

Influence of Microbes in Enduring Stress Created by Fluoride in *Oryza sativa*

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Abstract

There is an increasing global concern about fluoride (F^-) toxicity in agricultural soils as it adversely affects soil microbial activity and diversity. Since soil microorganisms contribute to plant health and plant nutrition, it is important to note the proper functioning of soil microorganisms in crop lands in the perspective of the fact that high F^- level in soils adversely affects the microorganisms which can in turn affect the crop production. The aim of the study is to evaluate the effect of microbial inoculation and soil F^- concentration on the nutrition of *Oryza sativa* using microbial isolates from F^- contaminated soils as inoculants. Besides, this study envisages the possibility of utilizing F^- tolerant microbes for the detoxification of crop lands polluted with F^- . Results revealed that inoculation of *Azotobacter* improved the nutrient content of plants under no added F^- . But combined inoculation of bacterium and arbuscular mycorrhizal fungi (AMF) improved nutrition under added F^- . F^- content in plant and grain apparently decreased in inoculation treatments. Mycorrhizal inoculation improved root colonization by AMF in the roots of rice.

Keywords: soil fluoride, *Oryza sativa*, nutrition, microbial inoculants

Резюме

Токсичността на флуорида (F^-) в земеделските почви, оказваща неблагоприятен ефект върху микробна активност и разнообразие, предизвиква нарастващ интерес в глобален мащаб. Почвените микроорганизми допринасят съществено за доброто хранене и развитие на растенията, а повишеното F^- ниво променя микробиома и от тук, снижава добивите от земеделските култури. Целта на изследването е да се оцени ефектът от микробната инокулация и концентрацията на F^- в почвата върху храненето на *Oryza sativa*, като за инокуланти се използват микробни изолати от F^- -замърсени почви. Това проучване предвижда възможността за използване на F^- -толерантни микроби за детоксикация на замърсени с посевни земи. Резултатите показват, че инокулацията на *Azotobacter* подобрява съдържанието на хранителни вещества в растенията без добавен F^- . Но комбинираната инокулация на бактерия и арбускуларни микоризни гъбички (AMF) подобрява храненето при добавен F^- . Съдържанието на F^- в растенията и зърното очевидно намалява при микробна инокулация. Микоризната инокулация подобрява колонизацията на корените от AMF в корените на ориза.

Introduction

Fluorine is highly reactive and most electronegative of all chemical elements (Greenwood and Earnshaw, 1984; Gillespie *et al.*, 1989) and is therefore never encountered in nature in elemental form. It is commonly found in its ionized form, fluoride (F^-) or reacted with another element. Major sources of F^- are mostly natural, i.e., F^- containing mineral rock like fluor spar or fluorite (CaF_2) cryolite (Na_3AlF_6), fluorapatite ($Ca_5(PO_4)_3F$) villiaumite (NaF), and topaz ($Al_2(SiO_4)F_2$). Fluoride containing rocks are considered as the reservoir of F^- (WHO, 1984). As F^- is strongly retained in most

agricultural soils due to its adsorption by clay minerals and Al^- and Fe^- oxyhydroxides, etc. in soils where phosphate fertilizer has been applied over several years, it has been found that there is a build-up of soil F^- .

Fluoride enters plants mainly through two pathways: initially F^- deposited over leaves enters through stomata, and secondly, through the soil and water into the roots by a passive diffusion process (Baunthiyal and Ranghar, 2014). Subsequently, it is transported via the xylem vessels through the apoplastic and symplastic pathways into the shoots

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(Pant *et al.*, 2008). F^- moves along the transportation stream to the tip and edges of the leaves, where it accumulates and can cause physiological, biochemical and structural changes and sometimes cell death (Domingos *et al.*, 2003). Elevated levels of F^- exposure reduce seed germination in plants (Wilde and Yu, 1998; Gupta *et al.*, 2009) by lowering the enzymatic activity and growth by slowing the rate of cell division and expansion (Chang and Thompson, 1966). Fluoride causes reduction in root length and shoot length in cluster bean due to unbalanced nutrient uptake by the seedlings in the presence of fluoride (Sabal *et al.*, 2006).

