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EVALUATION OF ZINC SOLUBILIZATION CAPACITY OF DIFFERENT MICROBIAL STRAINS AND THEIR EFFECT ON *BT* COTTON

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Abstract- The experiment was conducted to determine the degree of zinc (Zn) solubilization using different microbial isolates in lab experiment and their effect on growth and yield of *B*t cotton in pot culture experiment. Zinc solubilizing ability of seven microbial strains were determined using zinc oxide, zinc carbonate and zinc phosphate in both plate and broth media assays. Results of plate assay indicated that the microbial strain *Burkholderia cenocepacia* produced the highest clearing zone in zinc oxide containing medium. Whereas, *Pseudomonas striata*, *Bacillus megaterium* and *Trichoderma viride* formed the highest clearing zone with zinc carbonate containing medium as compared to other microbial isolates. Results of broth assay revealed that the microbial strain *Bacillus megaterium* showed significantly highest Zn solubilization followed by *Trichoderma viride* and *Pseudomonas striata* in $ZnCO_3$ amended media. Whereas, lowest Zn solubilization was observed in control with ZnO amendment. The maximum reduction in pH was also observed in different inoculated medium. Pot culture experiment indicated that inoculation of zinc solubilizing microbial isolates considerably enhanced the growth parameters, chlorophyll content and zinc in *Bt* cotton when compared to uninoculated control. The highest improvement in growth characters, chlorophyll content and zinc was noted in *Trichoderma viride, Pseudomonas striata* and *Bacillus megaterium* treated pots.

Keywords- Zinc sources, microbial isolates, clearing zone, zinc solubilization, *Bt* cotton, growth attributes, root characters, zinc uptake.

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Introduction

Zinc plays an important role in nutrition of both eukaryotic and prokaryotic organisms as cofactor or metal activator in various enzyme systems. Micronutrients are important for the growth of plants, animals and also for the microbes. Among these, Zinc is important for the enzyme activity such as carbonic anhydrase. Zinc is one of the important micronutrient essential for the growth and reproduction of plants. Further, Zinc is involved in carbohydrate metabolism, protein synthesis, gene expression, auxin (growth regulator) metabolism, pollen formation, maintenance of biological membranes, protection against photo-oxidative damage and heat stress and resistance to infection by certain pathogens.

The zinc deficiency scenario is somehow analogues to the phosphorus deficiency, since both the nutrients are deficient in Indian soils with an average of about 40 percent [11,18]. Application of water soluble zinc as zinc sulphate gets transformed into different forms like Zn(OH) and $Zn(OH)_2$ at a pH of 7.7 and 9.1 [20]; $ZnCO_3$ in calcium rich alkali soils [10]; $Zn_3(PO_4)_2$ in near neutral to alkali soils of high phosphorus application and zinc sulphide under reduced conditions. These forms of zinc become unavailable for plant growth. Thus, microorganisms are potential alternate that could cater plant zinc requirement by converting the unavailable zinc into available form in soil. The organic based zinc nutrition is best since its Zn use efficiency is more. Micronutrient deficiency problems can be solved by using the different microbial inoculants. In soil, it undergoes a complex dynamic equilibrium of solubilization and precipitation that is greatly influenced by the soil pH and micro flora and that ultimately affects their accessibility to plant roots for absorption [2]. As Sarathambal *et al*., [15] reported that the basic principle behind the solubilization of zinc is decreasing the pH and making zinc soluble and as a consequence the available zinc will get increased in the soil system. A term called zinc solubilizing microorganisms (ZSM) was coined for those that are capable of solubilizing the insoluble zinc compounds/minerals in agar plate as well as in soil. In our study, seven microbial species was used to tested the zinc solubilization in both plate and broth assay in laboratory. Further, these seven promising [13] Zn solubilizing microorganisms were tested for growth promotion and Zn uptake under pot culture experiment.

Materials and Methods

Laboratory experiment

Seven microbial isolates (*Burkholderia cepacia, Burkholderia cenocepacia, Pseudomonas fluorescens, Pseudomonas striata, Trichoderma viride, Trichoderma harzanium, Bacillus megaterium,*) were selected based on the zinc solubilizing zone formation were procured from Central Research Institute for Dry land Agriculture (CRIDA), Hyderabad, Department of Plant Pathology and All India Network Project on Soil Biodiversity-Biofertilizers Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani. The solubilization potential was evaluated in both plate and broth media assay.

