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Activated charcoal alleviates fluoride stress by restricting fluoride uptake and counteracting oxidative damages in the rice cultivar MTU1010

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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 H₂O₂, 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 (O₂⁻) and hydrogen peroxide (H₂O₂) 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

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detrimental to the consumer health, leading to neurological abnormalities and severe fluorosis (Banerjee et al., 2021).

Plants develop various protective metabolites such as osmolytes and enzymatic and nonenzymatic antioxidants for the prevention of negative consequences of F toxicity. Osmolytes are neutral, small molecules involved in the maintenance of the osmotic homeostasis of the cells. They are often referred to as compatible solutes and include sugars, glycine-betaine (gly-bet), and amino acids like proline (Pro). Such protective molecules act as scavengers of toxic free radicals which provide stability to the membrane lipids and proteins during exposure to F (Gadi et al., 2012). Paul et al. (2017) observed that in response to abiotic stresses, plants enhance the formation of osmolytes that in turn induces the survival capability of plants by scavenging free radicals, protecting the cell membrane, maintaining the homeostatic balance of cells and active conformation of cellular proteins. The protective role of osmolytes against F toxicity in other plants such as *Vigna* sp. and Bengal gram seedlings has been previously reported (Singh et al., 2021a; Dey et al., 2012).

Apart from the osmolytes, antioxidative machinery which comprises of both nonenzymatic and enzymatic antioxidants play a vital role in ameliorating the detrimental effects of F toxicity in rice seedlings. Superoxide dismutase (SOD), guaiacol peroxidase (GPOX), ascorbate peroxidase (APX), and catalase (CAT) are major players involved in the detoxification of excessive ROS. Singh et al. (2021a) demonstrated that the superoxide radical formed due to oxidative burst in fluoride-stressed *Vigna* seedlings is detoxified by the enzymatic action of SOD to less toxic product, i.e. H_2O_2 which is further converted to nontoxic molecules by CAT, APX, and GPOX. However, F toxicity prevents the CAT activity via the replacement of the hydroxyl molecule from the iron group present at the active site of the enzyme (Kumar et al., 2009). In absence of CAT, the above-mentioned enzymatic antioxidant activity was increased in rice seedlings on being treated with F (Chakrabarti and Patra, 2015). Along with enzymatic antioxidants, plants also form numerous nonenzymatic antioxidants like phenolics, ascorbic acid, and carotenoids. These secondary metabolites act as potential scavengers of harmful ROS enhancing the stability of the polyunsaturated acyl groups of the membrane lipids. The protective role of nonenzymatic antioxidants has been pointed out earlier in other crops such as *Vigna* sp., safflower seedlings, and watermelon exposed to F stress (Singh et al., 2021a; Ghassemi-Golezani and Farhangi-Abri, 2019; Ram et al., 2014). Another cytotoxic metabolite formed in tissues due to the excessive formation of ROS is MG which is efficiently detoxified by glyoxalase I (Gly I) and glyoxalase II (Gly II), together constituting the glyoxalase system (Hong et al., 2016).

Activated charcoal or activated carbon is characterised by a large number of tiny pores that are responsible for its huge porosity and surface area for surface adsorption. Such pores are quite minute in terms of their volume, often referred to as micropores. Activated charcoal is quite different from other forms of carbon due to the oxidation of the surface of the carbon and also due to the removal of all the impurities which are noncarbon in nature (Thomas, 2008). Therefore, activated charcoal demonstrates an elaborate and fine network of pores, thereby exhibiting a large intrinsic surface area that enables the adsorption of a range of substances on its surface (Thomas, 2008). This extraordinary adsorption power of activated charcoal has recently been exploited in several cases for the amelioration of the adverse effects of different forms of abiotic stress in several plant species. Activated charcoal also serves as a carbon source for the plant system, thus modulating different metabolic pathways that provide necessary energy for the essential development and growth of stressed plants. One such major metabolic pathway in plants is the tricarboxylic acid cycle (TCA) where two carbon containing metabolite, i.e. acetyl CoA is utilized by a series of enzymatic reactions to produce high energy molecules (Schnarrenberger and Martin, 2002). The catalytic action of isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH) produces one NADPH molecule each, whereas one $FADH_2$ molecule is released due to the enzymatic activity of succinate dehydrogenase (SDH). Pyruvate dehydrogenase (PDH) tends to catalyse the connecting step between glycolysis and TCA cycle, whereby pyruvate is converted to acetyl CoA along with the formation of one NADH molecule. Thus, it is quite evident that activated charcoal can act as a major carbon source which might eventually up regulate the TCA cycle in plants, thereby maintaining the formation of high energy molecules in stressed plants.

The ameliorative role of charcoal against diverse forms of abiotic stress in plants has not been widely investigated to date. Chou et al. (2017) demonstrated the abrogative role of activated charcoal under varying levels of salt (road salt) treatment in *Arabidopsis thaliana*. We envisaged that activated charcoal could mitigate fluoride toxicity in rice seedlings by affecting the osmolyte and antioxidant-mediated defense in rice. Our hypothesis was that the pores of activated charcoal could adsorb F^- ions, reducing their absorption via the plant roots. Thus, the prime aim of this manuscript was to authenticate the vital role played by activated charcoal in ameliorating the negative effects of F stress in rice seedlings. The protective role of charcoal, in inhibiting the uptake of F^- along with enhanced formation of protective molecules such as osmolytes and antioxidants, has been demonstrated here. Additionally, activated charcoal was shown to enhance the formation of protective metabolites and activity of enzymes of TCA

cycle that positively contributed toward the tolerance of rice. MTU1010 (also known as Cotton Dora Sannalu) is one of the very popular mega-rice varieties of India, which is extensively cultivated in many rice-growing states during both wet and dry seasons (<http://www.rkmp.co.in/content/mtu-1010>) due to its high yield, short duration, and desirable long slender grain type which altogether establish it as an important rice cultivar (Anila et al., 2018). Additionally, the effect of fluoride stress on MTU1010 has been demonstrated earlier from our laboratory (Singh and Roychoudhury, 2022). Based on such information, MTU1010 was selected for this work.

