOD was enhanced by 1.9- and 2.3-fold in bulb, 3.3- and 4.9- fold in root and 1.4- and 2.2-fold in shoot tissues during treatment with 100 and 150 mg L^{-1} NaF, respectively, as compared to that of their respective control. The activity of CAT was also elevated by 1.2-, 1.2-, and 2.0- fold in bulb, root and shoot, respectively, in NaF (100 mg L^{-1})-treated plants and by 1.8-, 1.3- and 2.5- fold in bulb, root and shoot, respectively, during treatment with 150 mg L^{-1} NaF. Similarly, the activity of APX was enhanced by 1.7- and 2.9- fold in bulb, 2.1- and 3.3- fold in root and 1.9- and 2.5- fold in shoot of plants exposed to 100 and 150 mg L^{-1} NaF, respectively, as compared to those of their respective control tissues. The activity of GPoX and GST was also induced by 2.7- and 1.8- fold in bulb, 2.8- and 2.0- fold in root and 2.4- and 2.1- fold in shoot in presence of 100 mg L^{-1} NaF, respectively. Application of 150 mg L^{-1} NaF induced the activity of GPoX by 4.1-, 4.0-, and 4.2- fold and the activity of GST by 3.0-, 2.3-, and 3.7- fold in bulb, root and shoot of stressed plants, with respect to those of their respective control tissues (Table 2).

Level of non-enzymatic antioxidants

The level of anthocyanins was induced by 1.4- and 1.8- fold in bulb, 1.4- and 2.1- fold in roots and 1.5- and 1.8- fold in shoot in presence of 100 and 150 mg L^{-1} NaF, respectively. F (100 mg L^{-1} NaF) treatment also induced the formation of flavonoids by 3.0-, 1.8- and 2.8- fold in bulb, root and shoot, respectively. The level of flavonoids was further elevated by 4.3-, 3.0- and 3.5-fold in bulb, root and shoot, respectively in NaF (150 mg L^{-1})-stressed plants, as compared to that of respective tissues of non-stressed plants. Enhancement by 1.8- and 2.5- fold in bulb, 2.4- and 2.2- fold in root and 1.6- and 2.0- fold in shoot tissues was noted for glutathione and ascorbate, respectively, in NaF (100 mg L^{-1})-stressed plants, whereas the level of the same were induced by 2.7- and 3.7- fold in bulb, 3.0- and 3.6- fold in root and 2.6- and 4.3- in shoot of NaF (150 mg L^{-1})-treated plants, respectively, as compared to that of non-stressed plants. The level of cysteine was induced by 1.9- and 2.5- fold in bulb, 2.0- and 2.8- fold in root and 1.5- and 2.2- fold in shoot in presence of 100 and 150 mg L^{-1} NaF, respectively, as compared to that of the respective tissues of non-stressed plants (Table 3).

Table 3: Anthocyanins, flavonoids, glutathione, ascorbate and cysteine content of onion tissues, grown in presence of 100 or 150 mg L^{-1} NaF

	Treatment	Anthocyanins (µM g^{-1} FW)	Flavonoids (µg g^{-1} FW)	Glutathione (mM g^{-1} FW)	Ascorbate (µg g^{-1} FW)	Cysteine (mM g^{-1} FW)
Bulb	Control	29.89 ± 1.28	128.55 ± 28.28	19.10 ± 3.92	35.21 ± 3.61	5.91 ± 0.45
	100 mg L^{-1} NaF	42.34 ± 2.04*	381.88 ± 25.81*	34.55 ± 4.03*	88.24 ± 5.66*	11.06 ± 0.54*
	150 mg L^{-1} NaF	54.26 ± 1.57	553.16 ± 39.05*	51.96 ± 4.14*	132.00 ± 4.74*	14.92 ± 0.50
Root	Control	23.16 ± 2.12	189.74 ± 27.92	13.53 ± 3.32	28.18 ± 6.21	3.62 ± 0.46
	100 mg L^{-1} NaF	31.73 ± 5.09*	335.04 ± 26.44*	32.16 ± 2.67*	62.48 ± 7.73*	7.21 ± 0.77*
	150 mg L^{-1} NaF	48.00 ± 1.94*	565.81 ± 40.11*	40.71 ± 4.67	103.09 ± 5.49*	10.01 ± 0.42
Shoot	Control	33.61 ± 1.96	162.74 ± 30.26	25.22 ± 3.13	37.03 ± 3.30	5.94 ± 0.41
	100 mg L^{-1} NaF	49.64 ± 1.56*	468.72 ± 29.80*	42.08 ± 4.54*	74.24 ± 7.54*	9.16 ± 0.44*
	150 mg L^{-1} NaF	60.82 ± 2.26*	563.76 ± 47.42	66.71 ± 4.01*	159.21 ± 6.30*	13.20 ± 0.61*

Data represented are the mean value (n = 3) ± standard error; '*' represents data with significant differences at $P \leq 0.05$, compared against respective control tissues (grown in water only).