To determine the zinc solubilization capacity of the microbial strains, they were subjected on PKV medium supplemented with D-glucose and different insoluble zinc compounds by following the protocol of Bapiri *et al*. [5]. The microbial isolates were inoculated into modified PKV medium (ingredients g L^{-1}) glucose-10.0 g; Ammonium Sulphate-1.0 g; Potassium Chloride-0.2 g; Dipotassium Hydrogen Phosphate-0.2 g; Magnesium Sulphate-0.1g; Yeast-0.2 g; distilled water -1000 ml, pH 7.0) containing 0.1% insoluble zinc compounds. In plate assay, the PKV medium was separately supplemented with insoluble zinc compounds at concentration of 0.1 %, as zinc oxide [1.244 g/L], zinc carbonate [1.913 g/L] and zinc phosphate [5.904 g/L]. After sterilization and plating, fresh cultures of microbial species were inoculated on the media using sterile toothpicks in three replications. The inoculated plates were incubated at 28°C for 3 days in dark for observing halo zone formation around colonies. Further, for broth assay, take 100

ml of liquid PKV medium in 250 ml Erlenmeyer flasks, which was separately supplemented with three insoluble zinc compounds at 0.1% zinc in the form of zinc oxide, zinc carbonate and zinc phosphate. Then, the media was steam sterilized for 30 minutes in autoclave. Thereafter it was inoculated with 0.1 ml aliquot of each microbial culture. Three flasks were maintained with an uninoculated control for each treatment. Experiments were done in three replications. The inoculated media was kept for three days. After 3 days the samples were withdrawn, centrifuged to remove the debris and cells. One ml of this solution was directly fed to Atomic Absorption Spectrophotometer (AAS) to determine the soluble Zn content. The pH of the isolates and the uninoculated samples were determined after 3 days of inoculation. The culture was filtered using Whatman No. 42 filter paper. The pH was measured using pH meter.

Pot culture experiment

Short term pot culture experiment was carried out to study the bio efficacy of promising zinc solubilizing microbial isolates using *Bt* cotton as test crop. The experiment was conducted Department of Soil Science and Agricultural Chemistry, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani during 2013. The soil used for pot culture experiment was zinc deficient (0.55 mg kg^{-1}) identical to that of *Typic Haplusterts* (Vertisol) of Parbhani series in Maharashtra. The soil was air dried, sieved and sterilized at 121^0 C for 1 hour for 3 consecutive days and filled in the pots of 8 kg holding capacity. Hoagland solution was used as a nutrient source. Tenth day after sowing 24hrs old fresh microbial isolates (*Burkholderia cepacia, Burkholderia cenocepacia, Pseudomonas fluorescens, Pseudomonas striata, Bacillus megaterium, Trichoderma viride, Trichoderma harzianum*) inoculated at 10 ml per pot as per the treatment. The experiment was laid out in a completely randomised design (CRD) with three replications. The growth parameters like plant height and number of leaves were recorded at 30, 60 and 90 days after sowing. The experiment was terminated after 90 days. Chlorophyll content was determined as per Hiscox and Israelstan [7]. Zn content in plant was determined as per procedure given by Jackson (1973) and uptake was calculated.

Statistical analysis

The data obtained from the laboratory experiment was statistically analysed by factorial completely randomised design whereas, data from pot culture was done by completely randomised design as per the methods described in "Statistical Methods for Agricultural Workers" by Panse and Sukhatme [12]. Appropriate standard error (S.E.) and critical differences (C.D.) at 5% level were worked out as and when necessary and used for data interpretation.

Results and Discussion

Laboratory experiment

In laboratory experiment, results of plate assay indicated that all the seven microbial isolates tested for the zinc solubilization produced a clear halo zone on PKV medium amended separately with three insoluble zinc compounds (zinc oxide, zinc carbonate and zinc phosphate) at 0.1% zinc concentration [Table-1]. The *Burkholderia cenocepacia* produced the highest clearing zone in zinc oxide containing medium (2.03 cm). Whereas, among the zinc carbonate containing medium, *Pseudomonas striata* (2.03 cm), *Bacillus megaterium* (1.86 cm) and *Trichoderma viride* (1.76 cm) formed the highest clearing zone as compared to other microbial isolates. However, zinc phosphate showed highest solubilizing potential in *Pseudomonas fluorescens* with clearing zone of 0.93 cm. The solubilization of zinc by these microbial strains from insoluble zinc sources might be a due to the different mechanisms such as proton extrusion, production of organic acids and production of chelating agents. Our results are also correlate with the findings of Agusto de Costa and Duta [1] they show that the such solubilization of zinc compounds mediated through production of organic acids and subsequent release of zinc in external environment and bioaccumulation of zinc inside cells of bacterial species.

