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Role of *Pseudomonas fluorescence* and *Pseudomonas aeuroginosa* on Antioxidant Parameters, Polyphenols and Total Flavonoids of Flouride (F) Hyperaccumulator Plant *Prosopis juliflora* under F Contaminated Soil

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Abstract

Fluoride pollution is a world wide problem. The presence of the toxic F is critically harmful effects on human health and also the environment. Among existing strategies to remediate F contaminates in soils, phytoremediation approach using F accumulating plants is much convincing in terms of F removal efficiency, but it has many limitations because of slow plant growth and decreased biomass under F-induced stress. Plant Growth Promoting Rhizobacteria (PGPR) play important role in agricultural systems, especially as biofertilizer. Therefore, two rhizobacterial strains *Pseudomonas fluorescence* and *Pseudomonas aeuroginosa* were used to determine the effects of inoculation on growth and antioxidant activity of *Prosopis juliflora* plants under F stress. These two strains, increases growth and uptake of F. Further, increase of superoxide dismutase, catalase and peroxidase activity was also recorded. *Pseudomonas fluorescence* significantly increases the biomass in comparison to *Pseudomonas aeuroginosa*. Present study suggests that the two PGPR strains may be used to improve the soil quality of F contaminated soil.

Key words: Antioxidant status, Plant growth promoting rhizobacteria, *Prosopis juliflora*

Introduction

Fluoride (F) non-metal is one of the main ecological worldwide problems. Fluoride is the most phytotoxic of the common air pollutants, and considered harmful for both humans and plants (Fornasiero 2001). Several reports suggest that, F is an essential element for the normal growth of plants and in higher concentration it is toxic for plants (Weinstein and Davison, 2004). Seed germination and early seedling growth are important phases for the successful growth and survival of plants and these physiological parameters of plants are affected by F stress (Weinstein and Davison 2004). Several physiological and biochemical processes are known to be affected by F such as chlorosis and necrosis of leaf, low nutrient uptake, reduction of plant biomass, and enzymatic activities (Gupta et al., 2009; Chakrabarti and Patra, 2013). Flavonoids substance is most important in plant (Haslam 1989; Rhodes 1994). Flavonoids are a various group of compounds that are produced by different biochemical pathway and possess a wide variety of biological activities. These are of particular interest because of their involvement in the responses of the plant to environmental different stress including: (1) deficiency in nutrients or (2) alterations attributed to ultraviolet (UV) rays or (3) modification by air pollutants (Robles et al. 2003). According to Winkel-Shirley (2001), it was found that flavonoids may play as yet uncharacterized roles in the UV stress response. Flavonoids are beneficial for the plant itself as physiological active compounds, as (1) stress protecting agents, (2) attractants or (3) feeding deterrents, and, in general, by their critical role in plant resistance (Treutter 2006). F has long been accepted as a potent metabolic inhibitor, which interferes with the metabolism of proteins, lipids, and carbohydrates. Although the different mechanism involved in the inhibition is not completely understood, F blocked enzymes that require such cofactors as Ca^{2+}, Mg^{2+}, and Mn^{2+} ions (Wilde and Yu 1998).

Plant growth-promoting rhizobacteria (PGPR) effect on plants by improving growth and enhancing root development, or increasing plant tolerance to various environmental stresses known as soil bacteria (Ahemad and Khan 2011; Bhattacharyya and Jha 2012). Metal phytoremediation, is often facilitated by

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soil microorganisms living in close association with plant roots (Shilev et al., 2001). This contribution of the rhizomicrobial population to phytoremediation of affected metal is usually referred to as rhizoremediation (Kuiper et al., 2004). In hyperaccumulators PGPR can increase the absorption of heavy metals through increasing plant biomass and *Pseudomonas fluorescens* and *Pseudomonas aeuroginosa* is a soil rhizosphere in habitant that can tolerate high concentrations of heavy metals in polluted waters (Wasi et al., 2010).