DISCUSSION

Unregulated discharge of toxic elements like mercury, arsenic, nickel, lead, cadmium, aluminium, etc., from the factories due to reckless anthropogenic activities has severely ruined the quality of the environment by contaminating the surrounding areas. A recent addition to this list is contamination with F-salt, whose level has significantly enhanced due to the release of pollutants like Teflon, pesticides, pharmaceuticals, and those from paint factories.[37] According to Bhattacharya and Samal, the major sources of F pollution with regard to Indian agricultural field are phosphate-fertilizers having < 1% to > 1.5% fluorine, untreated water and F emissions from factories like ceramic, glass, steel, zinc, and smelting factories.[2,3] Due to its high solubility, F-salt gets easily admixed in surrounding water bodies and further contaminate underground aquifers and agricultural land, severely impacting the quality and yield of crops.[10] With respect to Indian subcontinent, major onion producing states are highly contaminated with F-salt. In addition, slightly lower pH of soil required for optimum growth of onion further facilitates the uptake of F-salt via the roots.[38] Upon absorption from the soil, F-salt accumulates in the tissues of onion plants and initiates the formation of ROS by Mehler reaction, inciting oxidative stress.[39] Onion is one of the most important commercially valuable crops in India and is widely consumed by a large population all over the world. However, in spite of its wide demand and high values in Indian economy, the production of onion is significantly affected in India in recent times due to the enhanced level of F-salt in soil. Thus, this study was focused to demonstrate the adverse effects caused due to higher accumulation of F-salt in the bulb, root and shoot tissues of onion plants. Additionally, the role of protective metabolites and antioxidant enzymes was also analyzed to assess their role in reducing the symptoms of F-induced damages in tissues.

Prolonged exposure of plants to NaF (100 and 150 mg L^{-1}) greatly enhanced the accumulation of F^- ions in bulb, root and shoot, as compared to that of water-treated plants. Unregulated uptake of F^- ions via roots can be explained by the presence of chloride channel (CLC) in roots that acts as a F transporter. In addition, due to unrestricted translocation of F^- ions, higher F concentration was also noted in bulb and shoot. Our findings are in congruence with the earlier work of Jha et al. where they demonstrated that F uptake and accumulation in onion tissues show almost a linear trend with increasing F concentration in soil.[38] In addition, several studies in other crops like *Vigna* sp., *Cicer arietinum*, *Oryza sativa*, *Spinacia oleracea*, and *Cajanus cajan* have also showed higher accumulation of F^- ions in seedlings, when exposed to different concentration of F-salt.[15-17,29,40]

Higher accumulation of F^- ions in bulb, root and shoot induced the formation of ROS, i.e., H_2O_2 in tissues, eventually causing higher peroxidation of lipid membrane and formation of cytotoxic metabolites like MDA. Additionally, perforation in lipid membrane led to a higher leakage of electrolytes from the cells. F-

induced formation of ROS and MDA, together with electrolyte leakage in our experiment can be supported by the previous works of Yadu et al. in *Cajanus cajan* L. where they showed that treatment of seedlings with 75 mg L^{-1} NaF triggered the formation of H_2O_2 and MDA, eventually reducing cell membrane stability and higher electrolyte leakage.[17] During F stress, limited CO_2 fixation reduces carbon reduction by Calvin cycle along with the reduced level of oxidized $NADP^+$ which eventually causes higher formation of ROS by Mehler pathway.[39] Another major detrimental effect of F toxicity was noted on chlorophyll level which was significantly lowered in shoot with higher NaF concentration. Lower concentration of chlorophyll might be due to its higher breakdown or inhibited synthesis as shown earlier by Baunthiyal and Sharma in three different semi-arid plant species (*Acacia tortilis*, *Cassia fistula* and *Prosopis juliflora*), exposed to 10, 20 and 50 mg kg^{-1} F.[41] Data obtained in the present study can also be supported by the previous work of Sabal et al., Gupta et al., and Bhargava and Bhardwaj where they had shown lower chlorophyll content in *Cyamopsis tetragonoloba*, *Oryza sativa* and *Triticum aestivum* seedlings, respectively, exposed to F stress.[42-44]

