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Biopriming of *Pseudomonas aeruginosa* Abates Fluoride Toxicity in *Oryza sativa* L. by Restricting Fluoride Accumulation, Enhancing Antioxidative System and, Boosting Activities of Rhizospheric Enzymes

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Biopriming of *Pseudomonas aeruginosa* Abates Fluoride Toxicity in *Oryza sativa* L. by Restricting Fluoride Accumulation, Enhancing Antioxidative System and, Boosting Activities of Rhizospheric Enzymes

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Abstract: Plant-growth promoting bacteria (PGPB) are free-living microorganisms that actively reside in the rhizosphere and affects plants growth and development. These bacteria employ their own metabolic system to fix nitrogen, solubilize phosphate, and secrete hormones to directly impact metabolism of plants. Gaining a sustainable agricultural production under various environmental stresses requires a detailed understanding of mechanisms that bacteria use to promote plants growth. In the present study, *Pseudomonas aeruginosa* (MW843625), a PGP soil bacterium with a minimum inhibitory concentration (MIC) of 150 mM against fluoride (F) was isolated from agricultural fields of Chhattisgarh, India, and was assessed for remedial and PGP potential. This study concentrated on biomass accumulation, nutrient absorption, and oxidative stress tolerance in plants involving antioxidative enzymes. By determining MDA accumulation and ROS ($O_2^{\cdot-}$ and H_2O_2) in *Oryza sativa* L. under F (50 ppm) stress, oxidative stress tolerance was assessed. The results showed that inoculation with *P. aeruginosa* enhanced the ability of *Oryza sativa* L. seedlings to absorb nutrients, and increased the amounts of total chlorophyll (Chl), total soluble protein, and biomass. In contrast to plants cultivated under F-stress alone, those inoculated with *P. aeruginosa* along with F showed considerably reduced concentration of F in their roots, shoots, and grains. The alleviation of deleterious effects of F-stress on plants owing to *P. aeruginosa* inoculation has been associated with improved activity/ up-regulation of antioxidative genes (SOD, CAT, and APX) in comparison to only F subjected plants, which resulted in lower $O_2^{\cdot-}$, H_2O_2, and MDA content. Additionally, it has also been reflected from our study that *P. aeruginosa* has the potential to increase the activities of soil enzymes such as urease, phosphatase, dehydrogenase, nitrate reductase and cellulase. Accordingly, the findings of the conducted study suggests that *P. aeruginosa* can be exploited not only as an ideal candidate for bioremediation but also enhancing soil fertility and promotion of growth and development of *Oryza sativa* L. under F contamination.

Keywords: antioxidative defense; fluoride; plant growth promoting bacteria (PGPB); *Oryza sativa* L.; *Pseudomonas aeruginosa*; reactive oxygen species (ROS)

1. Introduction

Fluorine is 13th on the list of nature's most abundant elements and occurs mainly in the form of fluoride (F) compounds within the Earth's crust [1]. Fluoride enters *via* both natural as well as geogenic means in the food chain and acts as a toxic xenobiotic causing serious threat to the biotic components of the ecosystem [2]. The widespread use of groundwater has caused the water

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table to decrease significantly leading to extraction of water from deep bored pipes which results in the release of water containing F contaminants that seep from the mineral-beds and contributes towards endemic fluorosis in many regions occupied for agricultural purposes **[1,3**]. The factors responsible for F leaching from mineral beds include weathering of rocks owing to variation in the climate and ion exchange reactions [4]. Extensive extraction of minerals rich in F such as fluorite, apatite and cryolite has significantly contaminated the groundwater which is the principal source of irrigating food crops and eventually leads to F accumulation, growth retardation and loss of productivity [1]. India, including other Asian countries like China, Bangladesh, Pakistan, *etc*., is the chief cultivator of *Oryza sativa* L., and has been currently recognized as the hotspot of F contamination [5]. The World Health Organization (WHO) has warned against using F beyond the threshold limit of 1.5 mg L^{-1}. Ingestion of F above this limit has been widely revealed to cause severe fluorosis, including neurological diseases in living beings [6]. Considering plants, F toxicity impedes in water and minerals transportation which are essential requirements for their normal physiological and biochemical activities [7]. Fluoride is absorbed *via* vacuolar voltage gated chloride channels (CLC1 and CLC2) present in the roots and translocated by xylematic flow [1]. Prolong exposure of F to the plants results in several biochemical, physiological, and molecular alterations which could be acute or severe.

Fluoride negatively impacts metabolic activities, nutrients intake, germination potential, growth and development, photosynthesis, carbohydrate metabolism, respiration, nitrogen assimilation, protein synthesis, enzymes activities, and gene expression eventually leading to oxidative stress in plants [1]. Oxidative stress, following the overproduction of reactive oxygen species (ROS) *viz*.; hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radical ($OH^{\cdot}$), engenders membrane instability/ damage, accompanying release of several cytotoxic products [*eg*. malondialdehyde (MDA)] in the affected tissues. Stressed conditions in plants also accelerate protease activity which in turn causes protein inactivation and proteolysis [8]. Various enzymes associated with Krebs cycle such as succinate dehydrogenase, pyruvate dehydrogenase, and malate dehydrogenase; and enzymes involved in nitrogen acquisition; nitrate reductase, *etc*., are severely inhibited due to the F-stress in vulnerable varieties [9]. Plants possess strictly controlled antioxidative machinery to shield themselves from these extremely dangerous oxygen intermediates, regulating ROS generation and scavenging to prevent cellular damage [10]. The antioxidant defense system includes both enzymatic and non-enzymatic substances. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and several other enzymatic components regulate equilibrium of ROS in living beings [11,12]. The non-enzymatic elements, which are usually made up of lipids, phenolic compounds, ascorbic acid, tocopherol, flavonoids, proline, carotenoids, and glutathion, efficiently reduce oxidative damage by either working with the enzymatic players to achieve effective antioxidant defense through the consumption of H_2O_2 or by acting independently of them [10].

Several methods have previously been suggested to reduce F toxicity which involves exogenous application of chemicals like spermidine, melatonin, *etc*. [7]. Utilization of melatonin limited F uptake in *Oryza sativa* L. seedlings and lowered the damage caused due to it by restoring the defense machinery, and altering homeostasis of hormones [1]. Calcium compounds such as calcium hydroxide [$Ca(OH)_2$], calcium nitrate [$Ca(NO_3)_2$], and calcium chloride ($CaCl_2$) have also been incorporated as seed priming agents for the mitigation of F toxicity thereby enhanced germination percentage, root-shoot length, biomass, impeded Chl degradation and electrolyte leakage; and minimized MDA concentration and ROS [3]. Sodium nitroprusside (SNP) has also been deemed to be effective for reducing F toxicity in *Vigna radiata* L. and *Vigna mungo* L. by narrowing F uptake, rescuing membrane damage, and antioxidant enzyme activity thereby alleviating oxidative stress along with increased plant growth [13]. Likewise, supplementation of biochar with soil (50 g/kg soil) has also been considered as the remedy for minimizing toxicity of F in *Carthamus tinctorius* L. [14]. These days, nanotechnology has become potential tool in agriculture because it permits sustainable cultivation [15]. Pulsing silicon nanoparticles exogenously stimulated growth and also assisted in alleviating molecular injuries and production loss in *Oryza sativa* L. which were resulted due to the F toxicity [3].