2. Materials and methods

2.1. Plant growth condition and stress imposition

Rice seeds (cv. MTU1010) were acquired from Rice Research Institute (Chinsurah, West Bengal). The seeds were cleaned with 0.1% (w/v) HgCl_2 followed by vigorous washing with distilled water. The seeds were put on blotting paper after sterilization and were air-dried to reduce their moisture content to their initial value. The seeds were next kept on a sterile gauge in a Petri dish and allowed to germinate at 32 °C for 3 days. The germinated seeds were sown in the soil, containing 1.4% phosphorus, 1.4% nitrogen, 1.4% potash, 0.7% magnesium, 4% calcium, 1% sulphur, 15% cellulose, 60% moisture, 10% lignin, and carbon: nitrogen ratio as 25: 1, in plastic containers (10 cm in height and 6 cm in diameter) and were allowed to grow under normal sunlight and photoperiod as previously standardized by Banerjee et al. (2020). F stress was imposed through supplementation with 25 mg L^{-1} sodium fluoride (NaF) solution, either in the absence or presence of activated charcoal (5 mg g^{-1} soil) for 27 days. The seedlings were maintained in triplicates in four separate sets as follows:

Set 1: Seedlings grown in double distilled water

Set 2: Seedlings grown in 25 mg L^{-1} NaF solution

Set 3: Seedlings grown in presence of 5 mg activated charcoal g^{-1} soil

Set 4: Seedlings grown in presence of 25 mg L^{-1} NaF solution and 5 mg activated charcoal g^{-1} soil

After 30 days, the seedlings were harvested after taking their photograph. The harvested seedlings were stored for analysis at -80 °C. The concentration of NaF, i.e. 25 mg L^{-1} , causing severe oxidative damages, was fixed following the earlier work of Banerjee et al. (2019), whereas the concentration of activated charcoal was standardized by experimental trials.

2.2. Analysis of physiological parameters: germination percentage (%), fresh weight (FW), dry weight (DW), root length (RL), and shoot length (SL)

For the estimation of germination efficiency, the number of germinated seeds was counted and the germination

percentage was estimated. The FW was measured by taking 50 seedlings from each set, separately. The tissues were then dried at 80 °C for two days, following which the DW was measured. The RL and SL were measured by using an analytical ruler and were expressed in cm.

2.3. Estimation of fluoride accumulation in tissues and soil

For estimation of F level in plant tissues and soil, 0.2 g of seedlings or soil were extracted in 4 mL of TISAB solution (Total Ionic Strength Adjustment Buffer) and the level of F in homogenate was estimated following the earlier protocol of Singh and Roychoudhury (2020).

2.4. Analysis of damage parameters: Chlorophyll, Electrolyte leakage (EL), H_2O_2 , MDA, and MG

The content of total chlorophyll of the seedlings was calculated by following the previous protocol of Gupta et al. (2009). The leakage of electrolytes from the cells was measured using a conductivity meter, following the earlier work of Waqas et al. (2017). The potassium-iodide solution was used to react with endogenous H_2O_2 within the tissues followed by observing the absorbance of the reaction solution at 390 nm following the previous work of Wild et al. (2012). For estimation of MDA level, 0.5% (w/v) thiobarbituric acid dissolved in 20% (w/v) trichloroacetic acid was mixed with plant supernatant followed by monitoring the absorbance at 532 nm and 600 nm (Zeb and Ullah, 2016). For estimation of MG, the supernatant obtained by homogenising the seedling in 5% (v/v) perchloric acid was mixed with N-acetyl-L-cysteine and the absorbance of the final solution was observed at 288 nm, following the earlier work of Wild et al. (2012).

2.5. Activity of major enzymes of TCA cycle: PDH, IDH, SDH, and MDH

For estimation of PDH activity, the increase in absorbance of supernatant on being mixed with nicotinamide adenine dinucleotide (NAD), pyruvate, and CoA was recorded at 340 nm, following the earlier work of Singh et al. (2021b). The activity of IDH in tissues was determined by monitoring the rise in absorbance of the supernatant at 340 nm in presence of NAD and sodium isocitrate, according to the earlier work of Zhou et al. (2012). SDH activity was determined by monitoring the reduction of iodinitrotetrazolium (INT) at 458 nm following the previous work of Samanta et al. (2020). To estimate the MDH activity, the absorbance of the reaction mixture comprising the supernatant was recorded at 340 nm maintaining the earlier methods of Sil et al. (2018).

2.6. Estimation of osmolytes: Total sugars, Pro, gly-bet, and total amino acids

Total sugar was quantified following the previous work of Choudhury et al. (2010) by monitoring the absorbance

of the sample at 490 nm. Proline content was calculated following the previous methods of Campos et al. (2019). The amount of gly-bet in plant tissues was measured at 365 nm following the previous work of Grieve and Grattan (1983). For quantification of total amino acids, the supernatant obtained after tissue homogenization was treated with freshly prepared ninhydrin and absorbance of the reaction mixture was recorded at 570 nm (Singh et al., 2020).

2.7. Estimation of enzymatic and nonenzymatic antioxidants: SOD, CAT, APX, GPoX, ascorbic acid, total phenolic content (TPC), and carotenoids

SOD activity was determined from the earlier work of Alonso et al. (2001) and was expressed as U activity of enzyme mg^{-1} of protein, where U activity denotes the amount of enzyme required for 50% inhibition of the primary rate of reaction. For estimation of CAT activity, the amount of H_2O_2 decomposed was monitored at 240 nm, following the previous work of Velikova et al. (2000). For calculation of APX activity, the oxidation of ascorbate was checked at 290 nm (Singh and Roychoudhury, 2021). The activity of GPoX was estimated by monitoring the formation of tetraguaiacol in presence of guaiacol at 470 nm (Srinivas et al., 1999). Ascorbic acid was determined by treating the plant homogenate with 2% (w/v) sodium molybdate followed by recording the absorbance at 730 nm (Singh et al., 2020). TPC was measured colorimetrically at 760 nm using sodium carbonate and Folin-Ciocalteu reagent, as previously described by Basu et al. (2012). For quantification of carotenoids, 0.4 g of seedlings were homogenised in 5 mL of 80% (v/v) acetone, followed by observing the absorbance of the supernatant at 425 nm (Peterman et al., 1997).

