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Effect of Impregnated Valorised Biogenic Apatite with Microbial Culture Filtrates on Seed Germination and Seedling Growth of Mustard (*Brassica juncea* L.)

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ABSTRACT

Oliseed crops (*Brassica napus* and *B. juncea*) are the most important *rabi* oilseeds in India, following groundnut in the oilseed economy. The present study aimed to examine differential response of culture filtrates of phosphate solubilising microbes enriched with bonemeal to improve seed germination and seedling vigour of mustard. The culture filtrates of each organism was prepared by using PVK broth medium and using bonemeal as a P-source which again supplemented with chlorides and sulphates, respectively. The seeds of mustard were treated in each culture filtrate and kept overnight and then next day arranged in germination paper containing 200 seeds each. Seeds were soaked in sterilized water only serve as control. In experimental results maximum P-solubilization efficiency was shown by *A. niger* followed by *B. subtillis, B. amyloliquifaciens, V. laecani* and *T. harzianum* in broth. As the P-solubilization efficiency of microbes also increases gradually increases doses of bone meal. It is also found that the seed treated with culture filtrate obtained from Cl- and SO_4^{2-} media containing 5g and 15g of bone meal which were inoculated with organisms shown very reliable progress which was recorded on germination percentage, seedling vigour, seedling length and root length.

Keywords Mustard, Seed germination, Phosphate solubilization, Trichoderma, Bonemeal.

INTRODUCTION

Rapeseed (*Brassica napus*) and mustard (*Brassica juncea* L.) are the most important *rabi* oilseeds in India, following groundnut in the oilseed economy. These crops are the major edible oils in India's northern and eastern regions. It is widely cultivated in tropical and sub-tropical areas of the world. Globally, it is mainly cultivated in India, Canada, China, Pakistan, Poland, Bangladesh, Sweden and France which is covering about 35% area of total cultivated area of world. Globally, India shares with 16% in production and rank fifth in mustard production with fourth key consuming country of mustard oil (Darekar and Reddy 2018). Mustard cultivation and yield is depending on the many biotic and abiotic factors

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besides availability of nutrients in soils. However, readily available phosphoric nutrient to plant is not very frequent because of its immobile nature in soil. The role of phosphorus (P) in plant metabolism have a significant role, as it is involved in energy transmission, respiration, and photosynthesis. It plays an important structural role in nucleic acids, co-enzymes, phosphoproteins, and phospholipids (Solanki *et al.* 2018). In Indian agriculture, phosphorus has been referred to as the "king pin" and is one of the "big three" nutrients for crops. In crop production, it is frequently referred to as the "master key" component. It is involved in almost every major metabolic function in plants, including photosynthesis, energy transmission, signal transduction and macromolecular biosynthesis (Khan *et al.* 2010). Phosphorus (P) is meager available for plants directly because of its non-bioavailability form in soil. Microbial communities are helping to make available soil P due to their involvement in solubilization process and also in utilization by plants for their various metabolic activities (Vessey 2003). In areas where mustard is traditionally grown without P, poor growth and low yields are common features. Further, phosphorus improves seed size, stimulates proper seed filling and increases oil content (Solanki *et al.* 2018).

Phosphate solubilizing micro-organisms are distributed well in several ecosystems. Many microbes like- fungi (*Aspergillus, Penicillium, Mucor, Trichoderma)*, bacteria (*Bacillus, Pseudomonas, Micrococcus, Flavobacterium*) and actinomycetes (*Streptomyces*) have been isolated, which have consistent capacity to solubilize insoluble phosphorus such a rock phosphate, tricalcium phosphate, ((Bardiya and Gaur 1972, Gaur and Pareek 1974) and also from bones of dead animals. Phosphate solubilizing microbes promote seed germination and initial vigour of the plants by producing growth promoting substances (Kumar *et al.* 2020). Bio-priming helps seeds to germinate uniformly, even under adverse conditions. One conceivable approach of using phosphate solubilizing microbes by seed biopriming which helps in seedling development as well as root framework development (Comejo *et al.* 2014, Prasad *et al.* 2022). In addition to supplement of soluble form of phosphorus obtained from fish bones directly with seed bio-priming and/or soil treatment will play an important role. Though, most of the microbes are not worked well under adverse field conditions. Therefore, an experiment was conceived with aimed to study the effect of various microbes' culture filtrate alongwith valorised biogenic apatite (bone) produced in two different media and its application on seed germination and seedling growth of mustard.