In addition to above mentioned ameliorative methods, much attention has been given to bioremediation that involves living organisms or enzymes to detoxicate environment from various contaminants [16]. Although, rather slow and time-consuming, it outperforms traditional chemical treatments and, most importantly, maintains soil fertility [17]. Contamination of F in the ecosystem generates selective pressure for the advent of tolerant resistant bacteria against it. These tolerant strains can be used to inhibit uptake of F by plants by altering reactivity and mobility of F [18]. Bacterial remediation of F from contaminated areas can be made possible by utilizing indigenous or genetically modified microorganisms for environmental protection [19,20]. Bacterial resistance against F can be transient due to the horizontal gene transfer among two cells or stable because of chromosomal aberrations. Fluoride interacts with proton and forms hydrogen fluoride (HF), which gets absorbed inside the bacterial cells through diffusion and dissociates into H^+ and F^- ions [16]. These ions hinder activities of enzymes associated with glycolysis and F-ATPases, which in case of F-resistant bacteria are expected to be got mutated [21]. Furthermore, the cell wall of bacteria is composed of phosphate, sulfhydryl, and amine groups that can reduce F intake efficiently and thus help in its adherence to their surfaces [19,20,22]. Several other processes are also employed by bacteria like bioaccumulation, biosorption, and release of ligands like siderophores or biosurfactants to minimize F toxicity and its transport into the cells thereby affecting its availability in the environment. Plant growth promoting bacteria (PGPB) are essential for sustainable agriculture because they increase soil fertility, reduce pathogen growth and infection, boost biodiversity, and increase crop productivity [23]. Plant growth promoting bacteria can enhance the plant's rhizospheric environment by influencing soil enzyme activity. Fan et al. [24] highlighted the significant contribution of PGPB in sustaining the soil element cycle and crop yields. Soil microbial communities are essential for maintaining and supporting soil ecosystems. They serve as a vital connection between the aboveground and underground parts of plants in soil ecosystems [25]. Soil enzymatic activity is frequently utilized as a measure of overall soil microbial activity and fertility, as well as a potential indicator for assessing soil stress levels [26]. Several studies have reported the positive impact of PGPB on soil enzymatic activities. Based on their ability to increase plant output through rhizosphere management, numerous strains of beneficial soil bacteria have been identified and are currently being utilized in the biotechnology to enhance agricultural sustainability and food security [27]. Several bacterial strains *viz.*; *Bacillus subtilis, B. amyloliquefaciens, B. licheniformis, B. megaterium, Paenibacillus mucilaginosus, Pseudomonas fluorescens*, *P. putida*, *P. fluorescens*, *P. striata*, *P. azotoformans, P. chlororaphis, P. fluorescens, etc*., are employed as biofertilizer products in India, China, Vietnam, Cuba, Sweden, Sri Lanka, and many other countries [28]. *Pseudomonas*, a member of Proteobacteria, houses the most diverse group of bacteria having hundreds of different species [29]. They are the most researched bacterial species due to their presence in diverse environments including marine, freshwater, and terrestrial ecosystems, and closest association with higher life forms [30]. Prolific presence of *Pseudomonas* in the rhizosphere, and their remarkable growth promoting features like production of enzymes, metabolites, siderophores, phytohormones; better colonization over roots; solubilization of nutrients; and function as biocontrol agent makes them notable PGPB [31,32]. Thus, the current study aims at evaluation of plant growth promoting (PGP) properties of *P. aeruginosa* (MW843625), isolated from the F contaminated soil of agricultural fields, and its role in minimizing deleterious effects of F in *Oryza sativa L.* seedlings by decreasing the oxidative injuries and boosting activities of soil enzymes.

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2. Results and Discussion

2.1. Fluoride Resistance and Removal

The isolated bacterium identified as *P. aeruginosa* (Figure 1) revealed resistance to relatively higher concentrations of F as MIC of it was seen to be 200 mM. The resistance of *P. aeruginosa* to F has also been shown by Chouhan et al. [62], and Edward Raja et al. [63,64]. This bacterium has lowered down F content of growth media to a significant level. Maximum (89.66%) removal of F was achieved with the concentration of 60 mM, and this efficiency gradually declined with increase in the concentration of F (Figure 2). The most plausible explanation for this is that at lower concentrations, capacity of the bacterium for absorption of F is not fully utilized. However, as the concentration increases, removal efficiency considerably decreases, probably as a result of saturation and less availability of sorption sites on the bacterial surface [19,20]. Within bacterial cells, there are various mechanisms that provide tolerance, such as efflux pumps, intracellular sequestration, and detoxifying enzymes. In addition, an increase in the DNA repair mechanisms, the presence of F riboswitches, ion antiporters/transporters, and genetic mutations also play a role towards improving F resistance in bacterial cells [65]. Other organisms like *Staphylococcus lentus* and *Providencia vermicola* (KX941098) have also been reported which responded similarly towards removal of F from the media amended with different concentrations of it [19,20].

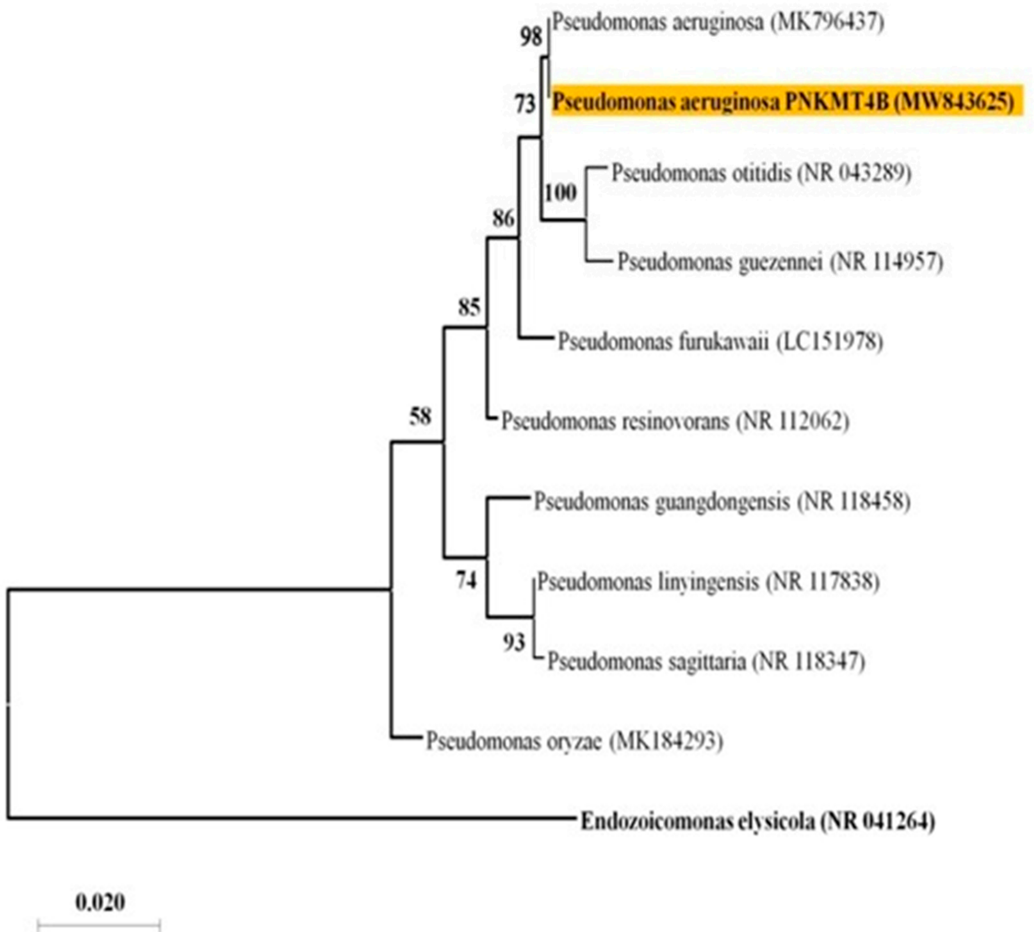

Figure 1. Phylogenetic tree of *P. aeruginosa* (MW843625) based on 16S rDNA.

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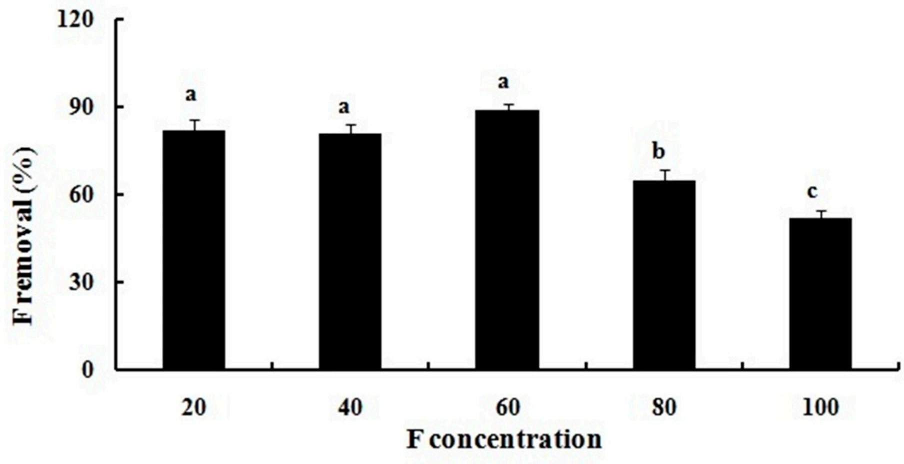

Figure 2. Percentage removal of fluoride by *P.aeruginosa* from growth media supplemented with different concentrations of fluoride.

2.2. Fluoride Biosorption Potential of P. aeruginosa

Surface topography of treated and untreated *P. aeruginosa* biomass was depicted in the SEM micrographs (Figures 3A i and ii). Due to the uptake of F and its adhesion on the bacterial surface, significant morphological alterations and discrete aggregates were documented. Surface of the F treated *P. aeruginosa* appeared comparatively thicker than the untreated biomass which was found to be porous. Further, surface distortion and lump formation were clearer in the F treated bacterial cells. Mukherjee et al. Thesai et al. and Shanker et al. [19,66,67] showed similar changes in the surface topographies of *Providencia vermicola* (KX926492), *Bacillus flexus* (KX646392), and *Acinetobacter* sp. (GU566361), respectively after F sorption. The EDX analysis also supported presence of F in the treated cells, which is marked by the F peak in the graph (Figures 3B i and ii). The presence of positive ions like sodium, magnesium, and calcium in the bacterial cells could be the cause of this uptake/ sorption of F [19].