Kumar *et al.* (2017) reported that F^- causes less development of plants by affecting several pathways associated with photosynthesis, respiration, protein synthesis, carbohydrate metabolism and nucleotide synthesis. In rice, plant height, leaf area index, panicle length, grain yield, grain length and kernel elongation were significantly affected by increasing doses of F^- (300 ppm F^-) in the form of hydrofluoric acid (Panda *et al.*, 2018). In another study with the same crop Gupta *et al.* (2009) observed that the fresh and dry weights and per cent of seedlings decreased monotonically with increasing F^- concentration.

Pigment content in plant is affected by F^- toxicity (Kumar and Rao, 2008; Mondal, 2017). Plant chlorophyll content is known to be markedly decreased by high concentrations of F^- (Kaur and Duffus, 1989; Elloumi *et al.*, 2016; Gadi *et al.*, 2016) while low concentrations (10 and 15 ppm) are reported to increase chlorophyll content in *Medicago sativa* and *Nicotiana tabacum* (Ibarra *et al.*, 1988). Fluoride toxicity in plants is normally indicated by marginal necrosis on foliage, which begins on the margins or tops of leaves and moves inward (Brewer, 1966a). Chlorosis, leaf tip burn and necrosis of leaf tip and leaf margin consequent on F^- toxicity has been reported earlier in crop plants (Miller, 1993; Fornasiero, 2001; Saint Anna-Santos *et al.*, 2012).

There is an increasing global concern about F^- toxicity in agricultural soils as it adversely affects soil microbial activity and diversity. Since soil microorganisms contribute to plant health and plant nutrition, it is important to note the proper functioning of soil microorganisms in crop lands in the perspective of the fact that high F^- level in soils adversely affects the microorganisms which can in turn affect the crop production. The application of microorganisms to soil in order to ameliorate F^- toxicity would be of considerable benefit. So far there have been no consolidated studies in this wetland

agro ecosystem in this direction. Therefore, this study aimed to evaluate the individual and interactive effects of microbial isolates from F^- affected soil in ameliorating F^- stress using rice (*Oryza sativa* L.) as test plants. Study also aimed to evaluate the effect of soil fluoride and microbial inoculation on the nutrition of *Oryza sativa*.

Materials and Methods

Three locations of the tropical wetland, Kut-tanad viz. Thalavadi, Monkompu and Muttar which had a low, medium and high level of soil F^- respectively (Roshni and Harikumar, 2021) were chosen for the study of soil microbial densities in relation to soil F^- . Dilution plating procedure was used to estimate the densities of cultivable bacteria (Wollum, 1982). Only the predominant bacterial cultures were characterized and identified (Buchanan and Gibbons, 1974). The isolates were maintained on agar slants at 4°C as stock cultures.

Arbuscular mycorrhizal spore density in soil

Spores or sporocarps of arbuscular mycorrhizal (AM) fungi were extracted from 50 g air-dried sub-samples of each soil sample in five replicates by wet-sieving followed by floatation centrifugation in 50% sucrose (Dalpé, 1993). The finest sieve used was 45 µm. The spores were collected on a grid-patterned (4×4 mm) filter paper, washed three times with distilled water to spread them evenly over the entire grid, and counted using a dissecting microscope. A sporocarp was counted as one unit. The number of spores is expressed as the mean of five replications.

For observation and identification of spore characters, spores of AMF were mounted on glass slides in polyvinyl alcohol-lactophenol (PVLG) and PVLG+Melzer's reagent (1:1, v/v) and then identified to species level using current taxonomic criteria (Schenck and Perez, 1990) and information published by INVAM (<http://www.invam.caf.wvu.edu>). The identified species were maintained on *Sorghum vulgare* plants.