MATERIALS AND METHODS

Collection and maintenance of P-solubilizing micro-organisms: Bacterial and fungal cultures were obtained from Division of Plant Pathology, ICAR-IARI, New Delhi. These isolates of bacteria and fungi were multiplied particularly on nutrient agar medium and potato dextrose agar medium and incubated at 25±1°C, respectively before use in broth.

Determination of phosphate solubilization efficiency of bacteria and fungi in broth : The Pikovskaya (1948) medium was prepared with slight modification which is composed of: 10 g of glucose, 0.5 g $(NH_4)_2SO_4$, 0.2g NaCl, 0.1g $MgSO_4.7H_2O$, 0.2 KCl, 0.002g $MnSO_4.7H_2O$, 0.002g $FeSO_4.7H_2O$, 0.5g yeast extract (per liter of distilled water). Further, another broth was prepared with same procedure and ingredients but chlorides (NaCl and KCl) were replaced with sulphates (0.245g Na_2SO_4 and 0.235g K_2SO_4). Phosphorus solubilization experiment was conducted on three doses of fish bones powder (5g, 15 g) in both media mentioned above with Chlorides and sulphates separately. The fish bones powder was obtained after pulverization from a coastal area farmer district Junagarh, Gujarat, India.

Each medium containing chlorides and sulphate was enriched with fish bones powder in two doses (5g and 15g) separately and both media were filled into the flasks of 250 ml then autoclaved at 15psi for one hour. All flasks were cooled, inoculated with individual microorganism in each flask and then incubated for 14 days at 37˚C (bacteria) and 27˚C (fungi). A same set of experiment was conducted without bone meal powder under similar conditions. After 14 days old culture filtrates of each microorganism was extracted from both group of organisms at the same time. Phosphorus solubilization estimation was done using permeates as determination of P_2O_5 concentration in

each sample which was measured by colorimetric vanadomolybdophosphoric acid method.

Colorimetric assay of determination of phosphorus : A Vanadomolybdophosphoric acid method was used for measuring the amount of soluble P_2O_5 concentration by using Thermofisher Nanodrop spectrophotometer at 420 nm. The formation of yellow vanadomolybdophosphoric acid on addition of ammonium molybdate and vanadium to the ortho-phosphate solution was observed. The formation of heteropoly acid was observed after Ammonium molybdate reaction under acid conditions, the intensity of yellow color indicates the concentration of orthophosphate present in the solution (Wyciszkiewicz *et al.* 2015).

Determination of solubilization index on PVK agar

All pure cultures of each micro-organisms were preliminarily screened on PVK agar for their apparent potential to solubilize Tricalcium phosphate (TCP) as insoluble inorganic phosphate sources. One liter (1 L) of PVK agar contained the following (g/L): 0.5g $(NH_4)_2SO_4$, 0.1g $MgSO_4.7H_2O$, 0.02g NaCl, 0.02g KCl, 0.003g $FeSO_4 \bullet 7H_2O$, 0.003g $MnSO_4H_2O$, 5 g $Ca_3 (PO_4)_2$, 10 g glucose, 0.5 g yeast extract, 15 g agar, and 1000 ml distilled water (Pikovskaya 1948). The PVK medium sterilized at 121˚C for 15 min and then lukewarm medium was poured into sterilized petri plates. A disc (5 mm) of mycelia from each fungal isolate transferred onto PDA and then incubate at 25±1˚C for 7 days. Fungal mycelia were cut from the edges of each actively growing colony using a sterile cork borer (5mm) and then placed at 25±1˚C for 7 days on PVK agar supplemented with 1% TCP containing petri plates with three replications. Plugs of fungal mycelia were placed on PDA which used as controls. While, in case of bacterial inoculation drop plate method was done. A clear zone on medium around the colony of each microorganism was measured after the 1st, 3rd, 5th, and 7th day of incubation and PS index was calculated according to the formula below (Premono *et al.* 1996).

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter + Clearing zone diameter}}{\text{Colony diameter}}$$

Treatment of mustard seed with culture filtrate by roll towel paper method : *Brassica juncea* cv of RL-1359 were chosen for treatment after surface sterilized with 70% ethanol, followed by 5% sodium hypochlorite and washed by sterilized distilled water. For each treatment, one hundred mustard seeds were selected then soaked in the respective culture filtrate and kept for overnight. Mustard seeds submerged in sterilized distilled water served as control. The treated seeds were incubated and rolled for 10 days in germination paper at 28±1°C in two replications and was irrigated with 20 ml sterilized water every-day in each germination paper.

Seed germination rate : After five and ten days of sowing the germination the average number of radicle and plumules that emerge was observed and computed using formula (IRRI 2011).

$$\text{Germination \%} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds in tray}} \times 100$$

Seedling length and weight: The shoot length was measured from the base of the primary leaf upto base of hypocotyl, and likewise, root length measured from the tip of the primary root upto the base of hypocotyl. Digital weighing machine was used to measure weight of the shoot and root.