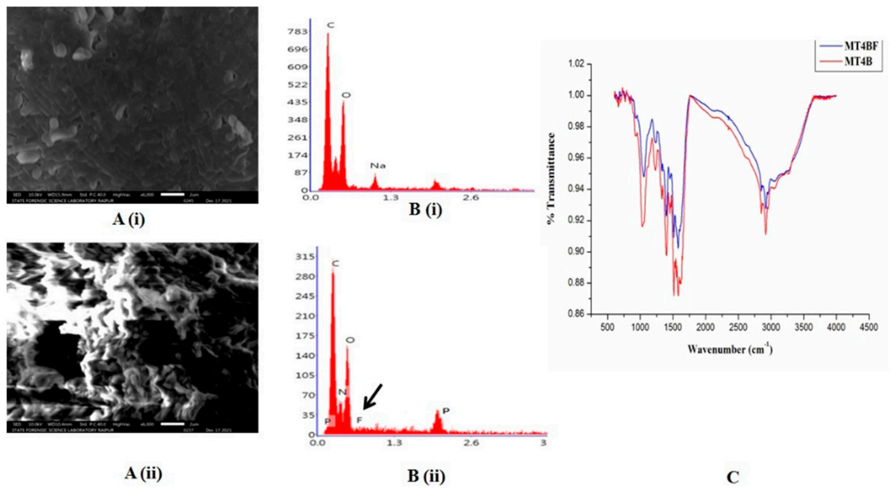

Figure 3. (A) Scanning electron microscopic images of *P. aeruginosa* (i) the untreated cells having non-significant structural alterations, (ii) F treated cells showing severe structural distortion; (B) Energy dispersive X-ray analysis of *P. aeruginosa* (i) absence of F peak in the untreated cells, (ii) presence of F peak confirms its uptake by the treated cells; (C) Fourier transform infrared spectra of untreated and treated *P. aeruginosa*.

Fourier transform infrared spectroscopy has been used to portray structural changes in the bacterial cells through identification of functional groups responsible for binding of F to their membranes (Figure 3C). Maximum stretching of a peak around 3300 cm^{-1} represented the O–H bond.

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While, sharper peaks at 1100 cm^{-1}, 1400 cm^{-1}, 1600 cm^{-1}, and 2900 cm^{-1} represented the primary amines (amine N-H stretch). Bands in the range of 1000-800 cm^{-1} related to the C-O-C and C-O-P stretching, suggested the presence of oligosaccharides and polysaccharides in the bacterial biomass [19]. It has been found that F was absorbed by the bacteria and gets accumulated once it enters inside the cell. Failure to synthesize any compound of metabolic pathways might be the possible explanation for insignificant shift in the transmittance of the raw and F loaded *P. aeruginosa* [19].

2.3. Plant Growth Promoting Attributes

The *in vitro* study of PGP traits of F-resistant *P. aeruginosa* displayed numerous characteristics of them, both with or without F (Table 1). Bacteria found in the soil may assist plants to grow better even under stress condition by increasing nutrient delivery, encouraging plants growth by synthesizing hormones like IAA, solubilizing inorganic P, accumulating or leaching metals, and reducing pathogen activity [68]. Usually, a bacterium has an immediate impact on growth and development of a plant by employing one or more of these processes [69]. One of these traits is the auxin (IAA), secreted by the PGPB which has a number of direct effects on growth and development of plants under stressful conditions [70,71]. The main effect of bacterially produced IAA is improvement in the development of lateral and adventitious roots, which leads to a rise in the absorption of minerals and nutrients [72,73]. Similar findings in concordance to the present results have been reported for different species of *Pseudomonas* that produced IAA and supported root growth in *Triticum aestivum* L. [74,75], *Brassica napus* L. [70], and *Medicago truncatula* L. [76] under stresses.

A sizeable amount of P available in the soil is inaccessible to plants because of its insoluble state. The action of soil microorganisms may help to improve this situation. Approximately, 1-50% soil dwelling bacteria are phosphate-solubilizing and can dissolve inorganic phosphates by producing organic acids, siderophores, and hydroxide ions [77,78]. According to Jiang et al. [79], phosphate solubilizing activity of *P. aeruginosa* boosted P availability in rooting media, which resulted in higher phosphate content in the inoculated *Oryza sativa* L. Furthermore, siderophores, which are chelating agents, enable bacteria to get beyond the nutritional iron (Fe) limitation in plants due to their high specificity and affinity for binding Fe^{3+} [80]. These bacterial siderophores foster plant growth by improving nutrition and preventing development of phytopathogens by sequestration of Fe from the environment [81–83].

It has also been demonstrated that the ammonia produced by the PGPB supplies N to their host plants, hence; encouraging root and shoot elongation and biomass production. Further, HCN acts as a biocontrol agent due to its ascribed toxicity against plant pathogen [84,85]. It has previously been shown that a wide variety of bacterial species, including *Alcaligenes, Aeromonas, Bacillus, Pseudomonas,* and *Rhizobium*, are capable of generating ammonia and HCN in sizable proportions [86].

Table 1. Plant growth promoting activities of *P. aeruginosa* both in absence and presence of fluoride. Data presented are means of 3 replicates ± SD.

PGPR traits	Without F	With F (60 mM)
Ammonia production (µg mL^{-1})	4.9^{a} ± 0.9	4.1^{a} ± 1.1
HCN production	+	+
Phosphate solubilisation (µg mL^{-1})	44.93^{a} ± 1.3	44.53^{a} ± 1.6
Siderophore production index	1.10^{a} ± 0.1	1.23^{a} ± 0.2
IAA production (µg mL^{-1})	18.60^{a} ± 0.62	15.95^{b} ± 0.52
Exopolysaccharide production (µg mL^{-1})	17^{b} ± 1.04	24^{a} ± 1.1

2.4. Rhizosphere Colonization of *P. aeruginosa*

Fluoride tolerant *P. aeruginosa* was investigated for their potency to colonize in the *Oryza sativa* L. rhizosphere. Bacteria was found to be present (4.2×10^4 CFU g^{-1}) in the inoculated soil sample but not in the control and F added soil, demonstrating successful colonization in the rhizospheric region. Similar results have also been published by Islam et al. [75] regarding *P. aeruginosa* and *Triticum aestivum* L.

2.5. Soil Enzymes

Soil enzymes activities are very sensitive to any xenobiotic pollution which indirectly reflects the ability of soil microbial populations to restore soils health [87]. The differences of soil enzymes activities in each treatment were compared and are shown in Table 2. Activities of soil urease, nitrate reductase, phosphatase, cellulase and dehydrogenase were estimated before the transfer of plantlets in the earthen pots and were calculated as 96.42 ± 2.04 μg N-NH_4^+ g^{-1} dw h^{-1}, 1.34 ± 0.06 μg NO_2^--N g^{-1} dw h^{-1}, 716.2 ± 24.3 μg pNP g^{-1} dw h^{-1}, 13.51 ± 1.39 μg D-Glu g^{-1} dw h^{-1}, and 65.34 ± 2.06 μg TPF g^{-1} dw h^{-1} respectively. Activities of these enzymes were again calculated after the harvesting of crop, and was found that in the F treated pots activities of all these enzymes were got reduced (Table 2). Several studies have documented the impact of F, usually in the form of NaF, on soil microbial activity and community composition [88,89]. Wilke [90] demonstrated alterations in the chemical characteristics and microbiological activities in the F-adulterated humus soil and concluded that nitrite reductase, alkaline phosphatase, dehydrogenase, and arylsulfatase activities were suppressed after F addition in the soils. Reddy and Kaur [91] provided more evidence that F inhibits activities of ATPase and soil peroxidase. Similarly, Rao and Pal [92] measured detrimental effects of F on soil microorganisms, reporting that high concentrations (380-1803 mg g^{-1} soil) of F impeded microbial growth and enzyme activity as well as breakdown of organic materials. Fluoride concentrations of 200-2000 mg g^{-1} soil have been shown to decrease denitrification, whereas; F values below 200 mg g^{-1} have been shown to limit soil respiration and dehydrogenase activity [93].

Data gathered after *P. aeruginosa* treatment showed significant improvement in the soil enzymes in both with and without F stressed conditions (Table 2). Under F stressed and bacterium inoculated condition, urease activity increased by 36%, nitrate reductase activity by 57%, phosphatase activity by 14%, cellulase activity by 41.6%, and dehydrogenase activity by 50% over the only F treated soil. Bacterial assisted remediation stimulates plant growth while simultaneously improves soil quality [94]. Yu et al. [87] observed that inoculation of *Pseudomonas* sp. GHD-4 increased bacterial diversity, decreased lead (Pb) concentration, and enhanced soil enzymes activities in the soil. According to Hidri et al. [95], inoculation of *Bacillus subtilis* enhanced soil quality by increasing activities of soil enzymes such as urease, alkaline phosphatase, β-glucosidase, and dehydrogenase when compared to the uninoculated control. Additionally, Marques et al. [96] determined that as zinc and cadmium levels increased, diversity of bacterial community decreased. In contrast, *Helianthus annuus*, inoculated with PGPR strains in the rhizosphere maintained a greater diversity of bacteria throughout the experimental period.