Experimental design

The experiment was performed in plastic bags (18 cm dia.) having a capacity of 3 kg soil. The bags were filled with sandy (Entisol) soil having a pH of 4.1, OC 5.20 g kg⁻¹, N 190 mg kg⁻¹, P 5.2 mg kg⁻¹ and K 191.5 mg kg⁻¹. The soil was steam-sterilized before filling and was seeded with rice (cv. Uma). Prior to seeding, the seeds were surface-sterilized using sodium hypochlorite (3%, v/v) for 5 min and washed three times with sterile water. When the plants reached about 15 cm in height,

the plant density was thinned to three per bag and inoculated with predominant nitrogen-fixing and P-solubilizing microorganisms, such as *Azotobacter chroococcum* Beijerinck (5.4×10^5 CFU ml⁻¹), and *Bacillus subtilis* (5×10^7 CFU ml⁻¹) respectively (Roshni and Harikumar, 2020). In mycorrhizal treatments, 50 ml of inoculum containing a consortium of AM chlamydospores (*Glomus dimorphicum* and *Glomus indicum*; approximately 200 spores) was uniformly mixed into the soil. The experiment consisted of 8 treatments (7 inoculated and 1 uninoculated control), two levels of F⁻ (F₀ - no added F⁻, F₁ - added with NaF to reach a final concentration of 40 mg kg⁻¹ soil) (Fig. 1). All the treatments were replicated three times. The soil and plant samples were taken at 138 days of growth for monitoring of various parameters.

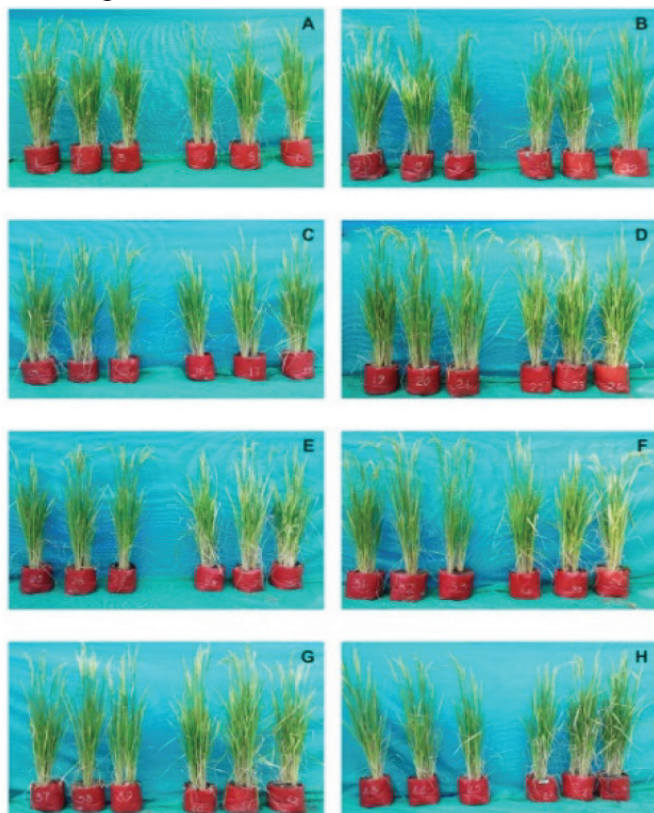


Fig. 1. Experimental design with *O. sativa*

Plant analysis

AM colonization in plant roots. The undamaged fine roots of the test plants were cut into 1 cm root segments. From these about 30 segments were selected at random. The root segments were first washed thoroughly in distilled water and then placed separately in 10% KOH and heated to 90°C for 15 – 30 min. They were then washed in distilled water and immersed in alkaline 3% H₂O₂ for 5 – 10 min. The roots were again washed in distilled water and acidified with 5 N HCl for 2 – 5 min. Then the root segments were stained with 0.05% trypan blue in lactophenol for 15 – 30 min and the excess stain

was removed with clear lactophenol (Phillips and Hayman, 1970). The root segments were mounted on clean microscopic slides in a mixture of glycerol and lactic acid (v/v). The root segments were gently squashed and covered by a glass cover slip and observed under a compound microscope (Nikon Eclipse E 400) using different magnification for AM fungal structures. Mycorrhizal colonization (%) was assessed by the gridline intersect method (Giovannetti and Mosse, 1980).