2.8. Estimation of Gly I and Gly II activity

For determining the activity of Gly I and Gly II, the protocol by Hasanuzzaman et al. (2019) was followed. Gly I activity was estimated by monitoring the rise in the absorption of reaction mixture for the production of S-D-lactoylglutathione (SLG) at 240 nm, whereas Gly II in terms of rise in absorbance at 412 nm for the formation of glutathione (GSH).

2.9. Localization of ROS, O_2^- , and cellular necrosis

For localization of ROS, leaf sample was treated with dichloro dihydrofluorescein diacetate-acetyl-ester following the earlier work (Kristiansen et al., 2009) and a fluorescence image was obtained using an epi-fluorescence microscope (Olympus, USA). Superoxide radical was visualized by staining the leaf sample with nitroblue tetrazolium (Banerjee et al., 2021). For visualizing cellular necrosis, leaves were treated with Evan's blue in presence of calcium chloride and were visualized with a stereomicroscope (Olympus, USA) (Vemanna et al., 2017).

2.10. Protein estimation

The concentration of protein in each sample was calculated by Bradford (1976) method using a standard curve prepared by known concentrations of bovine serum album. Equal amount of protein was used for all the associated assays.

2.11. Statistical analysis

Three replicates ($n = 3$) for each set were maintained in a completely randomized design. The data were graphically represented as mean \pm standard error (SE); statistical significance was calculated at $p \leq 0.05$, using one-way analysis of variance (ANOVA).

3. Results and discussion

Anthropogenic activities have largely contributed towards the enhancement in the level of xenobiotics in the environment, causing major harm to human populations due to the direct consumption of polluted water or food resources. Such xenobiotic components mostly consist of heavy metals, chemical wastes, radioactive substances, or other nonbiodegradable pollutants, released from factories, agricultural fields, household wastes, or pollutants released due to natural calamities like a volcanic eruption or flood (Hong et al., 2016). In recent times, the level of another xenobiotic element, i.e. F is gradually increasing at an alarming rate in the environment due to the effluents released from paint, dental and ceramic factories. In addition, washed out fertilizers from the agricultural field and household wastes also discharge a significant amount of F in the environment. F being highly soluble in water is easily admixed with the surrounding ground and surface water sources, polluting the water bodies. Use of such water for agricultural purposes contributes to the accumulation of F to the agricultural field which further gets accumulated in crops due to its high uptake via chloride channels (Banerjee et al., 2021). Till date, there is no report which focuses on the role of activated charcoal against F stress in any crop plant (Ali et al., 2017; Irshad et al., 2020). The prime object of this work was therefore to establish the efficacy of charcoal in overcoming the effects of F-induced damages in rice seedlings. We hypothesised that charcoal due to its large size and high porosity could adsorb F^- ions on its surface which would ultimately restrict the uptake of F^- ions within the tissues, lowering the oxidative damages in seedlings. The regulation of protective metabolites which could scavenge the ROS formed for maintaining the cellular homeostatic balance was also our focus of attention.

3.1. Charcoal maintains normal germination and growth of seedlings

In presence of charcoal, the growth of the seedlings was restored which was initially reduced due to the application of NaF solution (Figure 1). Exposure to NaF reduced

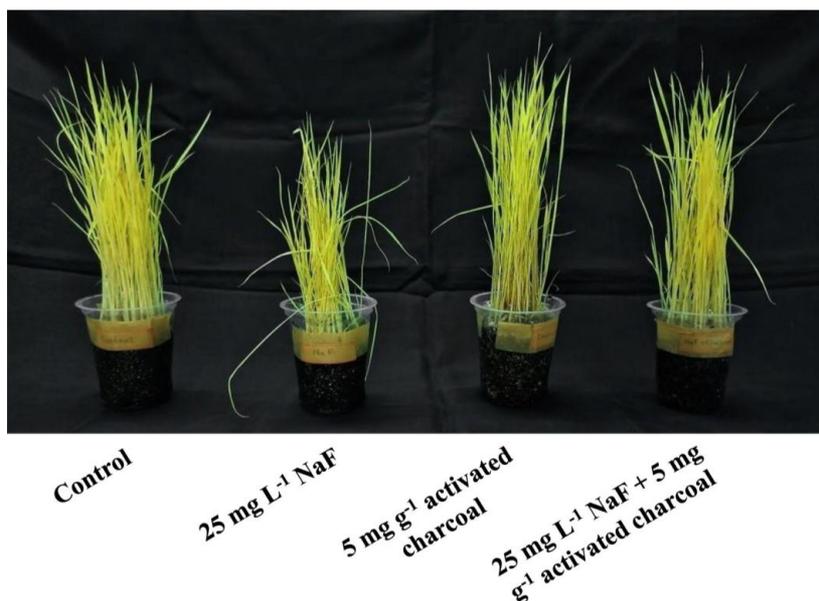


Figure 1. Efficacy of activated charcoal (5 mg g^{-1}) in maintaining the growth parameters of seedlings grown in either presence or absence of 25 mg L^{-1} NaF monitored for 20 days; water treated, i.e. nonstressed seedlings served as experimental control.

germination percentage by 1.2-fold, whereas other physiological parameters including FW and DW were also lowered by 1.2- and 1.4-fold, respectively. Additionally, the shoot and root length were also decreased by 1.4- and 1.3-fold, respectively which can be supported by the previous works of Singh et al. (2020) and Banerjee and Roychoudhury (2019b), where they observed that exogenous administration of NaF (25 mg L^{-1}) solution lowered the percentage of seed germination and also negatively affected the FW, DW, RL, and SL of seedlings. However, in presence of charcoal, seed germination was restored by 1.2-fold along with other physiological conditions, i.e. FW, DW, RL, and SL which were also restored by 1.2-, 1.4-, 1.3-, and 1.4-fold, respectively, in comparison to that of stressed seedlings (Table 1). Our results can be supported by the earlier work of Ghassemi-Golezani and Farhangi-Abriz (2019) where they showed that exogenous application of biochar maintained the DW with proper root and shoot growth.