2.6. Plant Growth and MSI

Root length, SL, FM, DM, and MSI were remarkably enhanced in the *P. aeruginosa* inoculated *Oryza sativa* L. than the positive control plants under non-F treated condition. Fluoride treatment markedly reduced the RL (47.82%), SL (62.56%), FM (root: 50.4%, shoot: 40.4%), DM (root: 43.8%, shoot: 38%), and MSI (31.18%) of *Oryza sativa* L. (Table 2). This might be because the developing tissues were absorbed inadequate amounts of nutrients. Membrane instability brought on by exposure of F affects lipid-protein interactions as well as functions of enzymes like H^+-ATPase [97]. Many other plant species also exhibited similar decrease in growth metrics [97–99]. Furthermore, F exposure makes the plasma membrane prone to lipid peroxidation and cytoskeleton instability, lowering MSI, thereby increasing electrolyte leakage from cells [100–102]. In a study conducted by

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Pelc et al. [103], an increase in F concentration inhibited seedlings growth in all the winter wheat cultivars. Further, Chahine et al. [104] also observed a decline in growth of Jesca (an African-Tanzania bean variety) when was exposed to elevated levels of F. However, in *P. aeruginosa* inoculated plants, RL and SL were improved by 29% and 48.3%, respectively as compared to those noted with F stressed seedlings. Similarly, it also increased FM (root: 30%, shoot: 23%), DM (root: 28%, shoot: 10%), and MSI (23%) as compared to F alone treated plants (Table 2).

Under stress conditions, PGPR can improve plant nutrition generally by employing one or more mechanisms like associatively fixing nitrogen, solubilizing phosphate, or producing siderophores, and by producing hormones or participating in enzymatic processes, that can encourage growth of rhizobial or mycorrhizal symbiosis, which in turn can improve plant growth and development [65,75,105,106]. The hormones released by the PGPB have a wide range of direct impacts on plant growth under variety of abiotic stresses [71]. Several researchers have previously reported matching findings of plant growth in response to bacterial inoculation along with different metal stress [18,34,74,107,108].

2.7. Total Chlorophyll

Fluoride stress in *Oryza sativa* L. seedlings caused a significant reduction (50%) in the total Chl content, while *P. aeruginosa* inoculation in the unstressed seedlings increased it in comparison to the control. Content of total Chl in the leaves is directly related with photosynthetic activity which occurs to reduce under abiotic stresses [109]. Chlorosis, inhibition in Hill activity, and decreased accumulation of photosynthetic pigments were previously being seen in the seedlings irrigated with F rich water [110,111]. However, inoculation of *P. aeruginosa* enhanced the total Chl content by 29% compared to those estimated in the F alone administered ones (Table 2). The higher total Chl content in response to *P. aeruginosa* treatment might be due to the boosted uptake of mineral nutrition [78]. In line, Wang et al., Islam et al., Elekhtyar, Samaniego-Gámez et al., Zhang et al., and Abd El-Mageed et al. also noted increased Chl content in plants treated with PGPB as compared to untreated ones under diverse abiotic stresses [75,109,112–115].

2.8. Fluoride Accumulation in the Tissues

Fluoride treated seedlings accrued 30 ± 3.65, 22 ± 2.75, 9 ± 1.54, and 2.9 ± 0.12 ppm F g^{-1} DM in roots, shoot, leaves and grains, respectively (Table 2). Roots, being in direct contact with the soil accumulated significantly more amount of F followed by shoot, leaves, and grains. Such alarming hike of xenobiotics in the crops can be a menace to human health. Interestingly, inoculating *P. aeruginosa* with F stressed seedlings caused a substantial reduction in its accumulation by 33%, 35%, 47%, and 74% in roots, shoots, leaves, and grains respectively. The sorption of F by the bacterium might be the cause of lesser accumulation of it in the tissues. Presence of cells of *P. aeruginosa;* at 4.2 × 10^4 CFU g^{-1} soil in the suspension of the inoculated *Oryza sativa* L. demonstrated that successful colonization of the bacteria probably caused reduced uptake of F in the tissues [116]. Similar decreases in the accumulation of toxic elements like As and Zn in *Vigna radiata* L., *Oryza sativa* L. and *Zea mays* L., respectively was reported by Pandey and Bhatt, Pandey et al. and Jain et al. [18,34,108].

2.9. Agronomical Attributes

Exposure of F inhibited process of reproduction, and development of panicles in the seedlings of *Oryza sativa* L. The PL, number of spikelet's per panicle, FG per panicle, GL, GB, and HGW of grains were significantly decreased by 34%, 51%, 72%, 37%, 40%, and 23%, respectively, while EG per panicle was increased by 86% in response to F when compared to control.

Fluoride inhibited grain hardening owing to its accumulation in the spikelet's [3]. On the other hand, when *P. aeruginosa* was added, all these agronomical parameters of the F stressed seedlings were nearly restored to control levels (Table 2). This could be due to the growth promotive effect of *P. aeruginosa* and also because of the reduced uptake of F in its presence. The

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results are in consistent with those of Khalid et al., Kumar et al., Amogou et al. , and Sedri et al. [117–120], who have discovered an upsurge in the grain production when various PGPRs were inoculated in the *Triticum aestivum* L. and *Zea mays* L.

2.10. Contents of Protein, Total Sugar, Zinc, and Iron in the Grains

The amounts of protein, total sugar, Fe, and Zn in the grains of F supplied seedlings were lowered by 1.8%, 30%, 22%, and 36% respectively (Table 2) than that determined in the grains of control plants. However, their contents were maintained up to the levels determined in the control tissues after being inoculated with *P. aeruginosa.* Plant growth promoting bacteria has been demonstrated to enhance exchange and uptake of mineral nutrients (both macro and micro). A change in the pH of the rhizospehric soil by organic acids, metal chelation by siderophores, and cycling of nutrients by the process of microbial mineralization probably boosts the availability of nutrients to plants [109,121].

Table 2. Comparative analyses of basic physiological parameters, F accumulation in tissues, yield attributes, ROS production, and antioxidants in *Oryza sativa* L. seedlings grown in DW *i.e.* Control, *P. aeruginosa* treated soil, F added soil, and *P. aeruginosa* + F added soil. Data presented are means of 3 replicates ± SD.

Traits	Parameters	Control	*P. aeruginosa*	F (50ppm)	F + *P. aeruginosa*
Soil enzyme activity (Post-harvest soil samples)	Urease (µg N-NH_4^+ g^{-1} dw h^{-1})	98.60^{c}± 1.18	129.88^{a}±1.38	67.55^{d}±0.60	105.88^{b}±0.99
	Nitrate reductase (µg N-NO_2^- g^{-1} dw h^{-1})	1.826^{c}±0.063	2.401^{a}±0.078	0.487^{d}±0.06	1.139^{b}±0.067
	Phophatase (µg pNPg^{-1} dw h^{-1})	721.4^{b}±20.3	795.6^{a}±19.5	621.6^{c}±22.3	726.4^{b}±21.6
	Cellulase (µg D-Glu g^{-1}dw h^{-1})	15.78^{b}±1.09	20.87^{a}±1.00	8.3^{c}±0.66	14.23^{b}±0.77
	Dehydrogense (µg TPF g^{-1} dw h^{-1})	68.45^{b}±4.11	96.65^{a}±3.26	26.76^{d}±2.26	54.55^{c}±2.65
Basic Physiological parameters	Root length (cm)	23.4^{a} ± 1.81	25.2^{a} ± 1.01	12^{c}± 1.87	17.4^{b} ± 2.07
	Shoot length (cm)	82.8^{a} ± 6.05	4.8^{a} ± 4.03	31^{c}± 2.64	59.6^{b} ± 3.28
	Fresh weight (mg)				
	-Root	90.23^{a}± 9.6	92.23^{a}± 6.2	45.12^{c}± 4.7	65.23^{b}± 8.9
	-Shoot	252.1^{a}±13.2	264.1^{a}± 10.2	151.23^{c}± 9.8	198.87^{b} ± 12.4
	Dry weight (mg)				
	-Root	41.05^{a}± 2.6	43.05^{a}± 3.31	23.2^{c}± 1.7	32.01^{b}± 4.7
	-Shoot	95.49^{a}± 5.2	99.49^{a}± 1.02	58.21^{c}± 3.8	65.23^{b}± 8.9