In broth assay [Table-2] after 3rd day of inoculation, *Bacillus megaterium* showed significantly highest Zn solubilization (294.33 mg L^{-1}) followed by *Trichoderma viride* (293.33 mg L^{-1}), *Pseudomonas striata* (285.67 mg L^{-1}) in $ZnCO_3$ amendments respectively. Whereas, lowest Zn solubilization was observed in control (80.0 mg L^{-1}) with ZnO amendment. As data presented in [Table-3], it was noted that available zinc in broth increased with the decrease in pH after 3 days of inoculation. At the end of third day, the maximum reduction in pH was observed in *Bacillus megaterium* (4.79) supernatant in zinc phosphate supplemented medium which was at par with *Trichoderma viride* in $Zn_3(PO_4)_2$ and *Bacillus megaterium* in $ZnCO_3$ supplemented medium and superior over other treatments. While, minimum reduction was noted in uninoculated control (7.14) treatment. Our results showed a different solubilization potential that were found among the different microbial isolates in ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$ containing media. As Bapiri *et al*., [5] reported that this might be related to differences in genomics and plasmid properties of strain that affected by the location from which they were isolated. Similarly, in our experiment all strains showed higher solubilising ability in $ZnCO_3$ containing medium. It might be due to because of they were isolated from calcareous soils, presently a higher potential than other Zn containing chemical. Substrates, making these adherence with the carbonate particles capable of solubilising zinc carbonate and also it is depends on the chemical properties of $ZnCO_3$ that easier than others affected by acidic exudates of bacteria [5]. Dissolution of the zinc carbonate, zinc oxide and zinc phosphate may be due to production of organic acids, like gluconic acid. The zinc phosphate solubilization by *Pseudomonas fluorescens* was investigated by Di Simine *et al*., [6]. They found that gluconic acid and 2 Keto gluconic acids produced in the culture broth helped in the solubilization of the zinc salts. Acidic environments, such as those of the present investigation, revealed that the expected mechanism by which gluconic acid is able to dissolve insoluble metal compounds is mainly by acidification, gluconic acid which is known as a metal-chelating agent. In acidic environments, such as *Bacillus megaterium*, *Pseudomonas fluorescens*, *Pseudomonas striata*, *Burkholderia cepacia* and *Burkholderia cenocepacia*, these microbial cultures showed drop in pH, which indicated that organic acid, might be involved. Thus, the obtainment of an elite culture or a consortium of strains capable of utilizing different unavailable insoluble forms of zinc and tolerant to higher zinc level may be useful to make zinc available in the soil system [16]. The fungi exhibited good solubilization potential as seen in case of *Fusarium* sp. and sterile mycelium [14].

Table-1 *Effect of various microorganisms and insoluble zinc compounds on clearing zone (cm)*

Microbial inoculants	Zinc source at 0.1%			
	ZnO	$ZnCO_3$	$Zn_3(PO_4)_2$	Mean
T_1 : *Burkholderia cepacia*	0.90	0.56	0.80	0.75
T_2 : *Burkholderia cenocepacia*	2.03	0.33	0.50	0.95
T_3 : *Pseudomonas fluorescens*	1.65	0.63	0.93	1.07
T_4 : *Pseudomonas striata*	1.93	2.03	0.73	1.56
T_5 : *Trichoderma viride*	1.86	1.76	0.90	1.51
T_6 : *Trichoderma harzianum*	1.23	2.0	0.80	1.34
T_7 : *Bacillus megaterium*	1.0	1.86	0.93	1.26
Mean	1.51	1.31	0.80	
	Z	T	Z x T	
SE±	0.013	0.020	0.035	
CD at 1%	0.037	0.056	0.098	

Table-2 *Effect of various microorganisms and insoluble zinc compounds on soluble zinc (mg L^{-1}) in broth*