These studies are considered for the F-resistant bacteria will be able to proliferate and promote plant growth in the presence of high level of toxic F. In the present studies leguminous species (*P. juliflora*) hyperaccumulator plant promoted us to assess the potential use of the F contaminated soil. It has been conducted on the effect of plant growth promoting bacteria on biochemical parameters and antioxidant effect of F hyperaccumulator plant *P. juliflora.*

Material and methods

Soil characteristics before harvesting:

The soil sample was analyzed for C, N, P, K, available Ca, Mg, DTPA extractable Fe, Mn, Zn, and Cu. The organic carbon was estimated by following Walkley and Black's method (1934). Available N was estimated by alkaline permanganate method (Subbiah and Asija, 1956). Available phosphorus were extracted by using Olsen's reagent and estimated through spectrophotometer after developing the blue colour by ascorbic method. Available Potassium was extracted with neutral ammonium acetate and estimated by flame photometer (Schollenberger, 1945). The available calcium and magnesium were estimated by using atomic absorption spectrophotometer after extraction with neutral normal ammonium acetate. The DTPA extractable Fe, Mn, Zn and Cu were estimated by using atomic absorption spectrophotometer (Lindsay, 1978).

Pot experimental design

Prosopis juliflora seeds collected from Central Arid Zone Research Institute (CAZRI) Jodhpur (Rajasthan) India. Seeds were surface sterilized with 20% H_2SO4 for 15 min and were rinsed with deionized sterile millipore water. Seeds were germinated in plastic pots (7-cm diameter and 12-cm height) with 1 kg of the sterilized testing soils. Each pot received eight seeds which were placed at 2-cm depth. Pots were rearranged in the greenhouse chamber. Treatment with F concentrations was 0, 25, 50, and 75 and 100 mg kg^{-1}. Each treatment was subjected to a different type of inoculation: control (no bacteria and no F conc.), PF (*Pseudomonas fluoresence*), and PA (Pseudomonas aeuroginosa) strains. Three replicates were used for each F level inoculation type treatment. Bacterial strain suspension (10^8 cfu mL^{-1}) in nutrient broth was used for the inoculation, by spraying soil surfaces (Marques et al., 2010), 10 days after germination. To the control pots, 10 mL of sterile distilled millipore water was added. Plants were harvested after 16 weeks, separated in roots and shoot and washed with tap water and deionized sterile water. Shoot and root length was determined by measuring scale.

Antioxidant activity

Extraction of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), 1 g of plant tissue was homogenized with 3 ml of 0.1 M of sodium phosphate (NaPO4) buffer (pH 7) in a pre-chilled mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C to collect supernatant for estimation of SOD, CAT, and POD. The activity was measured at 560 nm absorbance of SOD (Beauchamp and Fridivich 1971), CAT was determined by measuring the change of absorbance at 240 nm (Luck et al., 1974) and POD activity was determined by H_2O_2 at 420 nm absorbance through spectrophotometer (Putter et al., 1974).

Determination of polyphenols

The following reagents/buffers were used: 50% methanol 1 M Sodium carbonate Folin-Ciocalteau's reagent (1:10 dilute with double distilled water), the determination of polyphenols was done using the method of Mc Donald et al., 2001. The polyphenol content was determined at 30, 60, 90 and 120 days after inoculation of prosopis juliflora plants with fluoride stress and given microbes treatment. One gm tissue i.e. leaf of the plants were homogenized in 10 ml of 50% methanol. The supernatant was filtered and centrifuged at 5000 rpm in a cooling centrifuge (Remi cooling compufuge, CPR24) for 25 min at 4°C. The pellet was discarded and the supernatant was used for further assay, 0.5 ml of the plant extract was mixed with 5 ml of Folin's reagent and 4 ml of 1 M sodium carbonate. The mixture was allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm (UV-2450 double beam spectrophotometer). The standard curve was prepared using 0, 50, 100, 150, 200 and 250 $mg.l^{-1}$ gallic acid in methanol: water (50:50, v/v). Polyphenol content was

expressed as gallic acid equivalents (mg.g^{-1}fw) which is a common reference compound.