	Membrane stability index (%)	$72.66^{a} \pm 2.5$	$75.06^{a} \pm 2.5$	$50^{c} \pm 4.0$	$65^{b} \pm 1.0$
	Total Chlorophyll (mg g^{-1}FM)	$55.4^{a} \pm 3.35$	$57.5^{a} \pm 2.05$	$27.2^{c} \pm 2.41$	$38.78^{b} \pm 0.34$
F accumulation	Root (ppm g^{-1} DM)	Not done	Not done	$30^{a} \pm 3.65$	$20^{b} \pm 3.71$
	Shoot (ppm g^{-1} DM)	Not done	Not done	$22^{a} \pm 2.75$	$14.24^{b} \pm 3.02$
	Leaves (ppm g^{-1} DM)	Not done	Not done	$9^{a} \pm 1.54$	$4.71^{b} \pm 1.13$
	Grain (ppm g^{-1} DM)	Not done	Not done	$2.9^{a} \pm 0.12$	$0.74^{b} \pm 0.05$
Yield attributes	Panicle length (cm)	$21.8^{a} \pm 1.09$	$22.4^{a} \pm 0.06$	$14.2^{b} \pm 1.4$	$21.4^{a} \pm 0.89$
	Number of spikelets per panicle	$17^{b} \pm 1.6$	$20^{a} \pm 1.0$	$8^{b} \pm 1.14$	$15^{b} \pm 1.3$
	Number of filled grain per panicle	$95^{b} \pm 1.8$	$107^{a} \pm 1.2$	$26^{d} \pm 3.6$	$79^{c} \pm 6.9$
	Number of empty grains per panicle	$5^{c} \pm 1.3$	$3^{c} \pm 0.3$	$41^{a} \pm 2.3$	$18^{b} \pm 1.6$
	Grain Length (cm)	$0.8^{a} \pm 0.04$	$0.8^{a} \pm 0.02$	$0.5^{b} \pm 0.05$	$0.7^{a} \pm 0.05$
	Grain Breadth (cm)	$0.26^{a} \pm 0.01$	$0.26^{a} \pm 0.0$	$0.15^{c} \pm 0.01$	$0.2^{b} \pm 0.0$
	1000 grain weight (g)	$26.8^{b} \pm 1.3$	$28.9^{a} \pm 1.1$	$20.4^{c} \pm 1.14$	$26.8^{b} \pm 1.4$
Nutrient Contents	Protein (µg mL^{-1})	$80.03^{a} \pm 0.73$	$81.04^{a} \pm 0.81$	$78.5^{a} \pm 1.8$	$79.88^{a} \pm 2.78$
	Total Sugar (µg mL^{-1})	$836.6^{a} \pm 3.2$	$840.6^{a} \pm 2.7$	$581.4^{c} \pm 6.7$	$690.9^{b} \pm 3.7$
	Iron (ppm)	$43.4^{a} \pm 1.5$	$45.1^{a} \pm 1.03$	$33.6^{c} \pm 1.2$	$37.2^{b} \pm 1.6$
	Zinc (ppm)	$38^{a} \pm 3.78$	$40^{a} \pm 2.08$	$24^{b} \pm 4.58$	$34^{a} \pm 4.04$

2.11. ROS Generation

When *Oryza sativa* L. tissues (root/ leaf) were subjected to F stress, higher accumulations of $O_2^{\bullet-}$ and H_2O_2 were observed (root: 43% and 55%; leaf: 67% and 80%) compared to the corresponding controls. On the other side, treatment of F stressed seedlings with *P. aeruginosa* resulted in a lesser accrual of $O_2^{\bullet-}$ and H_2O_2 (root: 19% and 33%; leaf: 38% and 50%) than that estimated in tissues treated with F alone (Figures 4A and B). Induction of ROS that occurs as a result of F poisoning increases the production of $O_2^{\bullet-}$, as well as its subsequent by-products, such as H_2O_2 and OH·. Additionally, it has also been demonstrated that F can bind to sulfhydryl groups, which prevents proteins from being synthesized and incapacitates the activities of antioxidant enzymes [122]. As a result, an excessive accumulation of ROS is caused (2). Similarly, overproduction of ROS during F stress has also been noted in *Camellia sinensis* L. [123], *Eriobotrya japonica* L. [124], *Olea europaea* L. [99], and

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Triticum aestivum L. [125]. Normally, biological systems try to handle stress mediated over produced ROS involving a variety of ways under the guise of antioxidative defence mechanisms [126]. In addition, exogenous factors like PGPR, which can produce antioxidants in response to ROS, can also help to reduce negative effects of unfavorable environmental conditions. According to conducted studies, bacteria may be able to increase plants ability to withstand various stresses by inflicting physical and chemical changes in them, known as PGPR-induced systemic tolerance which was linked with increased levels of Chl a and b, as well as improved CAT, SOD, and other enzymes activities [127,128]. Further, $O_2^{\bullet -}$ and H_2O_2 productions in the *Oryza sativa* L. leaves were also determined under fluorescence microscope (Figures 5a and b). These observations clearly showed that exposure of F increased the intensity of ROS emission in leaves when compared to the untreated controls. Further, production of ROS was found to be declined noticeably in *P. aeruginosa* treated leaves to that of F alone subjected tissues, suggesting this bacterium successfully reduced F stress to a significant level. In parallel, Tak and Asthir [125], Banerjee et al. [3], Banerjee and Roychoudhury [129] and Singh et al. [65] also determined similar change in the ROS in F stressed leaves of *Triticum aestivum* L. *Oryza sativa* L. and *Lycopersicon esculentum* L., respectively.

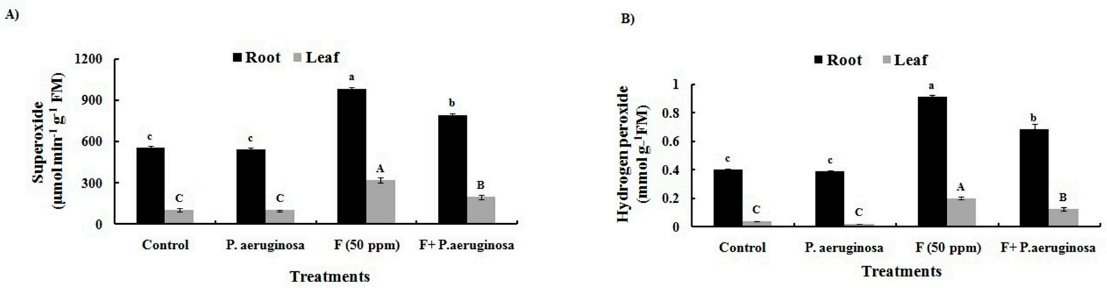

Figure 4. Changes in the levels of superoxide (A) and hydrogen peroxide (B) in *Oryza sativa* L. tissues under different treatments. All the experiments were carried out in three replicates (±SD). Differentalphabets on the bars indicate significant differences (P<0.05) in various treatments.

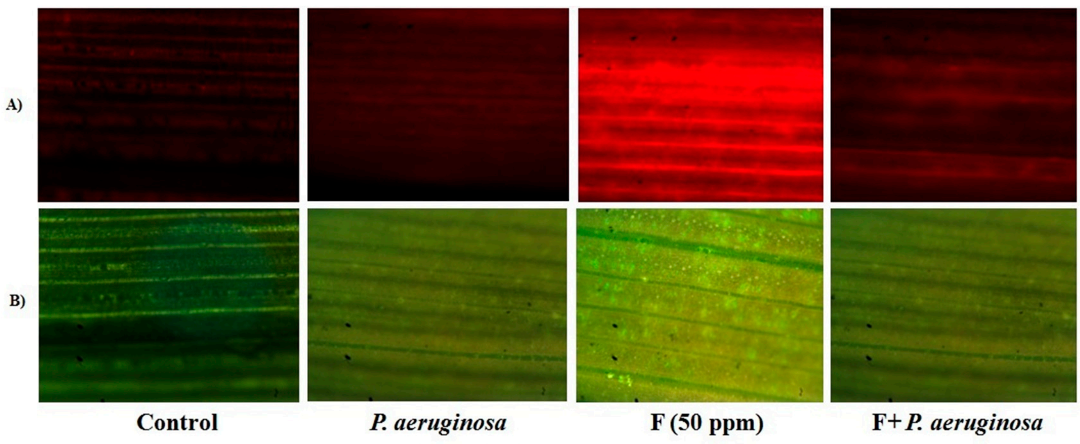

Figure 5. Localization of superoxide radical (A) and hydrogen peroxide (B) in the leaves of *Oryza sativa* L. treated with fluoride (F) and *P.aeruginosa* either alone or in combination.

2.12. Lipid Peroxidation

Lipid peroxidation was estimated as MDA level whose concentration was recorded to be increased by 45% in roots and 48% in leaves when seedlings were exposed to F alone. However,

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after addition of *P. aeruginosa*, amount of MDA in the roots and leaves reduced to 25% and 29% respectively, even in the presence of F (Figure 6). It is important to note that elevation in the ROS results in destruction of polyunsaturated fatty acids thereby increased concentration of MDA [130]. Similar change in MDA content has also been reported in *Helianthus annus* L., *Salicornia brachiata* L., *Camellia sinensis* L., *Cajanus cajan* L., *Triticum aestivum* L., and *Oryza sativa* L. [2,3,91,123,125,131].