Microbial inoculants	Zinc source at 0.1%			
	ZnO	$ZnCO_3$	$Zn_3(PO_4)_2$	Mean
T_1: Uninoculated control	80.0	88.33	83.33	83.88
T_2: *Burkholderia cepacia*	164.33	131.67	161.67	152.56
T_3 : *Burkholderia cenocepacia*	158.67	141.33	161.00	153.67
T_4 : *Pseudomonas fluorescens*	131.33	269.67	282.0	227.67
T_5 : *Pseudomonas striata*	129.33	285.67	205.0	206.67
T_6 : *Trichoderma viride*	187.33	293.33	240.0	240.22
T_7 : *Trichoderma harzianum*	160.0	243.33	234.33	212.56
T_8 : *Bacillus megaterium*	154.33	294.33	273.67	240.78
Mean	145.67	218.46	205.12	
	Z	T	Z x T	
SE±	1.40	2.30	3.98	
CD at 1%	3.89	6.36	11.03	

Pot culture experiment

Plant growth characters

The data pertaining to crop growth characters in cotton are presented in [Table-4]. Growth characters such as plant height and number of leaves increased significantly with inoculation of microbial strain *Trichoderma viride* with an increase of 26.42 and 36.57 per cent over other strains and uninoculated control treatment. These results are in conformity with Azarmi *et al*., [3] who reported that the *Trichoderma* inoculation is important for the success of seedling growth improvement. Different microorganisms produce plant growth regulators, which were considered another important mechanism often, associated with growth stimulation [22]. In the growth hormone, IAA production plays a crucial role in plant growth promotion. Better increment in plant height was also noted with *F. mosseal* + *A. laevis* + *T. viride* followed by combination of *F. mosseal* + *A. leavies* + *T. viride* + *P. fluorescens* in cotton [4]. Further, it may be due to certain plant growth hormones and secondary metabolites produced by *Trichoderma* [19].

Table-3 *Effect of various microorganisms and insoluble zinc compounds on change in pH in broth*

Microbial inoculants	Zinc source at 0.1%			
	ZnO	$ZnCO_3$	$Zn_3(PO_4)_2$	Mean
T_1: Uninoculated control	7.12	7.14	6.59	6.95
T_2: *Burkholderia cepacia*	7.21	6.48	5.03	6.24
T_3 : *Burkholderia cenocepacia*	6.90	5.93	5.16	6.00
T_4 : *Pseudomonas fluorescens*	6.22	5.30	5.14	5.55
T_5 : *Pseudomonas striata*	7.02	5.68	6.08	6.26
T_6 : *Trichoderma viride*	7.12	6.05	4.91	6.02
T_7 : *Trichoderma harzianum*	6.88	6.69	6.05	6.54
T_8 : *Bacillus megaterium*	7.02	4.92	4.79	5.57
Mean	6.93	6.02	5.47	
	Z	T	Z x T	
SE±	0.06	0.10	0.17	
CD at 1%	0.17	0.28	0.49	

Shoot and root characters

Shoot and root dry weights were found to be significantly increased with the inoculation of microbial strain *Trichoderma viride* followed by *Pseudomonas striata* and *Bacillus megaterium*. It is envisaged from the data [Table-4] that biomass of all the inoculated plants of *Bt* cotton increased significantly in terms of dry shoot and root weight after 90 days of inoculation. It may be due to more absorption of nutrients *via* increase in root surface area. Maximum increment in shoot biomass was recorded in *F. mosseae* + A. *leavis* + *T. viride* followed by combination of *F. mosseae* + *A. leavies* + *T. viride* + *P. fluorescens* [4]. Our results are also corresponds with Shanmuhaiah *et al*., [17] they reported that the inoculated strains of *P. fluorescens* produced several plant growth promoting phytohormones including indole – 3 acetic acid. Also the introduction of *Trichoderma* strains with or without pathogen did not affect existing beneficial populations which might be results of slightly deleterious effect of this strain causing increased root leakage / damage, which allows a greater population of aggressive rhizosphere and root colonizers such as *P. fluorescens* and *T. viride*.

Chlorophyll content

It is evident from the given data [Table-5] that the highest increase in chlorophyll content due to the inoculation of *Trichoderma viride* was observed but found at par with *Bacillus megaterium*, *Pseudomonas striata* and *Trichoderma harzianum* and superior over the control. Increase in chlorophyll content indicates the increase in rate of photosynthesis, which can be ascribed to more absorption of nutrients. Similar kind of growth and development of crops was observed by Yadav and Aggarwal [23] who noted that inoculation of *G. Mosseal* + *T. viride* + *P. fluorescens* showed significant increase in chlorophyll content over control. Similar results were also corroborate with Azarmi *et al.,* [3] they reported that leaf chlorophyll was significantly increased in the *Trichoderma sp*. fortified treatment. Further, Singh *et al.,* [19] reported that the total chlorophyll content was also significantly higher in *cryseobacterium* sp PSR-10 inoculated plants under both the sterilized and unsterilized soil system.