Nutrients in plant tissue. The content of N in plant tissues was determined by the colorimetric method proposed by Snell and Snell (1949). The Jackson (1973) method was used to determine the concentration of P in plant tissues. The K content in samples was measured by a flame photometer (Elico CL 345) (Faithfull, 2002).

Fluoride in plant tissue. Harvested rice plants were washed and rinsed with deionized water (F⁻ content 0 ppm) and dried for 48 hours at 80°C. Samples were then ground and stored in clean dry, tightly closed plastic bottles. A 0.5 g sample of powdered plant tissue was ashed for 1 h at 550°C with 4 g of sodium-potassium carbonate mixture, after which the temperature was raised to 950°C for 30 min. After cooling, the ashed material was dissolved in 20 ml 1M HCl and filtered, made up to 50 ml with deionized water, and stored in plastic flasks until analyzed (Troll *et al.*, 1977). For potentiometric measurement of total F⁻, the diluted solution was mixed with a total ionic strength adjustment buffer (TISAB) solution (1:10) to dissociate fluoride complexes, stabilize the pH, and maintain constant ionic strength (Adriano and Doner, 1982). The TISAB buffer contained 57 ml acetic acid, 58 g NaCl and 4 g CDTA per litre, adjusted to pH 5.2 with 6M NaOH and diluted to 1 litre with distilled water (Lezovic, 1969). Measurement was done using ion-selective electrode coupled to an ion analyser (Orion-4 star).

Statistical analysis

Analysis of variance (ANOVA) and multivariate ANOVA (MANOVA) were used to determine the effect of each treatment and the interaction between them. If the ANOVA/MANOVA detected significant differences, means separation was accomplished through the Tukey's Honestly Significant Difference (HSD) test at P<0.05.

Results

Effect of microbial inoculation and soil F⁻ on nutrition of rice

In general, all the inoculation treatments improved the plant N content under F₀ and F₁ levels (Table 1).

Table 1. Effect of microbial inoculation and soil fluoride on nitrogen content in rice

Treatment	N (mg kg ⁻¹)			
	Plant		Grain	
	F0	F1	F0	F1
Control	7700 ± 173.21 ^{ab}	7733.33 ± 233.33 ^{ab}	10333.33 ± 233.33 ^{ab}	10333.33 ± 328.3 ^{ab}
<i>Azotobacter</i>	8900 ± 115.47 ^{ce}	8933.33 ± 166.67 ^{ce}	12033.33 ± 145.3 ^{ce}	11900 ± 200 ^{ce}
<i>B. subtilis</i>	8166.67 ± 120.19 ^{ac}	8200 ± 152.75 ^{acd}	11033.33 ± 120.19 ^{ac}	10933.33 ± 218.58 ^{ac}
AMF	7733.33 ± 240.37 ^{ab}	7433.33 ± 176.38 ^a	10300 ± 321.46 ^{ab}	9966.67 ± 284.8 ^a
<i>Azotobacter</i> + <i>B. subtilis</i>	9100 ± 0.00 ^{de}	8766.67 ± 120.19 ^{ce}	12333.33 ± 120.19 ^{de}	11766.67 ± 133.33 ^{ce}
<i>Azotobacter</i> + AMF	8933.33 ± 233.33 ^{ce}	8766.67 ± 88.19 ^{ce}	12300 ± 57.74 ^{de}	11800 ± 173.21 ^{ce}
<i>B. subtilis</i> + AMF	8600 ± 100.00 ^{bce}	8266.67 ± 202.76 ^{acd}	11433.33 ± 120.19 ^{bcd}	11033.33 ± 290.59 ^{ac}
<i>Azotobacter</i> + <i>B.</i> <i>subtilis</i> + AMF	9466.67 ± 88.19 ^e	8600 ± 346.41 ^{bce}	12633.33 ± 145.3 ^e	11366.67 ± 405.52 ^{bcd}