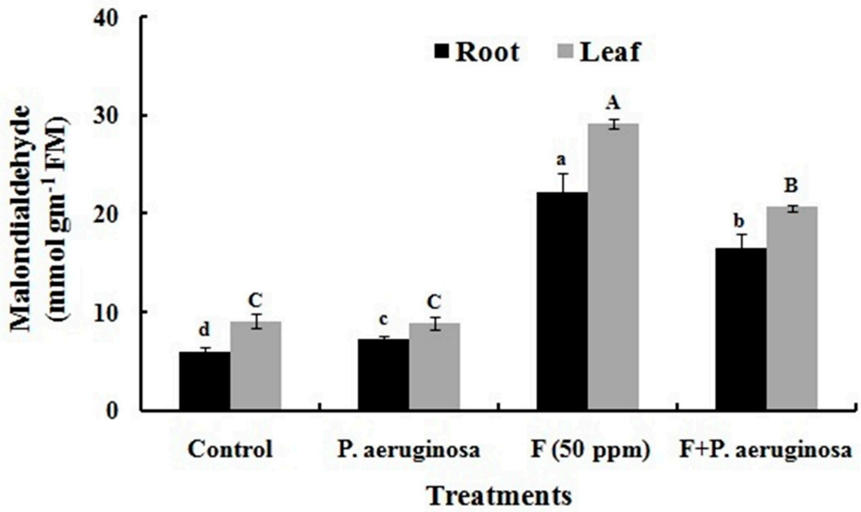

Figure 6. Accumulation of malondialdehyde in the roots and leaves of *Oryza sativa* L. of various treatments. All the experiments were carried in three replicates (±SD). Different alphabets on the bars indicatesignificant differences (P<0.05) in various treatments.

2.13. Antioxidant Enzymes

Activities of SOD, CAT, and APX were considerably suppressed by prolonged exposure of F (leaf: 2.4, 4, and 2 folds, respectively; root: 1.5, 1.9, and 1.25 folds respectively) (Figures 7A, B and C). This might be as a result of binding of F ion with the functional groups of amino acids surrounding the active regions of the enzymes as well as their displacement or competition with magnesium, Fe, or copper ions of enzymes [133]. However, *P. aeruginosa* in close association with seedlings during F treatment significantly decreased the ROS production and overall oxidative damage by increasing the activities of SOD, CAT, and APX (Figures 7A, B and C). Several reports suggested that the plants having higher antioxidant content are more resistant to oxidative damage [134,135]. In agreement, Ma et al. [136] reported that the bacterial inoculated *Ricinus communis* L. and *Helianthus annuus* L. when grown in soils polluted with Ni displayed greater CAT and peroxidase (POX) activity. Similarly, Wang et al. [112] demonstrated that inoculation of an As-resistant *Agrobacterium radiobacter* with *Populus deltoids L.* (LH05-17) caused increased growth, Chl content, soluble sugar, and activities of SOD and CAT. Yet, another study discovered that the *Pisum sativum* L. infused with Ni and Zn tolerant *Rhizobium* sp. RP5 dramatically increased the antioxidants activities in the roots and nodules, and detoxified ROS up to an extent [137]. Further, genes of SOD, CAT, and APX were also analyzed following RT-PCR. Analysis revealed a down-regulation in the expression levels of their genes in F stressed tissues, while were up-regulated considerably in the presence of *P. aeruginosa* (Figures 7A, B and C). Expressions of these genes showed comparable patterns with their spectrophotometric data, demonstrating that the transcriptional regulation of genes played a major role in regulating activities of protective enzymes. Similar changes in the expressions of above genes have also been determined in the *Vigna radiata* L., and *Zea mays* L. under different abiotic stresses [18,34]. Khan et al. [138] demonstrated that

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inoculation of *Bacillus pumilus* enhanced growth of *Oryza sativa* L. under salinity and high boron conditions by upregulating expressions of antioxidants genes thereby cell protection.

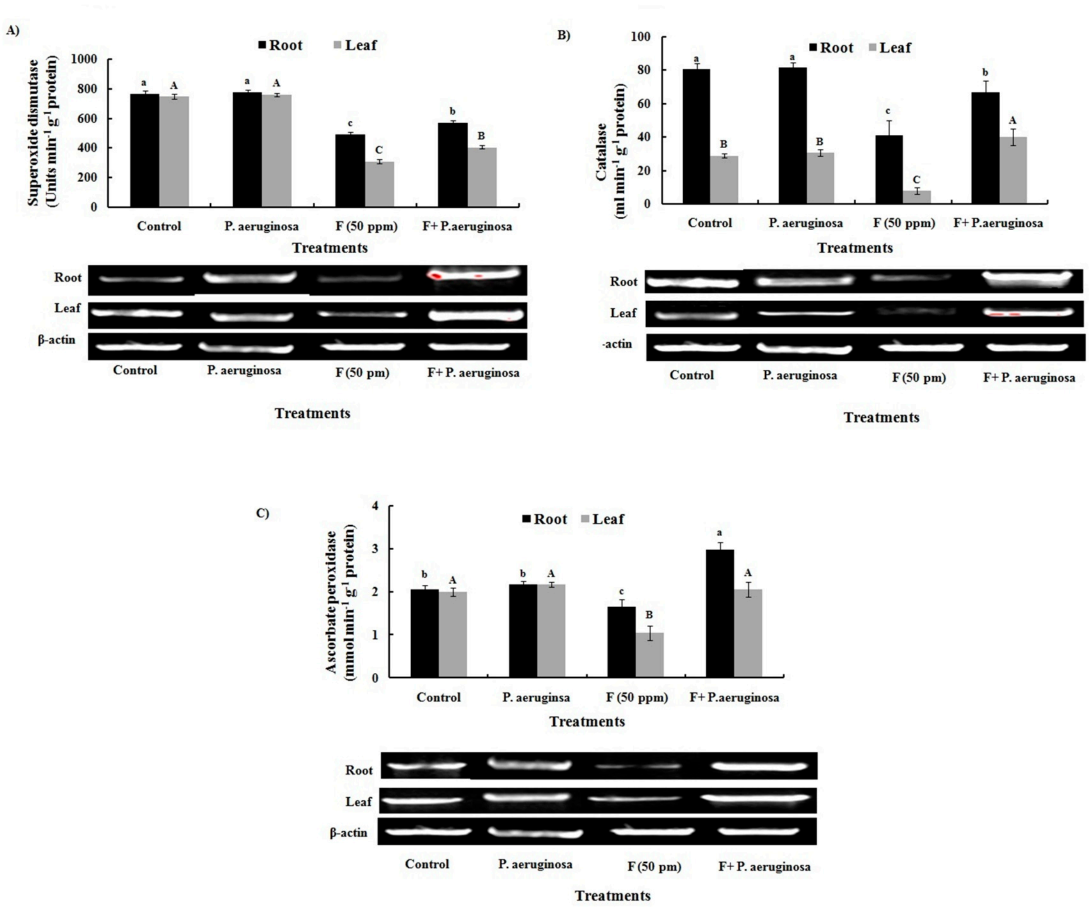

Figure 7. Spectrophotometric and gene expression analyses of (A) superoxide dismutase, (B) catalase, and (C) ascorbate peroxidase in the tissues of *Oryza sativa* L. under different treatments. Each bar represents mean ± SD of three independent observations. Data followed by different alphabets are statistically significant at P<0.05.

3. Materials and Methods

3.1. Bacterial Strain Isolation and Identification

One of the F tolerant bacteria (FTB), namely MT4B was isolated from the F contaminated soil of agricultural fields of Rajnandgaon District (21° 30′ N, 82° 0′ E, 250-300 msl) of Chhattisgarh, India [18,33]. The isolated FTB was determined to be Gram negative, rod shaped, having green color colony and was identified as *Pseudomonas aeruginosa* based on the 16S rRNA gene sequencing. The sequence obtained was submitted to the NCBI GeneBank DNA database and bacterial accession number MW843625 was obtained [33]. Further, the phylogenetic analysis of the strain was executed by aligning the obtained sequences with related reference sequences retrieved from the GenBank database using Clustal Omega software. The neighbor joining method with MEGA X software was employed with bootstrap values generated from 100 replicates for distance analysis and phylogenetic tree construction.

3.2. Fluoride Resistance and Removal Assays

The minimum inhibitory concentration (MIC) of F for *P. aeruginosa* was determined by the agar dilution method. This strain was streaked on Luria Bertani (LB) agar plates amended with various concentrations (5, 10, 25, 50, 75, 100, 125, 150, 200, 250, 275 mM) of F and incubated at

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35°C for 24 h. The least dose of F that inhibited growth of the isolate was considered as the MIC [18]. The ability of *P. aeruginosa* to remove F was ascertained by exposing it to a spectrum of F concentrations (20, 40, 60, 80, and 100 mM) selected on the basis of MIC value. The LB broths with varying concentrations of F were seeded with the bacterial suspension and incubated for 24 h at 35°C in shaking condition (100 rpm). Thereafter, bacterial cultures were centrifuged at 6000 rpm for 10 min at 4°C and the supernatants were collected. Now, concentration of F was determined using a F ion selective electrode (Orion 9609, Thermo Scientific, USA) by mixing the collected supernatants (10 mL) with 1 mL of TISAB (Total ionic strength adjustment buffer) solution (pH 5.5) and then percentage of F removed by the *P. aeruginosa* was calculated using the formula given below [19];

$$\text{Percentage Removal} = \left[\frac{\mathbf{Ci-Cf}}{\mathbf{Cf}}\right] \times \mathbf{100}$$

Where, Ci and Cf is the initial and final concentration of F in the culture media

3.3. Determination of F Biosorption by P. aeruginosa

Scanning electron microscopy (SEM, JSM-IT300, JEOL, USA) was used to superficially characterize the biomass in order to ascertain how the shape and spatial orientation of cells have been changed after exposure to F [34]. Bacterial cells are not a natural conductor, therefore; before SEM analysis, the cells were coated with platinum to increase their surface conductivity. Energy dispersive X-ray (EDX, JEOL, USA) was carried out to determine presence of F within the bacterial biomass [19].