Table-4 *Effect of different microbial inoculants on growth characters of Bt cotton*

Treatments	Growth characters			
	Plant height (cm)	Number of leaves	Shoot dry weight (g)	Root dry weight (g)
T_1: Control	38.07	13.67	10.57	1.12
T_2: *Burkholderia cepacia*	42.40	15.67	12.07	1.27
T_3: *Burkholderia cenocepacia*	41.67	15.00	11.93	1.26
T_4: *Pseudomonas fluorescens*	45.57	18.00	14.30	1.19
T_5: *Pseudomonas striata*	47.80	18.00	17.50	1.43
T_6: *Trichoderma viride*	48.13	18.67	19.13	1.56
T_7: *Trichoderma harzianum*	44.53	16.33	13.70	1.29
T_8: *Bacillus megaterium*	46.60	18.00	18.30	1.52
Grand Mean	44.34	16.66	14.68	1.33
SEm±	2.62	1.05	0.86	0.06
CD at 5%	7.65	3.07	2.52	0.17
CV %	7.23	7.72	7.21	5.50

Zinc content and uptake

Zinc content and uptake of *Bt* cotton was significantly affected due to the inoculation of different microbial inoculants [Table-5]. Results observed that the strain *Trichoderma viride* significantly improved plant zinc content (61.74 mg kg^{-1}) and uptake (1.181 mg pot^{-1}) followed by *Pseudomonas striata* (1.049 mg pot^{-1}) and *Bacillus megaterium* as compared to strain *Burkholderia cenocepacia* and un-inoculated control. The magnitude of increase in zinc content with inoculation of *Trichoderma viride* being about 26.10 per cent, respectively than that of control treatment. Similar findings are concurrent with the results obtained by Vaid *et al.,* [21] who found that bacterial inoculation enhanced the Zn concentration and its uptake in wheat. When plants are treated with *Trichoderma*, root system produces some organic acids in the rhizosphere such as gluconic, citric and or fumaric acids which decreases soil pH leads to increased solubility of the insoluble compound and an availability of micronutrients as well as an increase in plant nutrient uptake. Similar results are also reported by Azarmi *et al.,* [3].

Table-5 *Effect of various microorganisms on chlorophyll and zinc content in Bt cotton*

Treatments	Chlorophyll content			Zinc in plant	
	Chlorophyll a (mg g^{-1} fw*)	Chlorophyll b (mg g^{-1} fw*)	Total Chlorophyll (mg g^{-1} fw*)	Zn content (mg kg^{-1})	Zn uptake (mg pot^{-1})
T_1: Control	2.17	0.90	3.09	48.96	0.517
T_2: *Burkholderia cepacia*	2.28	1.00	3.32	58.30	0.704
T_3:*Burkholderia cenocepacia*	2.24	0.93	3.29	52.97	0.633
T_4:*Pseudomonas fluorescens*	2.26	1.05	3.43	54.58	0.781
T_5: *Pseudomonas striata*	2.34	1.20	3.55	59.97	1.049
T_6:*Trichoderma viride*	2.37	1.26	3.60	61.74	1.181
T_7:*Trichoderma harzianum*	2.30	1.17	3.43	56.39	0.772
T_8: *Bacillus megaterium*	2.35	1.23	3.62	57.28	1.049
Grand Mean	2.29	1.09	3.41	56.27	0.835
SEm±	0.05	0.06	0.14	2.05	0.04
CD at 5%	0.15	0.17	0.43	6.00	0.111
CV %	2.78	6.65	5.29	4.47	5.61

*fw- fresh weight of plant tissue

Increment in growth and nutrient uptake by plant was might be due to the result of partly Zn solubilization and IAA and gluconic acid production by the microbial cultures. Further, Jayant Raman [9] noted that the treatment combination such as *Azotobacter chrococcum*, *Pseudomonas striata* and *Trichoderma viride* had the highest content and uptake of Zn.

Conclusion

In laboratory experiment, the microbial strains namely, *Bacillus megaterium* and *Trichoderma viride* showed highest Zn solubilizing ability by lowering down the pH due to acid production. Whereas, in pot culture experiment, microbial strain *Trichoderma viride and Pseudomonas striata* showed significant enhancement in growth characters such as plant height, number of leaves, chlorophyll content, dry weight of shoot and root. Also these inoculations increased the zinc content and uptake by *Bt* cotton crop.

Conflict of Interest: None declared

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