Estimation of total flavonoids

The total flavonoids estimation by following reagents/buffers were used: 95 % ethanol, 10 % aluminum chloride, 1M Potassium acetate. The determination of flavonoids was done using the method of Chang et al. (2002). The flavonoid content was determined at 30, 60, 90 and 120 days. 1 gm leaves were homogenized in 25 ml of 95% ethanol and centrifuged in a cooling centrifuge (Remi cooling compufuge, CPR 24) at 5000 rpm for 25 min at 4˚C. The pellet was discarded and the supernatant was used for further assay. The flavonoid content was determined by aluminum chloride colorimetric method. 0.5 ml of extract was mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was read at 415 nm with a double beam UV-2450 spectrophotometer. Quercetin was used to make the standard curve. Flavonoid synthesis varies and is induced by some ecological factors such as pollution, as evidenced by biotic stressors (Treutter 2006). Ryan et al. (2002) showed that photoprotection plays a predominant role for flavonoids, which was evidenced by a high quercetin/kaempferol ratio in Petunia leaves.

Statistical analysis

Standard deviation (SD) and Spearman's correlation were calculated by statistical package (SPSS 17.0) to examine difference between each treatment at ($P \leq 0.05$).

Result and Discussion

Soil Analysis

The soil had relatively micronutrients were low levels. Concentrations of other metals were also low: Fe (4.96 mg kg^{-1}), Mn (4.22 mg kg^{-1}), Cu (0.53 mg kg-1) and Zn (0.12 mg kg^{-1}). Meanwhile the Concentrations of macronutrients were also low: N (167 mgha^{-1}) P (9.12 mg kg^{-1}), K (16.4 mg kg^{-1}), and Ca (123 mg kg^{-1}), Mg (16.9 mg kg^{-1}), organic carbon (0.65 kg ha^{-1}), E.C (2.36 ohms) and pH (8.8) that was highly salinity.

Table 1. Soil physiochemical characteristics

pH	E.C	OC	N	P	K	Ca	Mg	Fe	Zn	Mn	Cu
8.2	1.34	0.55	127	6.12	12.4	113	11.9	1.96	0.82	3.22	0.73

(E.C-dSm^{-1}) (O.C %), (N, P, K, Ca, Mg-kgha^{-1}), (Fe, Zn, Mn, Cu-mgkg^{-1})

Growth parameters under given treatments

The plants grown on different treated with P.F showed the best growth in (table 2) the values were even greater than the control as compare to P.A. Fluoride cause reduction in root length (12.7 cm) at 100mgkg^{-1} NaF soil and shoot length (10.4 cm) at 100 mgkg^{-1} NaF soil due to unbalanced nutrient uptake by seedlings in the presence of F in soil (Sabal *et al*., 2006). *Pseudomonas fluoresecnce* increased as the rate of (30.86-24.96 cm at control to 25 mgkg^{-1} NaF) root and shoot respectively and the largest reduction was observed in plants treated with 100 mgkg^{-1} NaF. Root dry weight and shoot dry weight increased at (0.165 to 0.293 gm) at 25 to 100 mgkg^{-1} NaF and (0.187 to 0.296 gm) at 25 to 50 mgkg^{-1} NaF respectively and total chlorophyll largest reduction at also 100 mgkg^{-1} NaF.

Table 2. Effect of *Pseudomonas fluorescence* (P.F) and *Pseudomonas aeuroginosa* (P.A) on growth contents of F hyperaacumulator plant *Prosopis juliflora* (under NaF ppm stress).