Mean values within the column followed by the same letter(s) are not significantly different at P<0.05 according to Tukey's HSD

Under F0 level, the highest increase was observed in treatments received with *Azotobacter* inoculation alone. The nitrogen content in rice grains also followed the same pattern as that of plant tissue. Under both F0 and F1 level of F⁻, the P content in the plant tissue and grain increased significantly (P<0.05) in inoculated treatments compared to the uninoculated control. However, the combined inoculation of *Azotobacter* + *Bacillus* + AMF gave better results in improving the P content in both plant and grain (Table 2).

The K content in the tissue and grain was significantly more in most inoculated treatments under F0 and F1 levels of F⁻ (Table 3).

When the K content of plants raised under F0 and F1 levels were compared, the plants grown under F0 levels had more K content in their tissue and grains than treatments without added F⁻ (F1).

Table 2. Effect of microbial inoculation and soil fluoride on phosphorus content in rice

Treatment	P (mg kg ⁻¹)			
	Plant		Grain	
	F0	F1	F0	F1
Control	1000 ± 57.74 ^{ab}	933.33 ± 66.67 ^a	1333.33 ± 88.19 ^{ab}	1200 ± 57.74 ^a
<i>Azotobacter</i>	1100 ± 57.74 ^{ac}	1100 ± 0.00 ^{ac}	1466.67 ± 88.19 ^{ac}	1433.33 ± 33.33 ^{ac}
<i>Bacillus subtilis</i>	1366.67 ± 33.33 ^{cd}	1300 ± 57.74 ^{bcd}	1800 ± 57.74 ^{ce}	1733.33 ± 66.67 ^{bce}
AMF	1333.33 ± 66.67 ^{cd}	1066.67 ± 88.19 ^{ac}	1766.67 ± 88.19 ^{ce}	1500 ± 57.74 ^{acd}
<i>Azotobacter</i> + <i>Bacillus subtilis</i>	1466.67 ± 66.67 ^{de}	1300 ± 115.47 ^{bcd}	1900 ± 100 ^{def}	1766.67 ± 145.3 ^{ce}
<i>Azotobacter</i> + AMF	1300 ± 57.74 ^{bcd}	1066.67 ± 33.33 ^{ac}	1733.33 ± 88.19 ^{bce}	1400 ± 57.74 ^{ac}
<i>Bacillus subtilis</i> + AMF	1733.33 ± 33.33 ^{ef}	1466.67 ± 66.67 ^{de}	2300 ± 57.74 ^{fg}	2000 ± 57.74 ^{ef}
<i>Azotobacter</i> + <i>Bacillus</i> <i>subtilis</i> + AMF	1900 ± 57.74 ^f	1600 ± 57.74 ^{df}	2500 ± 57.74 ^g	2066.67 ± 66.67 ^{ef}

Mean values within the column followed by the same letter(s) are not significantly different at P<0.05 according to Tukey's HSD

Microbial inoculation and F⁻ dynamics

Under both F0 and F1 levels, the F⁻ content in plant and grains were significantly (P<0.05) decreased (Fig. 2 and Fig. 3).