3.2. Retention of F^{-} ions in the soil in presence of charcoal

In absence of charcoal, 40.1-fold higher accumulations of F^{-} ions were noted in seedlings whereas, in presence of charcoal, the level of endogenous F^{-} ions within the tissues was reduced by 2.2-fold, in comparison to the stressed-alone seedlings. The level of F in soil in absence of charcoal was 30.8-fold higher in comparison to that of control soil, whereas application of charcoal further elevated the soil F level by 1.4-fold, in comparison to that of soil treated with NaF solution alone (Figure 2). Thus, it can be presumed that the F^{-} ion was adsorbed on to the

surface of charcoal applied to soil that ultimately resulted in lower uptake of F^{-} ions via roots. Therefore, the level of F^{-} ions was significantly higher in soil treated with 5 mg g^{-1} activated charcoal, but lower within the seedlings. Similar work was also undertaken by Ghassemi-Golezani and Farhangi-Abriz (2019) where they showed that in presence of biochar, the amount of F^{-} uptake was reduced by roots which resulted in lower accumulation of F^{-} ions in safflower plants. They also showed that the level of F in soil was reduced upon treatment with biochar due to its higher adsorption capacity that resembled our data.

3.3. Charcoal-mediated lowering of oxidative damages in seedlings

Greater accumulation of F^{-} ions decreased the level of chlorophyll in leaves by 1.4-fold which can be justified by the greater activity of chlorophyllase or reduced availability of Fe^{2+} ions that plays a vital role in the synthesis of chlorophyll, as shown earlier by Singh and Roychoudhury (2021). Additionally, the level of ROS such as H_2O_2 was also induced by 2.0-fold which in turn led to the perturbation of lipid membrane that resulted in a 2.9-fold increase in the leakage of electrolyte from cells along with 2.5-fold and 1.4-fold higher accumulation of cytotoxic metabolites, i.e. MDA and MG, respectively (Table 1). The higher formation of ROS due to excessive accumulation of F^{-} ions in tissues can be justified by previous observation of Yadu et al. (2016) where F^{-} ions were shown to initiate ROS formation through Mehler reaction, involving the oxidised form of NADP⁺ serving as an electron acceptor via reduction of ferredoxin during

Table 1. Physiological parameters (germination percentage, FW, DW, RL, and SL) and the level of molecular damages (chlorophyll loss, electrolyte leakage, H₂O₂, MDA, and MG) in seedlings grown in presence of 25 mg L⁻¹ NaF, either in presence or absence of 5 mg g⁻¹ activated charcoal. Data (mean value of triplicate sets) with significant difference at p ≤ 0.05 are represented by '*'. Mean ± SE.

	Parameters	Control	25 mg L ⁻¹ NaF	5 mg g ⁻¹ activated charcoal	25 mg L ⁻¹ NaF + 5 mg g ⁻¹ activated charcoal
Physiological parameters	Germination percentage (%)	96.2 ± 1.3	79.1 ± 1.5*	98.0 ± 2.3	92.3 ± 2.1
	Fresh weight (mg)	302.4 ± 4.1	249.0 ± 2.7*	305.1 ± 2.9	297.3 ± 2.7
	Dry weight (mg)	121.0 ± 3.2	84.1 ± 1.9*	127.4 ± 3.9	115.0 ± 2.1
	Root length (cm)	7.1 ± 1.4	5.2 ± 0.7*	6.9 ± 1.4	7.0 ± 1.4
	Shoot length (cm)	20.9 ± 1.5	15.4 ± 1.2*	22.1 ± 2.1*	21.4 ± 1.7
Damage parameters	Chlorophyll (µg g ⁻¹ FW)	64.4 ± 3.1	46.2 ± 0.50*	71.8 ± 3.6*	62.4 ± 2.2
	Electrolyte leakage (%)	12.8 ± 1.2	37.5 ± 1.9*	10.3 ± 1.5	16.7 ± 1.4
	H ₂ O ₂ (µg g ⁻¹ FW)	1.2 ± 0.2	2.4 ± 0.7*	0.8 ± 0.1*	1.5 ± 0.6
	MDA (µg g ⁻¹ FW)	0.7 ± 0.1	1.8 ± 0.1*	0.7 ± 0.3	0.8 ± 0.1
	MG (mg g ⁻¹ FW)	19.9 ± 1.1	28.3 ± 0.4*	19.0 ± 1.2	20.1 ± 0.2

electron transfer in photosynthesis. Similar reports were also published earlier by Ram et al. (2014), Yadu et al. (2017) and Singh and Roychoudhury (2020) where they reported that higher accumulation of F⁻ ions within the tissues of watermelon, *Cajanus cajan* and rice seedlings, respectively led to the enhanced formation of ROS which hampered the integrity of lipid membrane, thus resulting in higher leakage of electrolyte and formation of cytotoxic metabolites in cells.

Activated charcoal treatment in presence of NaF solution enhanced the level of chlorophyll by 1.3-fold, whereas the level of H₂O₂ was reduced by 1.6-fold that eventually protected the lipid membrane, as reflected by 2.2-fold lower electrolyte leakage and 2.2-fold and 1.4-fold lower formation of MDA and MG, respectively in plant tissues (Table 1). Lesser uptake of F⁻ ions lowered the H₂O₂ accumulation which in turn lowered other abnormal symptoms such as chlorophyll degradation, higher electrolyte leakage, and MDA and MG formation. Similar results were also stated by Irshad et al. (2020) where application of biochar reduced the level of cadmium and arsenic in rice plants, thereby protecting the photosynthetic

machinery (chlorophyll a and b), lowered the formation of H₂O₂ and MDA and also checked the leakage of cellular electrolytes that coincided with our observation.

3.4. The activity of Kreb's cycle enzymes was restored in presence of charcoal

In presence of NaF, the activity of Kreb's cycle enzymes like IDH, PDH, MDH, and SDH was significantly lowered by 1.6-, 1.7-, 3.3- and 1.6- folds in F stressed seedlings in comparison with the nonstressed seedlings (Figure 3). Reduced activity of Kreb's cycle enzymes can be justified by the earlier research of Singh et al. (2021b). According to Samikkannu et al. (2003), reduced activity of Kreb's cycle enzymes lowered the availability of carbon in the arsenic-stressed cells, reducing their survival capability. Our observation was concurrent with the earlier observation and the decline in plant growth could be attributed to hindered carbon metabolism due to lowered activity of Kreb's cycle enzymes.