PARAMETERS						
Treatment	**RL(cm)**	**SL(cm)**	**RDW(gm)**	**SDW(gm)**	**RFW (gm)**	**SFW (gm)**
Control	30.86±7.85	20.60±1.03	0.212±0.00	0.256±0.00	0.255±0.00	0.294±0.01
Control 25 ppm	26.40±0.69	16.66±5.08	0.167±0.00	0.187±0.00	0.191±0.00	0.218±0.03
25 ppm + P.F	34.30±0.69	24.96±0.40	0.280±0.00	0.288±0.00	0.248±0.00	0.305±0.01
Control 50 ppm	24.83±5.31	14.63±5.13	0.180±0.00	0.189±0.00	0.218±0.01	0.237±0.00
50 ppm + P.F	28.33±0.62	20.30±2.07	0.285±0.00	0.296±0.00	0.258±0.00	0.316±0.00
Control 75 ppm	25.33±3.92	14.66±1.61	0.185±0.01	0.192±0.00	0.235±0.00	0.258±0.00
75 ppm + P.F	24.50±0.04	19.50±0.06	0.288±0.00	0.315±0.00	0.259±0.00	0.328±0.01
Control 100 ppm	12.76±0.01	10.43±0.23	0.203±0.01	0.195±0.00	0.247±0.00	0.269±0.00
100 ppm + P.F	19.36±0.75	18.53±0.63	0.293±0.00	0.322±0.00	0.288±0.00	0.349±0.01

Control 25 ppm	26.33±2.54	14.76±1.09	0.165±0.00	0.196±0.00	0.210±0.02	0.210±0.02
25 ppm + P.A	30.23±4.09	22.80±0.51	0.256±0.00	0.247±0.00	0.248±0.00	0.283±0.00
Control 50 ppm	21.73±1.67	12.76±0.05	0.181±0.00	0.206±0.01	0.229±0.01	0.221±0.01
50 ppm + P.A	27.43±0.63	19.13±0.28	0.267±0.00	0.270±0.00	0.257±0.01	0.281±0.00
Control 75 ppm	19.06±1.09	11.86±1.15	0.188±0.00	0.221±0.00	0.244±0.01	0.237±0.00
75 ppm + P.A	24.86±1.27	16.96±0.23	0.281±0.00	0.277±0.00	0.265±0.01	0.343±0.02
Control 100 ppm	17.26±0.40	10.60±0.11	0.204±0.00	0.260±0.05	0.248±0.00	0.259±0.00
100 ppm + P.A	23.96±0.40	16.03±1.78	0.283±0.00	0.291±0.00	0.284±0.01	0.346±0.01

Values are mean of three replicates and ± SD

Antioxidant enzyme mechanism:

The antioxidant activities effect on *P. juliflora* treated of plants to different rhizobacteria (both with and without treatment) led to an increase in the activity of CAT, POD and SOD. The activity of enzymes increased with increase in duration of F stress as well as doses of rhizobacteria (P.F and P.A). Significant relation was observed between the given of microbial treatment in the leaves of *P.juliflora* and activity of CAT, POD and SOD (Table 3).The oxidative stress induced by F in plants appear to be an indirect effect of fluoride toxicity to plant (Kumar *et al*., 2009). Similar results are obtained in plants stressed by the same metal, Cu (Demirevska- Kepova *et al*., 2004), or other metals such us Mn, Pb, Ni and Cd (Gomes-Junior *et al*., 2006).

Table 3: Effect of *Pseudomonas fluorescence* (P.F) and *Pseudomonas aeuroginosa* (P.A) on CAT, SOD and POD in *P. juliflora* leaves after 120 days treatment with under NaF ppm stress.