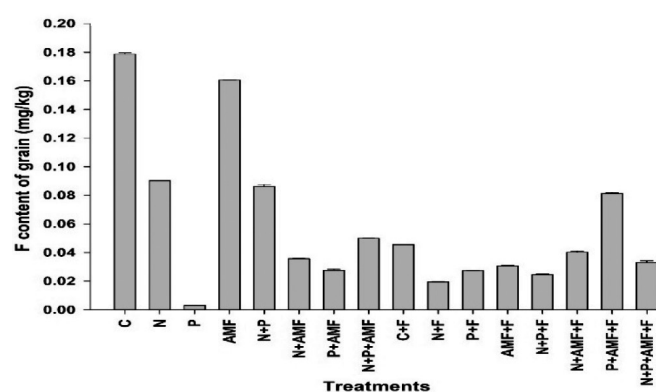
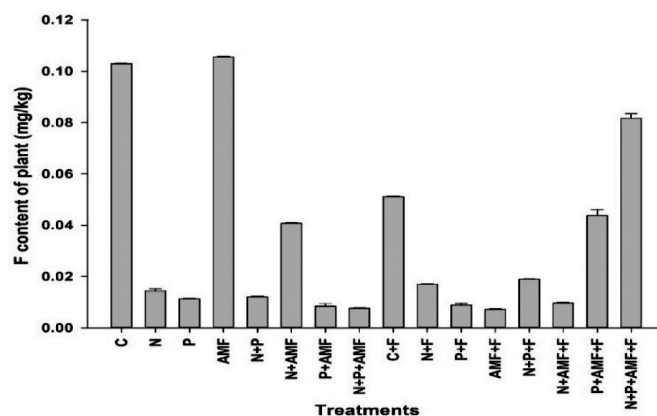
**Fig. 2.** Fluoride content of grain

Table 3. Effect of microbial inoculation and soil fluoride on potassium content in rice

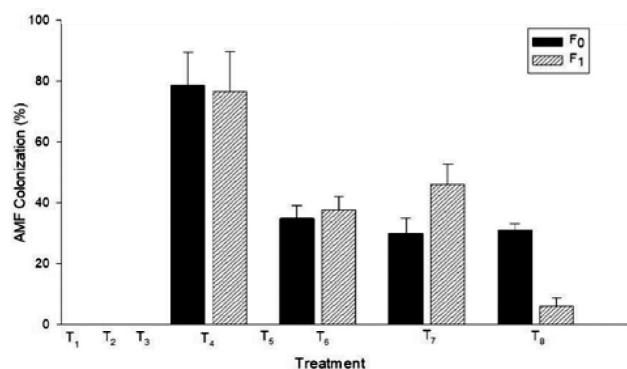
Treatment	K (mg kg ⁻¹)			
	Plant		Grain	
	F0	F1	F0	F1
Control	8866.67 ± 317.98 ^{acd}	8433.33 ± 611.92 ^{ac}	1700 ± 57.74 ^{acd}	1633.33 ± 120.19 ^{ac}
<i>Azotobactor</i>	9400 ± 288.68 ^{acd}	6966.67 ± 166.67 ^a	1800 ± 57.74 ^{acd}	1333.33 ± 33.33 ^a
<i>Bacillus subtilis</i>	10066.67 ± 440.96 ^{acd}	9466.67 ± 835.33 ^{acd}	1933.33 ± 88.19 ^{acd}	1833.33 ± 166.67 ^{acd}
AMF	9566.67 ± 440.96 ^{acd}	8033.33 ± 841.3 ^{ab}	1833.33 ± 88.19 ^{acd}	1666.67 ± 66.67 ^{acd}
<i>Azotobactor</i> + <i>Bacillus subtilis</i>	11800 ± 472.58 ^{de}	9466.67 ± 698.41 ^{acd}	2266.67 ± 88.19 ^{de}	1833.33 ± 120.19 ^{acd}
<i>Azotobactor</i> + AMF	9200 ± 907.38 ^{acd}	8166.67 ± 1197.68 ^{ab}	1766.67 ± 176.38 ^{acd}	1566.67 ± 233.33 ^{ab}
<i>Bacillus subtilis</i> + AMF	10433.33 ± 606.45 ^{bcd}	8533.33 ± 633.33 ^{ac}	2000 ± 115.47 ^{bcd}	1633.33 ± 120.19 ^{ac}
<i>Azotobactor</i> + <i>Bacillus</i> <i>subtilis</i> + AMF	14600 ± 288.68 ^e	11666.67 ± 120.19 ^{ce}	2800 ± 57.74 ^e	2233.33 ± 33.33 ^{ce}

Mean values within the column followed by the same letter(s) are not significantly different at P<0.05 according to Tukey's HSD

**Fig. 3.** Fluoride content of plant

Mycorrhizal colonization and soil F⁻

Comparison of the mean mycorrhizal colonization between F0 and F1 treatments showed an increase to the tune of 10% in F1 treatment. The highest mycorrhizal colonization in the inoculated treatment was noted in AMF alone inoculation (T4) in both F0 and F1 treatments while in the treatments of combined inoculation with other microorganisms it was generally low (Fig. 4).