Plants maintain an abundant repertoire of various metabolites and enzymes that serve as antioxidants to scavenge the ROS generated in bulk amounts during harsh environmental conditions. Osmolytes such as total amino acids and proline serve as protective metabolites in plant tissues by scavenging ROS, maintaining fluidity of cell membrane, and regulating cellular osmotic balance. During F (100 and 150 mg L^{-1} NaF)-toxicity, the level of total amino acids and proline was significantly induced in plant tissues via which plants somehow managed to overcome F toxicity. However, the rise in the level of osmolytes was still not sufficient enough to maintain normal growth of the plants, thereby resulting in stunted growth. Higher level of osmolytes during F stress, as observed here, can be supported by the earlier work of Singh and Roychoudhury where they demonstrated higher synthesis of osmolytes in response to F-toxicity in rice plants, linked with induced activity of Δ^1-pyrroline-5-carboxylate synthetase (P5CS).[20] Additionally, higher levels of proline and total amino acids in NaF treated *Triticum aestivum* due to higher P5CS activity was also reported by Tak and Asthir.[45]

Another major group of protective machineries operating in plants are the enzymatic antioxidants such as SOD, CAT, APX, GPoX, and GST that altogether play a pivotal role in scavenging of ROS and thus reducing the toxic effects of abiotic stress. In case of F-stress (100 and 150 mg L^{-1} NaF), the activity of all the major enzymatic antioxidants was enhanced in bulb, root, and shoot of onion plants which showcase the role of enzymatic antioxidants. Data obtained in this study can be justified by the earlier work of Banerjee and Roychoudhury, where they showed that exogenous application of NaF (25 mg L^{-1}) induced the activity of enzymatic antioxidants in rice seedlings.[19] Similar studies conducted by Kumar et al. showed higher SOD and GPoX activity in F exposed mulberry leaves.[46] The same results were also obtained by Chakrabarti and Patra in F treated mustard, spinach, radish, and coriander seedlings.[47] However, in spite of such significant enhancement in the activity of enzymatic antioxidants, the level of ROS was still high in onion plants which suggested that the escalated activity of enzymatic antioxidants was insufficient to combat the higher formation rate of ROS that eventually led to substantial oxidative damages in the tissues.

In addition to enzymatic antioxidants, non-enzymatic antioxidants also play a pivotal role in protecting plants against abiotic stress, allowing proper maintenance of the integrity of lipid membrane along with regulating the osmotic balance of cells. In case of non-enzymatic antioxidants, viz., anthocyanins, flavonoids, ascorbate, glutathione, and cysteine, similar trend was noted as that of osmolytes and enzymatic antioxidants. In F-exposed onion plants, the level of all the above mentioned non-enzymatic antioxidants was significantly elevated. Higher level of non-enzymatic antioxidants in F-exposed onion plants is corroborated by the earlier work of Singh and Roychoudhury where they reported higher formation of non-enzymatic antioxidants in rice plants in response to increasing concentration of F.[20] They further reported that higher formation of non-enzymatic antioxidants can be linked with the higher activity of phenylalanine ammonia lyase, the major enzyme involved in the synthesis of non-enzymatic antioxidants. Similarly, the higher level of non-enzymatic antioxidants in several other F-stressed plants such as Bengal gram seedlings and *Triticum aestivum* was also reported by Dey et al. and Bhargava and Bhardwaj, respectively.[40,44]

Overall, the current work clearly demonstrated the toxic effects of two different concentration, viz., 100 and 150 mg L^{-1} NaF on bulb, root, and shoot of onion plants. Onion represents one of the most important horticultural crops, having growing demand for cultivation and production with the progression of time. However, there is no such elaborate report that showcase the toxic effect of F stress on onion. The data obtained from this work clearly showed that exposure of onion to F led to its higher uptake via root and unregulated translocation in other tissues such as bulb and shoot. F-induced oxidative damage led to perforation in cell membrane, causing electrolyte leakage and formation of MDA along with reduction in chlorophyll level. To combat the effects of F toxicity and F-induced oxidative damages, onion plants significantly induced the formation of osmolytes and non-enzymatic antioxidants along with activity of enzymatic antioxidants. However, this rise in the formation of protective metabolites was not sufficient enough to scavenge the enormous level of ROS formed in the

tissues. Earlier work of Singh et al. also established that in case of the susceptible rice cultivar (MTU-1010), the increment in the level of protective metabolites was insufficient to reduce the toxic effects of F stress, as compared to that of the tolerant variety (Khitish).[15] Based on this work, onion could also be regarded as highly susceptible to F stress, so that appropriate mitigating strategies should be adopted to reduce the uptake and accumulation of F^- ions in onion tissues, so as to ensure proper growth and yield of this highly demanding horticultural vegetable crop.

DECLARATIONS

Ethical approval

The work does not involve human or animal studies. Hence, no approval is required.

Consent to participate and consent to publish

Competing interest

The authors declare that there is no conflict of interest in publishing this manuscript.

AUTHOR CONTRIBUTIONS

Ankur Singh performed all the experiments, generated the data and drafted the manuscript. Prof. Aryadeep Roychoudhury supervised the entire work, arranged all resources and fundings, and made necessary modifications within the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data will be made available upon justified requests.

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