Activated charcoal inhibited the uptake of F⁻ ions via roots and also acted as an adequate supply of carbon source for the enzymes of the Kreb's cycle that eventually

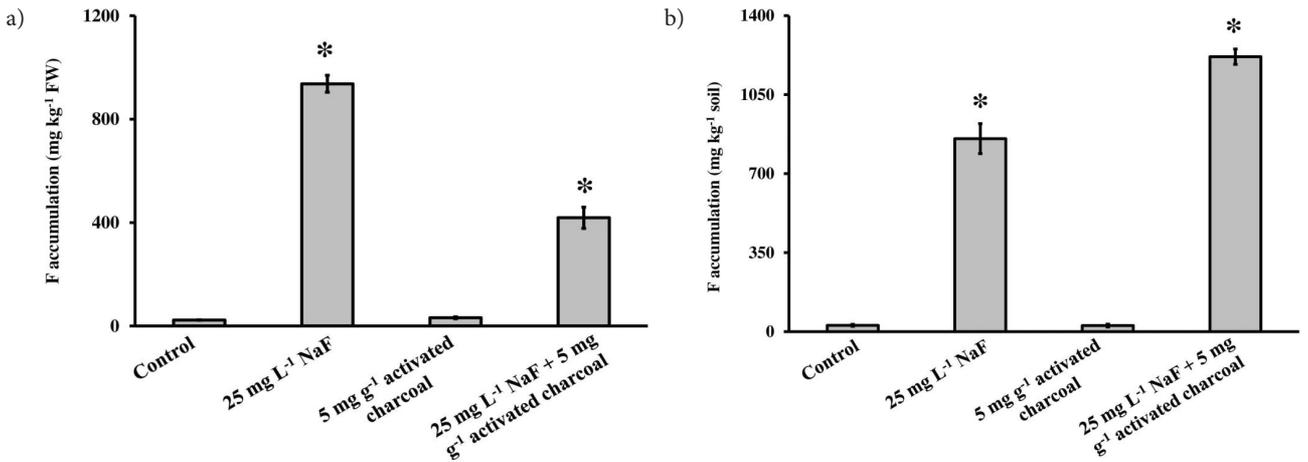


Figure 2. Accumulation of F in the plant tissues (a) and in soil (b) in presence of 25 mg L⁻¹ NaF, either in absence or presence of 5 mg g⁻¹ activated charcoal. Data (mean value of triplicate sets) with significant difference at $p \leq 0.05$ are represented by '*'. Bars are SE.

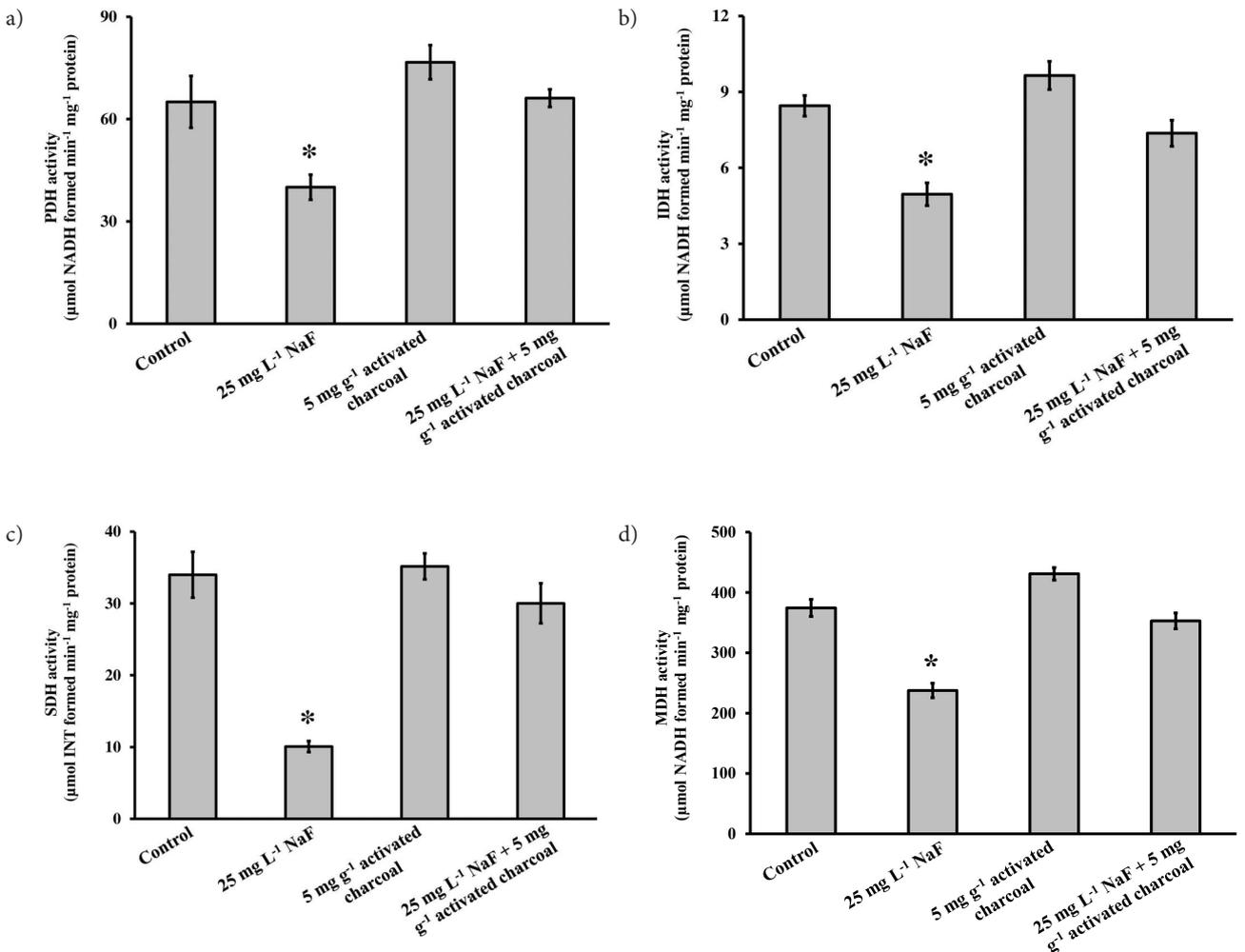


Figure 3. Activity of the enzymes of Krebs's cycle, PDH (a), IDH (b), SDH (c) and MDH (d) in rice seedlings grown in presence of 25 mg L⁻¹ NaF, either in absence or presence of 5 mg g⁻¹ activated charcoal. Data (mean value of triplicate sets) with significant difference at $p \leq 0.05$ are represented by '*'. Bars are SE.

up regulated the activity of PDH, IDH, SDH, and MDH by 1.6-, 1.5-, 3.0-, and 1.5- folds, respectively in comparison to the seedlings that were F-treated (Figure 3). Singh et al. (2021b) showed that exogenous application of calcium compounds forms conjugate with F^- ions that resulted in lower translocation of F^- ions via roots, protecting the activity of the enzymes controlling the energy biosynthesis steps of Krebs's cycle. Such earlier work can be used as a supportive data for our present observation where activated charcoal improved the functioning of Krebs's cycle pathway by providing a sufficient carbon pool and restricting fluoride uptake.