**Fig. 4.** Effect of AM inoculation in the percentage mycorrhizal colonization in rice

Discussion

Few microorganisms have been identified capable of degrading F⁻ under *in vitro* conditions (Kuntze *et al.*, 2011; Zhuo *et al.*, 2014). It is known that bacteria have different mechanisms to deal with toxic compounds (Banerjee *et al.*, 2016). In the case of F⁻, bacteria possess a special type of chemical proteins called putative F⁻ transporters that help them to reduce the toxic effect of F⁻ in the bacterial cell (Baker *et al.*, 2012). These genes are in fact riboswitches which are special types of metabolic binding RNA that are activated by high F⁻. Earlier reports show that bacterial species such as *Acinetobacter* sp are tolerant to elevated F⁻ concentration. However, in this study, the bacterial species *Bacillus subtilis* showed its predominance in most F⁻ contaminated soils indicating its tolerance to a wide range of F⁻ levels. This further corroborates the finding of Kumar *et al.* (2010), who isolated these species of bacteria from F⁻ endemic areas of Birbhum District of West Bengal, India.

It is generally observed that the AMF abundance and diversity decreases with persistent abiotic stress (Millar and Bennett 2016; Lin *et al.*, 2020). However, there are certain species of AMF which can endure stress. For example, *G. mosseae* showed best stress tolerant results followed by *G. fasciculatum*, *G. intraradices* and *G. aggregatum* in a study conducted to assess the effects of different *Glomus* fungi on the growth of *Ocimum basilicum* grown under salinity stress (Yadav, 2017). The present study showed a higher frequency of occurrence of *G. indicum* and *G. dimorphicum* indicating that these fungi are not much affected by F⁻ concentration in soil.

This substantiates the earlier finding of Yadav (2017) that *Glomus* sp. are relatively tolerant to stress. More in-depth studies are required to examine the actual mechanism that shapes AMF community in F⁻ contaminated soils.

Irrespective of F⁻ levels, microbial inoculation improved NPK nutrition by rice (Singh *et al.* 2020), which had its reflection on tissue nutrient content. Inoculation of *Azotobacter* improved the nutrient content of plants under no added F⁻ but P content increased in treatments with combined inoculation of bacterium and AMF. A good number of research papers have witnessed the role of AMF in the uptake of relatively immobile nutrients like P in a manner beneficial to crop plants (Tarraf *et al.*, 2017; Frew, 2019). In general, the F⁻ content in plants and grains apparently decrease in inoculation treatments probably due to its removal by immobilization into microbial cells. Polanco-Calvo and Zwazek (2011) reported that mycorrhizal association drastically decreased F⁻ uptake by jack pine seedlings and shoot F⁻. In another study, Sharma *et al.* (2014) noted a significant increase in plant growth and leaf area in tea plants on application of AMF even in the presence of F⁻.

Mycorrhizal inoculation improved the percentage colonization by AMF, which showed a spurt in combined inoculation treatments. Competition between the organisms for C source could be a possible reason for the low level of colonization in these treatments. Yadav (2017) reported that F⁻ toxicity in soil could reduce the spore count and root colonization by *Glomus* fungi.

Conclusion

It can be concluded that the microbial isolates from F⁻ contaminated soils could be used as potential inoculants not only in alleviating fluoride stress but also for improving the nutrition and mycorrhizal percentage colonization of rice grown under F⁻ stress.

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