ANTIOXIDANT ENZYME ACTIVITY			
Treatment	**Peroxidase (µg g^{-1}fw)**	**Catalse (µg g^{-1}fw)**	**Superoxidase(µg g^{-1}fw)**
Control	0.023±0.00	0.033±0.00	0.166±0.00
Control 25 ppm	0.120±0.01	0.156±0.00	0.172±0.01
25 ppm + P.F	0.132±0.00	0.221±0.00	0.232±0.00
Control 50 ppm	0.140±0.00	0.143±0.00	0.180±0.00
50 ppm + P.F	0.159±0.00	0.325±0.00	0.236±0.00
Control 75 ppm	0.176±0.00	0.166±0.00	0.183±0.00
75 ppm + P.F	0.179±0.00	0.440±0.02	0.258±0.00
Control 100 ppm	0.183±0.00	0.185±0.00	0.182±0.00
100 ppm + P.F	0.214±0.00	0.441±0.03	0.262±0.01
Control 25 ppm	0.115±0.00	0.141±0.00	0.173±0.00
25 ppm + P.A	0.114±0.00	0.228±0.00	0.184±0.00
Control 50 ppm	0.125±0.00	0.156±0.00	0.174±0.01
50 ppm + P.A	0.118±0.01	0.236±0.00	0.198±0.00
Control 75 ppm	0.135±0.00	0.243±0.02	0.178±0.01
75 ppm + P.A	0.129±0.01	0.325±0.01	0.222±0.01
Control 100 ppm	0.140±0.00	0.239±0.04	0.191±0.00
100 ppm + P.A	0.136±0.02	0.370±0.02	0.248±0.00

Values are mean of three replicates and ± SD

Total polyphenols estimation

Total polyphenol content in the samples used in this study ranged from 56.45 to 156.78 mgkg^{-1} (Fig. 1). Highest total polyphenol content 156.78 mgkg^{-1} and the lowest total polyphenol content 78.9 mgkg^{-1}.The average total polyphenol content was 119.70 mgkg^{-1} leaves. However, there was no significant difference between the polyphenol content in plant. This decrease in polyphenol content is not considered beneficial.

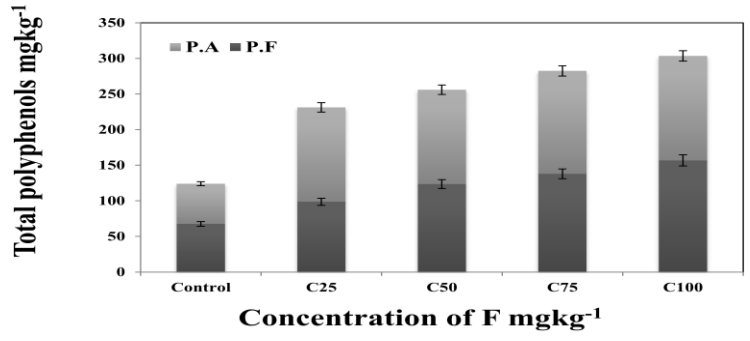

Figure 1. Effect of *Pseudomonas fluorescence* (P.F) and *Pseudomonas aeuroginosa* (P.A) on total polyphenols under different concentration of NaF

Total flavonoids analysis

Total flavonoids contents were in the sample range 55.34 mgkg^{-1} at 100 mgkg^{-1} NaF to 76.14 mgkg^{-1} (Fig. 2) in control same as in further case of P.F and 67.87 mgkg^{-1} in P.A to 56.44 mgkg^{-1}. Coberly and Rausher (2003) indicated that flavonoids are consideration to function in plant stress responses but not all, species. Some investigators postulated that antioxidant flavonoids exert protective functions during droughts. Flavonoids also help plants to live on soils that are rich in toxic metals such as Aluminum (Barcelo and Poschenrieder 2002). Reddy and Kaur (2008) showed higher concentrations of sodium fluoride decreased photosynthetic pigments (chlorophylls and carotenoids) content in Salicornia brachiata while, anthocyanin content increased significantly. It is suggest that F compounds inhibit or activate some important enzymes for flavonoid synthesis. In this study, it is clear that variation in leaf flavonoid profiles in polluted plants is a response to F pollutants and may have a protecting defensive position against F pollution.