3.5. Charcoal positively regulated the formation of endogenous osmolytes

Osmolytes play a remarkable role in maintaining proper homeostasis of cells and protecting the integrity of cell membranes. With regard to osmolytes, the level of total soluble sugar, Pro, total amino acids, and gly-bet was triggered by 1.7-, 1.7-, 1.9-, and 1.1- folds, respectively as compared to the control seedlings. The elevated levels of such osmolytes exerted a contributory role in abating the detrimental effects of F toxicity (Table 2). The higher level of sugar and Pro in stressed seedlings could be justified by the earlier works of Gadi et al. (2012) where the level was enhanced in NaF-treated *Vigna* seedlings. The enhanced level of gly-bet, Pro and total amino acids during F stress could also be justified by the recent work of Banerjee and Roychoudhury (2019b).

Treatment of rice seedlings with activated charcoal in conjunction with NaF further aggravated the level of the above-mentioned osmolytes. Charcoal-treated stressed seedlings showed 1.8-, 1.4-, 1.3-, and 1.3- folds higher concentration of total soluble sugar, Pro, gly-bet, and amino acids, respectively (Table 2). Gohari et al. (2021) earlier showed that the level of Pro was enhanced in salt-stressed grapevine on exogenous application of Pro-functionalized carbon quantum dot that maintained the osmotic balance of the cells, pH level of the cytosol and flow of water within the cells. Selim et al. (2021) showed that treatment of arsenic-stressed C3 plants (barley) and C4 plants (maize) with elevated CO_2 level can induce the amount of soluble sugars and amino acids by acting as a carbon source. Higher levels of soluble sugar and amino acids thus participate in the synthesis of other protective metabolites providing the capability to overcome the negative effects of arsenic stress

3.6. Charcoal enhanced the activity of the enzymatic antioxidants

Enzymatic antioxidants participate in converting the toxic ROS formed during stress to nontoxic metabolites. Almost 2.0-fold higher SOD activity was noted in the stressed seedlings which indicated that the up regulated SOD activity was a means to detoxify the superoxide

radical to a less toxic metabolite, i.e. H_2O_2 during stress. Ghassemi-Golezani and Farhangi-Abri (2019) observed a concentration-dependent rise in SOD activity in F-stressed safflower seedlings. F-toxicity lowered the activity of CAT by 1.9-fold, whereas the activity of other H_2O_2 detoxifying enzymatic antioxidants, i.e. APX and GPoX was enhanced by 1.9- and 1.4- folds, respectively (Table 2). Thus, in absence of sufficient CAT activity, most of the H_2O_2 generated during stress was detoxified by the other enzymatic antioxidants. Reduced CAT activity and enhanced activity of the above-mentioned peroxidases can be justified by the previous works of Kumar et al. (2009), where F-toxicity in the leaves of mulberry inhibited the activity of CAT due to the displacement of the hydroxyl group in the iron moiety of the enzyme. Additionally, they pointed out that enhanced activity of peroxidase enzymes served as an intrinsic mechanism to evade F-induced oxidative damages.

In presence of charcoal, the activity of all the above-mentioned enzymatic antioxidants, including SOD, CAT, APX, and GPoX was enhanced by 1.5-, 2.6-, 1.6-, and 1.3- folds, respectively in the stressed seedlings, in comparison to the stressed seedlings without charcoal treatment (Table 2). Thus, activated charcoal strengthened the defence machinery by not only escalating SOD and all peroxidases, but also restoring the CAT activity via preserving the active conformation of CAT during fluoride stress. Our observation is in line with the previous report of Ghassemi-Golezani and Farhangi-Abri (2019) where exogenous application of biochar induced the activity of enzymatic antioxidants in the root and leaf of F-stressed safflower plants.

3.7. Activated charcoal retained the level of nonenzymatic antioxidants

F toxicity significantly hindered the formation of nonenzymatic antioxidants by reducing the level of ascorbic acid, TPC, and carotenoids by 1.3-, 1.1-, and 2.0-folds, respectively (Table 2). A similar result was also obtained by Singh et al. (2020) where the level of the nonenzymatic antioxidants, viz., carotenoids and TPC was lowered in presence of NaF. Banerjee and Roychoudhury (2019b) showed that the level of ascorbic acid was reduced in the susceptible variety of rice seedlings, in comparison to the tolerant cultivar, which is in accordance with the present result. In addition to the scavenging of ROS generated, the nonenzymatic antioxidants also play an important role in plant development and growth by tweaking cellular processes like cell elongation, mitosis, cell death, and senescence (de Pinto and De Gara, 2004). Lower levels of carotenoids can also be supported by the previous works of Sachan and Lal (2018) where the application of fluoride stress eventually decreased the level of carotenoids in

Table 2. Osmolytes (total sugars, Pro, gly-bet, and total amino acids), activity of enzymatic antioxidants (SOD, CAT, APX and GPoX), nonenzymatic antioxidants (ascorbic acid, total phenolics, and carotenoids) and activity of glyoxalase enzymes (Gly I and Gly II) in seedlings grown in presence of 25 mg L⁻¹ NaF, either in presence or absence of 5 mg g⁻¹ activated charcoal. Data (mean value of triplicate sets) with significant difference at $p \leq 0.05$ are represented by “*”. Mean \pm SE.