Fourier transform infrared (FTIR) spectroscopy was carried out following the methodology of Pandey and Bhatt [34] to demonstrate interaction between F and functional groups present in the untreated and F laden biomass of *P. aeruginosa*. For this, freshly grown culture of *P. aeruginosa* was centrifuged at 6000 rpm for 15 min; pellet was collected, and dried in an oven at 60°C. Now, dried biomass was grounded and then homogenized with potassium bromide in 1:1 (w/w) ratio. Lastly, spectral scanning of the sample was performed using FTIR spectrometer (Nicolet iS10, Thermo Scientific, USA) in the range 4000-400 cm^{-1}.

3.4. Plant Growth Promoting Activities of P. aeruginosa Under F-Stress

Pseudomonas aeruginosa was screened for multiple PGP traits *viz.;* indole 3-acetic acid (IAA), ammonia, hydrogen cyanide (HCN), siderophore production, and solubilization of phosphorus (P), both in the absence and presence of F (60 mM; as maximum F removal was seen at this concentration). The quantitative analysis of IAA was performed by following the method of Gordon and Weber [35]. This strain was examined for ammonia production by adding 0.5 mL of Nesseler's reagent to 10 mL of freshly grown cultures in peptone water, and absorbance was recorded at 425 nm using a UV-Vis spectrophotometer (Lambda-25, Perkin Elmer, USA) [36]. Production of HCN was determined in accordance with the methodology of Castric (1975) [37] by visualizing yellow to brown color change of Whatman filter paper No.1 drenched with 0.5% (v/v) picric acid solution, placed inside the Petri plate lids. Siderophore production was monitored following the chrome azurol-S (CAS) analytical method [38] by observing yellow to orange halos around the bacterial colonies. Likewise, phosphate solubilizing activity of the isolate was quantitatively assayed in the National Botanical Research Institute's phosphate growth medium (NBRIP) containing tricalcium phosphate, following the method of Fiske and Subbarow [39].

3.5. Model Plant and Experimental Design

Soil utilized for sowing seeds of model species was collected from the School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh, India. The collected soil was tested for the presence of F, if any. Further, the soil was autoclaved to exterminate the microorganisms present in it, so as to examine the effect of *P. aeruginosa* alone over growth of the plant. *Pseudomonas aeruginosa* cultured in the LB broth was used as a bioinoculant after its dilution with 0.9% (w/v) saline. The inoculant was mixed with soil at a cell count of 10^6 CFU g^{-1} soil [34]. Now, fresh healthy seeds of *Oryza sativa* L. (Var. MTU1010) procured from the Indra Gandhi Agricultural University, Raipur, Chhattisgarh, India, were sorted, and surface sterilized with 70% (v/v) ethanol and 0.1% (v/v) sodium hypochlorite solution for 3 min, separately, followed by multiple washing with sterile distilled water (DW). Sterilized seeds were soaked in DW for 24 h and sown in the conditioned soil. Four treatments were designed: (i) Seeds were sown in the soil lacking both F and *P. aeruginosa*: as control, (ii) Seeds were sown in the soil having *P. aeruginosa* as bioinoculant, (iii) Seeds were sown in the soil having 50 ppm F, and (iv) Seeds were sown in the soil having 50 ppm F and *P. aeruginosa* as bioinoculant.

For the present study, 50 ppm dose of F was chosen based on the results of pilot experiment performed following 10, 20, 30, 40, 50, and 60 ppm of F concentrations, which revealed 50% reduction inthe germination percentage (GP) of *Oryza sativa* L. seeds treated with 50 ppm F, as compared to that recorded with the control. Initially, seeds were sown in sterilized plastic disposable glasses (with 30 g soil) each having10 seeds for 25 consecutive days for germination and proper growth of seedlings by irrigating with sterile DW (10 mL) every day. Thereafter, seedlings were transplanted with utmost care into the bigger sized (5 kg soil containing capacity) earthen pots filled with exactly similar conditioned soil. Timely irrigation of the pots was done with sterilized DW till maturation of plant and ripening of grains. All the sets were maintained at normal photoperiod (10-12 h) and sunlight (5-6 h). After complete maturation and ripening of seeds, the roots, leaves and grains were harvested and stored at -80^{o}C until completion of decided experiments.

3.6. Rhizosphere Colonization by P. aeruginosa

The colonization of *P. aeruginosa* in the rhizosphere was deduced by following the method of He et al. [40]. Rhizospheric soil (1g) was collected from the pots of all the four treatments, suspended separately in 10 mL sterile DW and kept in shaking condition for 30 min. The resulting suspensions were used as inoculums and were spread in the LB agar plates supplemented with F. Now, plates were incubated for 72 h at 35^{o}C and bacterial growth was observed.

3.7. Determination of Soil Enzymes Activities

Soil was analyzed for activities of various enzymes (urease, nitrate reductase, phosphatase, cellulase, and dehydrogenase) before sowing the seed and after harvesting the crop. Soil urease (EC 3.5.1.5) activity was determined by Shcherbakova method [41] by using urea as a substrate The concentration of released N-NH_4^+ after adding Nessler's reagent was measured by taking the absorbance at 400 nm and expressed as μg N-NH_4^+ g^{-1} dw h^{-1}.

Soil nitrate reductase (EC 1.7.99.4) activity was determined using Kandeler method [42] by incubating soil with KNO_3 as a substrate for 24 h. Activity was measured as released NO_2^- after adding coloring reagent (sulfanilamide and 0.1 g of N-(1-naphthyl) ethylenediamine dihydrochloride) by measuring the absorbance at 520 nm and expressed as μg N-NO_2^- g^{-1} dw h^{-1}.

Soil cellulase (EC 3.2.1.4) activity was determined by the Pancholy and Rice [43] by adding sodium acetate buffer (50mM, pH 5.5) and carboxymethylcellulose in the soil. Afterwards, concentration of reducing sugars was measured by Somogyi-Nelson method [44] and by taking absorbance at 520 nm. Activity was expressed in the unit μg D-Glu g^{-1} dw h^{-1}.

Soil phosphatase (EC 3.1.3.2) activity was determined by the procedure of Tabatabai and Bremner [45] using disodium *p*-nitophenyl phosphate as a substrate. Enzyme activity was measured spectrophotometrically at 400 nm by measuring the amount of *p*-nitophenyl phosphate liberated and expressed as μg pNP g^{-1} dw h^{-1}.

Dehydrogenase (EC 1.1.1.x) activity was measured by the Thaimann method [46]. Soil was incubated with triphenyl tetrazolium chloride, as substrate. The content of triphenylformazan was measured with a spectrophotometer at 546 nm and activity was expressed as μg TPF g^{-1} dw h^{-1}.

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3.8. Assessment of Growth Attributes and Membrane Stability Index

Germination percentage and, root and shoot length (RL and SL) of mature plant was calculated from the meanof five seedlings and in three replicates. Fresh mass (FM) was estimated instantly after harvesting, while dry mass (DM) was recorded after drying the tissues in an oven at 70°C for 24 h [47]. By following the procedure of Rady [48], MSI of plant tissues was derived. Two sets of each treatment, in three replicates, were prepared by taking 1 g of plant tissues (leaves and roots) and imbibing them in test tubes with 10 mL of MilliQ water. The first set was kept in a water bath maintained at 40°C for 30 min, while the second set was kept at 100°C for 10 min. Thereafter, electrical conductance of each set was monitored. The data assisted with the first set was given the code C1, while the second set was coded as C2. At the end, below formula was followed to compute the MSI (%).

$$\text{MSI (\%)} = \left(1 - \frac{C1}{C2}\right) \times 100$$

3.9. Determination of Total Chlorophyll

The leaves (0.2 g) from three randomly selected plants of each treatment were acquired, homogenized using chilled acetone (80%, v/v) and centrifuged at 5000 rpm for 10 min to settle down all the debris [49]. Absorbance of the supernatant was recorded at 645 and 663 nm. Later, the total Chl content was calculated using below equation and expressed as mg g^{-1} FM;

$$\text{Total Chl} = \frac{20.2 \times A645 + 8.02 \times A663}{\text{Fresh mass of leaves}}$$

3.10. Measurement of F Content in Plant Tissues

For the estimation of F concentration, 1 g of tissues (roots, shoots, leaves and grains) in triplicates were dehydrated in an oven at 70°C for 24 h, powdered, and digested with aquaregia [Conc HNO3: HCl (1:3, v/v)]. The digested samples were then centrifuged (5000 rpm, 15 min) and supernatant was collected. The supernatant acquired was incorporated with TISAB buffer (1:1, v/v) and were analyzed with F Ion Selective Electrode [2].