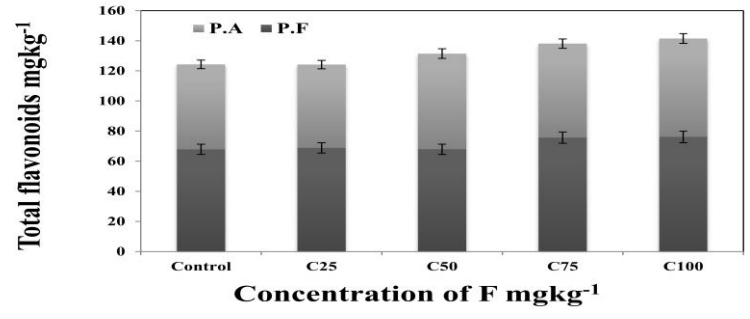

Figure 2. Effect of *Pseudomonas fluorescence* (P.F) and *Pseudomonas aeuroginosa* (P.A) on flavonoids under different concentration of NaF

Conclusion

Plant growth support abilities of *Pseudomonas fluorescence* and *Pseudomonas aeuroginosa* were shown in the present study, and thus, these heavy metal resistant bacteria improve F accumulation abilities by *P. juliflora*. The *P. juliflora* plant can become stable the soil during its growth cycle, as metals are mainly retained with F in the root area. The increase in shoot and root biomass observed for inoculated plants grown in the F contaminated soils potentiates the application of the produced biomass for energy purposes after harvest of the above ground tissues. The roots will remain in the soil, adding organic matter and nutrients to it, which can help further increase immobilizing activity of the F. At the same time, an even with high translocation to the shoots, may be large amounts of the F would be extracted from the soil, this soil less polluted.

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Abbreviations

F, fluoride; P.A, *Pseudomonas aeruginosa*; P.F, *Pseudomonas fluorescens*; RL, root length; RDW, root dry weight; SL, shoot length; SDW, shoot dry weight; N, nitrogen; P, phosphorus; K, potassium; Fe, iron; Mn, manganese; Zn, zinc; Cu, copper; DTPA, diethylene triamine penta acetic acid.

Reference

1. **Beauchamp, C. and Fridivich, I.** (1971). Superoxide dismutase improved assay and an assay applicable to acrylamide gels. Analy. Biochem., **44(1)**:267-87.

2. **Barcelo, J. and Poschenrieder, C.** (2002). Fast root growth responses, root exudates, and internal detoxification as clues to the mechanism of aluminium toxicity and resistance, a review. Environ. Experim.Bot., **48**:75-92.

3. **Chakrabarti, S. and Patra, P.K.** (2013). Effect of fluoride on superoxide dismutase activity in four common crop plants. Fluoride., **46(2)**:59-62.

4. **Demirevska-Kepova, K., Simova-Stoilova, L., Stoyanova, Z., Hölzer, R. and Feller, U.** (2004). Biochemical changes in barley plants after excessive supply of copper and manganese. Environ. Exp. Bot., **52**:253-266.

5. **Fornasiero, R.B.** (2001). Phytotoxic effects of fluorides. Plant Science **161**:979-85.

6. **Glick, B.R.**(2010). Using soil bacteria to facilitate phytoremediation Biotech. Advan., **28**:367-374.

7. **Gomes-Juniora, R.A., Moldesa, C.A., Delitea, F.S., Gratãoa, P.L., Mazzaferab, P., Leac, P.J. and Azevedoa, R.A.** (2006). Nickel elicits a fast antioxidant response in *Coffea arabica* cells. Plant Physiol. Biochem., **44**:420-429.

8. **Gupta, S. and Banerjee, S.** (2009). Fluoride accumulation in paddy (*Oryza sativa*) irrigated with fluoride contaminated ground water in an endemic area of the Birbhum District, West Bengal, **42**:224-227.

9. **Gupta, S., Banerjee, S. and Mondal, S.** (2009). Phytotoxicity of fluoride in the germination of paddy (*Oryza sativa*) and its effect on the physiology and biochemistry of germinated seedlings. Fluoride., **42(2)**:142-6.