	Parameters	Control	25 mg L ⁻¹ NaF	5 mg g ⁻¹ activated charcoal	25 mg L ⁻¹ NaF + 5 mg g ⁻¹ activated charcoal
Osmolytes	Total sugars (mg g ⁻¹ FW)	6.2 \pm 1.0	10.8 \pm 1.2*	12.6 \pm 1.5	19.5 \pm 1.2*
	Pro (μ g g ⁻¹ FW)	31.2 \pm 4.0	54.5 \pm 4.6*	45.4 \pm 3.8	75.3 \pm 4.0*
	Gly-bet (mg g ⁻¹ FW)	72.4 \pm 5.1	135.8 \pm 5.9*	131.3 \pm 4.8	184.8 \pm 4.4*
	Amino acids (mg g ⁻¹ FW)	5.4 \pm 0.2	6.1 \pm 0.3*	5.0 \pm 0.5	7.9 \pm 0.2*
Activity of enzymatic antioxidants	SOD (U mg ⁻¹ protein)	4.1 \pm 0.4	8.0 \pm 0.4*	5.7 \pm 0.5	12.1 \pm 0.2*
	CAT (μ M H ₂ O ₂ decomposed min ⁻¹ mg ⁻¹ protein)	8.7 \pm 1.0	4.5 \pm 1.2*	10.2 \pm 0.7	11.9 \pm 0.6
	APX (μ M ascorbate oxidized min ⁻¹ mg ⁻¹ protein)	105.6 \pm 12.4	203.6 \pm 8.7*	124.1 \pm 9.8	328.6 \pm 6.2*
	GPoX (μ M tetraguaiacol formed min ⁻¹ mg ⁻¹ protein)	80.1 \pm 3.6	115.0 \pm 5.6*	47.6 \pm 5.2	148.2 \pm 4.1*
Nonenzymatic antioxidants	Ascorbic acid (μ g g ⁻¹ FW)	13.6 \pm 0.8	10.5 \pm 1.1*	12.3 \pm 0.5	21.4 \pm 1.1*
	TPC (μ g g ⁻¹ FW)	43.7 \pm 4.6	39.5 \pm 4.8*	77.7 \pm 2.7*	84.1 \pm 3.1
	Carotenoids (μ M g ⁻¹ FW)	5.5 \pm 0.4	2.7 \pm 0.8*	6.3 \pm 1.1	7.7 \pm 1.2
Activity of glyoxalase enzyme	Gly I (μ M SLG formed min ⁻¹ mg ⁻¹ protein)	140.2 \pm 9.7	217.1 \pm 9.8*	130.8 \pm 4.2	319.7 \pm 11.3*
	Gly II (μ M GSH formed min ⁻¹ mg ⁻¹ protein)	12.7 \pm 0.7	19.7 \pm 1.0*	14.0 \pm 0.8	35.0 \pm 1.4*

barley seedlings resulting in higher chlorophyll damage. Yuan et al. (2010) stated that salt stress diminished the level of TPC in 5 and 7-day old radish seedlings which compromised their tolerance capability. Such observation is also concurrent with our observation of reduced TPC level in F-stressed rice seedlings.

In presence of charcoal, the concentration of ascorbic acid, TPC and carotenoids was enhanced by 2.0-, 2.1-, and 2.8- folds, respectively, as compared to NaF-treated

seedling which provided better assistance in the scavenging of ROS, enhancing the tolerance capability of F-stressed seedlings (Table 2). Ghassemi-Golezani and Farhangi-Abriz (2019) observed that the exogenous application of biochar allowed restoration in the level of nonenzymatic antioxidants which is in line with our observation. This established the intrinsic capacity of activated charcoal as a potent agent in mitigating F toxicity via the modulation of ascorbic acid, TPC, and carotenoids during fluoride stress.

3.8. Charcoal triggered the activity of the enzymes of glyoxalase system

In NaF-treated seedlings, the activity of both Gly I and Gly II was up regulated by 1.5-fold in comparison to that of control seedlings which can be regarded as an adaptive strategy to restrict the accumulation of endogenous MG within the tissues (Table 2). Higher Gly activity in rice seedlings can be justified by the previous research of Singh and Roychoudhury (2020), showing enhancement in the activity of enzymes comprising the glyoxalase system in NaF-treated seedlings.

Treatment of seedlings with NaF solution in presence of activated charcoal further escalated the activity of Gly I and Gly II by 1.4- and 1.7- folds, allowing more efficient detoxification of the accumulated MG in seedlings. Earlier demonstration by Hasanuzzaman et al. (2019), showing

up regulation of Gly I and Gly II activity via exogenous Si application during nickel-induced oxidative stress in rice, is in congruence with the present result. Additionally, the higher activity of glyoxalase enzyme can be justified by the fact that Gly I and Gly II largely regulate the level of GSH in plant tissues which is the major component of various antioxidative enzymes such as APX that in turn lowers the level of ROS (Yadav et al., 2008).

3.9. Charcoal-induced lowering of ROS, O₂⁻, and cellular necrosis in leaves

Localization studies led to the fact that extensive accumulation of ROS and O₂⁻ along with cell necrosis occurred in the leaves of NaF-treated seedlings (Figure 4). Yadu et al. (2016) stated earlier that higher deposition of F⁻ ions led to the induced formation of ROS via Mehler pathway that eventually triggered other abnormalities