3.11. Determination of Agronomical Attributes

Agronomical parameters like panicle length (PL), spikelet count per panicle, number of filled and empty grains (FG and EG), hundred grain weight (HGW), and grain length/ breadth (GL/GB) were estimated. Panicle length was calculated by measuring five randomly selected panicles, in three replicates, for each treatment. To quantify FG and EG, grains were initially separated by hand threshing and winnowing, and then counted manually. To compute HGW, weight of 25 grains was first recorded, and then extrapolated to determine the HGW [3]. In order to compute length and breadth of a single grain, ten numbers of grains were randomly selected from each set of treatments and arranged horizontally and vertically. The length and breadth of the arranged grains were then measured using a scale manually, and registered data was then divided by ten to determine the measurement of individual grain [3].

3.12. Determination of Protein, Total Sugar, Zinc, and Iron in the Grains

Content of protein in the grains was estimated using the protocol of Bradford [50] and data was expressed in terms of μg ml^{-1}. For the estimation of total sugar, methodology of Dubois et al. [51] was followed and the values were expressed as μg ml^{-1}. To quantify zinc (Zn), and iron (Fe), methodologies of Malavolta et al. [52] and, Kalaimaghal and Geetha [53] respectively were followed. Samples were prepared and concentrations were estimated in ppm following an Atomic absorption spectroscopy [3].

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3.13. Generation of ROS

Concentration of $O_2^{\cdot -}$ was spectrophotometrically estimated following the methodology of Sangeetha et al. [54] by taking absorbance at 540 nm. Content was calculated by its ability to diminish nitro blue tetrazolium (NBT), and its values were represented as μmol min^{-1} g^{-1} FM. Hydrogen peroxide was assayed following the protocol of Velikova et al. [55]. Using an extinction coefficient of 0.28 μM^{-1} cm^{-1} its amount was calculated and the results were expressed as μmol g^{-1} FM.

3.14. Fluorescence Microscopy

Production and intensities of both $O_2^{\cdot -}$ and H_2O_2 in the mature leaves of *Oryza sativa* L. were imaged using Confocal Fluorescence Microscope 2000 LED (Leica, Germany) after incubating them in the dihydroethidium (DHE), and 2,7- dichlorodihydrofluorescein diacetate (H_2DCFDA), respectively [56].

3.15. Lipid Peroxidation

Methodology of Hodges et al. [57] was adopted to determine peroxidation of lipids by calculating amount of MDA. The 3 mL of 20% (w/v) trichloro acetic acid supplemented with 0.5% (w/v) thiobarbituric acid was used to homogenize 0.2 g of tissues (roots and leaves). The mixtures were heated for 30 min at 95°C in a water bath, and then chilled for 15 min in a freezer. The homogenate was then centrifuged for 15 min at 10000 rpm. By subtracting the non-specific absorbance at 600 nm, absorbance of the supernatant was calculated at 540 nm. The quantity of MDA was determined using an extinction coefficient of 155 mM^{-1} cm^{-1}, and data was represented as mmol g^{-1} FM.

3.16. Enzyme Extraction

Using a chilled mortar and pestle, fresh roots and leaves (0.2 g) were individually homogenized in 2 mL of cold potassium phosphate buffer (50 mM, pH 7.0) amended with 1 mM EDTA. The supernatant obtained after centrifuging the homogenate at 12000 rpm for 15 min at 4°C was utilized for the assessment of antioxidative enzymes with the exception of APX activity, which involved grinding the tissues separately in a homogenizing solution that also contained 2 mM ascorbate along with the other components.

3.17. Enzyme Assays

The activity of SOD (EC 1.15.1.1) was assessed using the procedure of Marklund and Marklund [58] by calculating the percentage inhibition of pyrogallol auto-oxidation at 420 nm. In a test tube, 2.74 mL of Tris-HCl buffer (50 mM, pH 8.2) containing 1 mM of both diethylenetriaminepentaacetic acid and EDTA was taken, to which 0.2 mL of enzyme extract was added. The 60 μL aliquot of pyrogallol (0.2 mM in 10 mM HCl) was added to start the reaction, and after six min of incubation, a change in absorbance at 420 nm was noted and its activity was expressed as units of SOD min^{-1} g^{-1} protein.

The method proposed by Chance and Maehly [59] was used to measure the activity of CAT (EC 1.11.1.6). By observing decrease in absorbance at 240 nm, breakdown of H_2O_2 was estimated. The assay mixture included 60 μL of enzyme extract and 37.5 mM potassium phosphate buffer (pH 6.8). The H_2O_2 (200 μL of 60 mM) was added to start the enzymatic reaction, and change in absorbance was monitored for 5 min at intervals of 15 sec. The extinction coefficient of 0.039 M^{-1} cm^{-1} was used to quantify CAT activity, which was expressed as μmol min^{-1} g^{-1} protein.

According to Nakano and Asada [60], activity of APX (EC 1.11.1.11) was estimated by determining the rate of ascorbate oxidation at 290 nm. The reaction mixture contained 2.3 mL of 0.025 M potassium phosphate buffer (pH 7.0), 10 μL of enzyme extract, 190 μL of EDTA, and 500 μL of ascorbic acid. Initial absorbance was measured at 290 nm immediately after adding 10 μL of H_2O_2 (0.1 M) to the assay mixture. Final absorbance was recorded after 20 min of incubation. Activity was measured using an extinction coefficient of 0.0028 M^{-1} cm^{-1} and expressed as mmol min^{-1} g^{-1} protein.

3.18. Gene Expression Analysis

Extraction of RNA was carried out following hot phenol method of Verwoerd et al. [61]. After extraction, RNA quality and concentration were recorded using a Nanodrop Spectrophotometer (ND1000, Thermo Scientific, USA). Agarose gel electrophoresis (1.2%, w/v) was performed to validate RNA quality by observing two bands of rRNA (18s and 28s rRNA). Now, cDNA was synthesized by employing Hi-cDNA Synthesis Kit (HiMedia, India),

Exploiting set of gene-specific primers for each enzyme, a 20 μL reaction volume for reverse transcriptase-PCR was developed. By employing a house keeping gene β-actin templates were normalized. Distinct primers for SOD (forward 5′-CTGATCTAGAGGGAAGTCA-3′; reverse 5′-TGTATGGGAGCATGCTACT-3′), CAT (forward5′-CTATTGGAAGATTATCATCT-3'; reverse 5'-AGAATTCTTGATTTTTCTA-3') and APX (forward 5'- TGGCACTCTGGGTACTTT-3'; reverse 5'- GATTTGAGGGACCATGGACT-3') were created using software prim er3 (http://frodo.wi.mit.edu). Polymerase chain reaction was performed by setting the PCR program cycle as: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 60 sec; annealing at 49-65°C for 45 sec; extension at 72°C for 60 sec; followed by final extension at 72°C for 5 min. The amplicons were electrophoresed for 40 min at a constant voltage of 50 V in 1.5% (w/v) agarose gel. By observing the fluorescence intensities of the bands under a Gel-Doc (BioRad, USA), degree of gene expression was determined.

3.19. Statistical Analysis

Data presented are mean values of three replicates ± SD and were analyzed using SPSS software (Version 20.0). One-way analysis of variance (ANOVA) was adopted followed by Duncan's multiple range tests to measure the significant difference between the means of all the treatments at $P<0.05$.

4. Conclusions

Pseudomonas aeruginosa (MW843625), isolated from the soil of agricultural fields of Chhattisgarh, India, having relatively higher concentration of F, was found to be a potent accumulator of F. Characterization of this isolate showed changes in surface morphology and elemental composition of its biomass in the presence of F ions which confirmed its uptake by the bacterium. Furthermore, *P. aeruginosa* displayed a number of PGP traits and was found to be significantly increase root-shoot length and biomass of F stressed *Oryza sativa* L. Bioinoculation of *P. aeruginosa* also resulted in significant improvements in soil enzyme activities, leading to an enhanced microecological environment. Additionally, it successfully decreased uptake and accumulation of F in mature tissues of *Oryza sativa* L., leading to appreciable level of F tolerance. The agronomical attributes and yield of stressed seedlings treated with bioinoculant showed significant improvements, and reduced F accumulation in the grains. Inoculation of *P. aeruginosa* reduced oxidative damage by increasing levels/ gene expressions of antioxidants (SOD, CAT and APX) and also facilitated the production of photosynthetic pigments. Based on the findings, *P. aeruginosa* can be utilized as a bio-tool for F remediation, and as microbial inoculant for improved agricultural practices in F contaminated soil. Additionally, focused field studies following the involvement of recombinant technology are necessary to determine the F bioremediation potential of this isolate and also to enhance the effectiveness of *P. aeruginosa* on various crops under F stress condition.

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