10. **Jha, S.K., Nayak, A.K. and Sharma, Y.K.** (2009). Fluoride toxicity effect in onion (Allium cepa L) grown in contaminated soils. Chemosphere., **60**:1493-1496.

11. **Kuiper, I., Lagendijk, E.L., Bloemberg, G.V. and Lugtenberg, T.J.J.** (2004). 'Rhizore-mediation: a beneficial plant-microbe interaction' Mol. Plant Microbiol. Interact., **17**:6-15.

12. **Kumar, K.A., Varaprasad, P. and Rao, A.V.B.** (2009). Effect of fluoride on catalase, guiacol peroxidase and ascorbate oxidase activities in two varities of mulberry leaves (*Morus alba L.*)' Res. J. Earth Sci., **1**:69-73.

13. **Lindsay, W.L. and Norvell, W.A.** (1978). Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Science Amer. J., **42:**421-428.

14. **Luck, H**. (1974). *In*: Methods in Enzymatic Analysis 2 nd Ed. Bergmeyer Academic Press New York., 885.

15. **Marques, A.P.G.C., Pires, C., Moreira, H., Rangel, A.O.S.S. and Castro, P.M.L.** (2010). 'Assessment of the plant growth promotion abilities of six bacterial species using *Zea mays* as indicator plant' Soil Biol. Biochem., **42**:1229-1235.

16. **Putter, J.** (1974). *In*: Methods of Enzymatic Analysis 2 nd Ed. Bergmeyer Academic Press New York., 685.

17. **Rhodes, M.J.C.** (1994). Physical role for secondary metabolites in plants: Some progress, many outstanding problems. Plant. Mol. Biol., **24**:1-20.

18. **Robles, C., Greff, S., Pasqualini, V., Garzino, S., Bousquet-Melou, A., Fernandez, C., Korboulewsky, N. and Bonin, G.** (2003). Phenols and flavonoids in Aleppo pine needles as bioindicators of air pollution. J. of Environ. Qual., **32**:2265-71.

19. **Sabal, D., Khan, T.I., and Saxena, R.** (2006). Efeect of fluoride on cluster bean (*Cyamopsis Tetragonoloba*) seed germination and seedling growth Fluoride., **39**:228-230.

20. **Schollenberger, C.J. and Simon, R.H.** (1945). Determination of exchange capacity and exchangeable bases in soil-ammonium acetate method. Soil Sci*.,* **59:**13-24.

21. **Shilev, S.I., Ruso, J., Puig, A., Benlloch, M., Jorrin, J. and Sancho, E.** (2001). 'Rhizospheric bacteria promote sunflower (*Helianthus annus L.*) plant growth and tolerance to heavy metals' Minerva Biotech., **13**:37-39.

22. **Subbiah, B.V. and Bajaj, J.C.** (1962). A soil test procedure for assessment of available nitrogen in rice soils. Curr.Sci., 31:196.

23. **Walkley, A. and Black, C.A.** (1934). An examination of different methods for determining soil organic matter ana a proposed modification of the chromic acid titration method. Soil Sci., **37:**29-38.

24. **Wasi, S., Tabrez, S. and Ahmad, M.** (2010). Suitability of immobilized *Pseudomonas fluorescens* SM1 strain for remediation of phenols, heavy metals, and pesticides from water. Wat. Air. Soil Pollut., **220**:89-99.

25. **Weinstein, L.H. and Davison, A.W.** (2004). Fluorides in the environment: effects on plants and animals Wallingford, Oxon, UK: CABI Publishing.

26. **Winkel-Shirley, B.** (2001). Flavonoid biosynthesis: A colorful model for genetics, biochemistry, cell biology and biotechnology. Plant Physiol., **126**:485-93.

27. **Wilde, L.G., and Yu, M.** (1998). Effect of fluoride on superoxide dismutase (SOD) activity in germinating mung bean seedling. Fluoride., **31**:81-8.