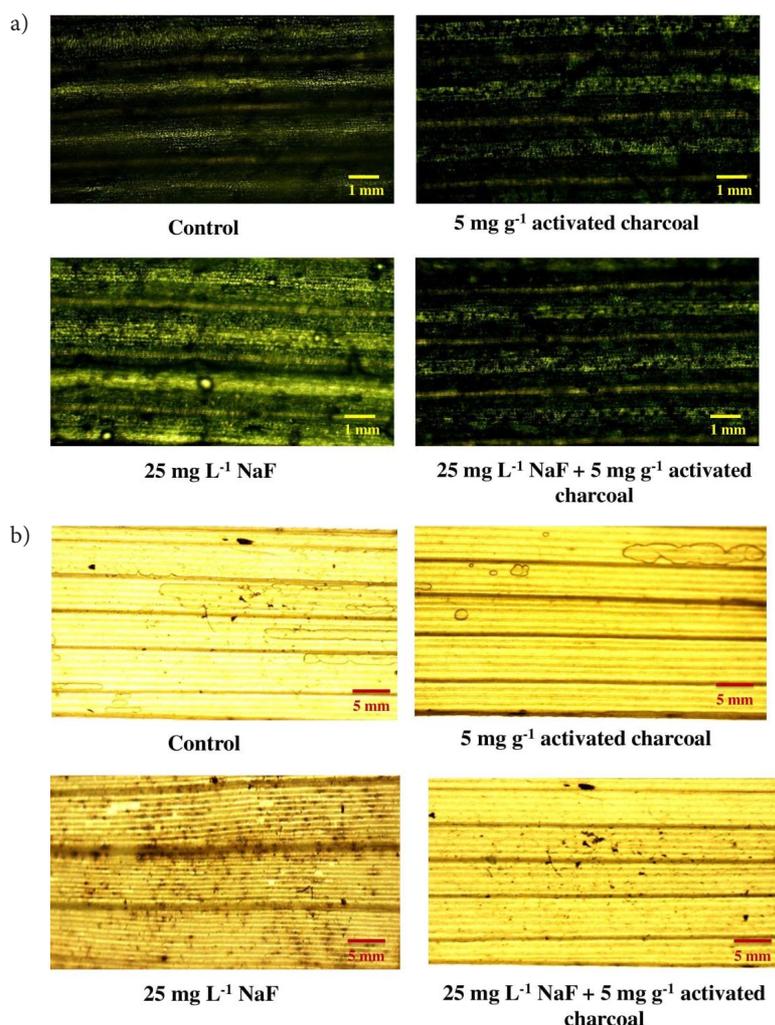


Figure 4. Localization of ROS (a) and O₂⁻ (b), and cellular necrosis (c) in the leaves of rice seedlings grown in presence of 25 mg L⁻¹ NaF, either in absence or presence of 5 mg g⁻¹ activated charcoal.

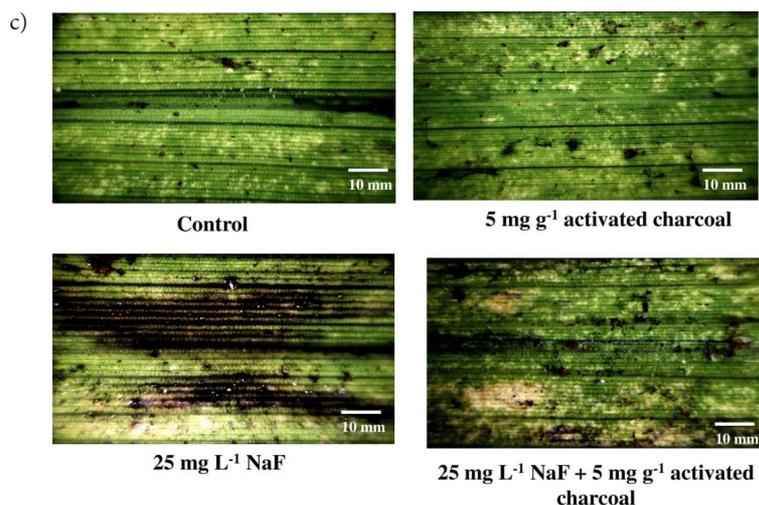


Figure 4. (Continued).

including cellular necrosis. The development of large necrotic patches in the stressed leaves can be justified by the higher formation of ROS due to the substantial accumulation of F⁻ ions. However, in presence of activated charcoal, the ROS and superoxide generation in the leaves was significantly reduced that prevented cell death, thereby enhancing the tolerance level of the seedlings. Our observation can be justified by the recent work of Banerjee et al. (2021) where lesser necrotic patches along with the reduced accumulation of ROS were noted in the leaves of the tolerant rice cultivars, in comparison to the susceptible varieties. Our observation can also be justified by the earlier work of Sharma and Kaur (2019) where they demonstrated higher accumulation of propidium iodide and oxidation of dichlorodihydro fluorescein diacetate-acetyl-ester in the roots of NaF-exposed *Spirodela polyrhiza* that signified higher cell death and ROS accumulation, respectively in the roots of stressed plants, in comparison to the control plants. Additionally, higher induction of ROS and superoxide ions in the leaves of the salt-sensitive rice cultivars was reported by Kaur et al. (2016). They further noted that the level of the above-mentioned cytotoxic radicals was lowered in the tolerant variety.

4. Conclusion

The actual aim of this work was to demonstrate the efficacy of activated charcoal in ameliorating the toxic effect of F-induced damages in rice seedlings. The level of F in the agricultural field is increasing at an alarming rate, so it is necessary to manage this emerging xenobiotic pollutant and come up with an effective technique that will maintain the growth and development of rice in fluoride-contaminated soil. The present investigation showed that charcoal treatment can be efficiently used for lowering the accumulation of F⁻ ions in the tissue biomass, since the surface pores act as potent adsorbents that eventually

retain F⁻ ions in the soil only, preventing uptake in rice plants. Exogenous application of charcoal also maintained the growth and development of seedlings by preserving the chlorophyll level and inhibiting the formation of ROS that protected the cell membrane from electrolyte leakage and lipid peroxidation, as evident from lowered MDA levels. The cytotoxic metabolite, MG was also detoxified due to up regulated Gly I and Gly II activity. Activated charcoal also served as an efficient carbon source that rescued the activity of the enzymes of Krebs' cycle during fluoride stress which was necessary to meet the energy demand of the plants for higher tolerance level. The induction of the protective metabolites, viz., enzymatic and nonenzymatic antioxidants and osmolytes, was well-programmed and they acted in concert to scavenge the harmful adducts formed in the cells, and preserve the integrity of cell membrane for maintaining the cellular osmotic balance. Taken together, our work proved the efficacy of activated charcoal treatment in abrogating the negative effects of F-induced oxidative damage in rice and also inhibiting the transportation of F⁻ ions via roots in the aerial parts. Although this research was performed at the laboratory level, it highlights the importance of activated charcoal administration in field condition as an inexpensive and environment-friendly strategy to mitigate F toxicity in rice, improving overall growth and productivity.

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Author contributions

AS performed all the experiments and drafted the manuscript. SC served as technician for this work. AR supervised the entire work, critically analyzed all data and made necessary changes within the manuscript. Both the authors reviewed the manuscript.

Consent for publication

Not applicable

Declarations

The authors declare no competing interests.

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