Seed vigour index: The seed vigour index was calculated based on product of germination (%) and seedling length (cm) after five days of incubation using the method (Abul-Baki and Anderson 1973):

Seed vigour index= Germination (%) × Seedling length (cm)

Statistical analyses: All the data were statistically analyzed using standard deviation using OPSTAT software.

RESULTS AND DISCUSSION

The most crucial macronutrient for crop development and growth is phosphorus, and phosphate-solubilizing fungi and bacteria are essential for boosting the

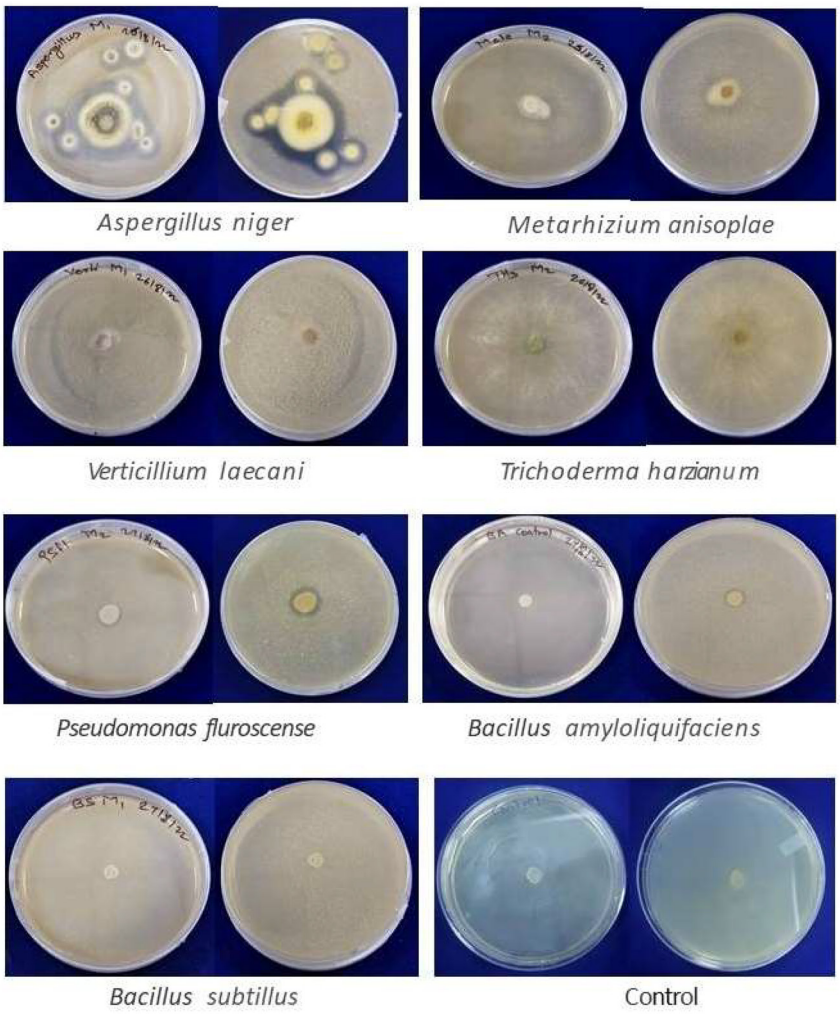

Plate 1. View of frontside and opposite side of each microorganism colony exhibiting clear zone formation for TCP solubilization on PVK medium.

availability of P to plants. Seven distinct microbes i.e *Aspergillus niger, Metarhizium anisoplae, Verticillium laecanii, Psuedomonas fluroscence, Bacillus amyloliquifaciens, Trichoderma harzianum* and *Bacillus subtilis* isolates were selected. The microbial colonies on PVK agar media was identified as P- solubilizing fungi and bacteria based on formation of clear zone around microbial colony. On PVK medium, the seven isolates displayed a halo zone around the colony, showing that they can mineralize organic phosphorous. The utilization of phosphates from culture media by the microbes to proliferate themselves, causes this clear zone effect.

P-solubilization index

The clear zones became visible in *A. niger, M. anisoplae, V. laecanii, P. fluroscence, B. amyloliquifaciens* (Plate 1), while, *T. harzianum* and *B. subtilis* exhibited a slight clear zones around colonies after 7th day of inoculation on same PVK agar medium (Kumar *et al.* 2020). P-solubilization index was observed maximum in *A. niger* followed by *P. fluroscence, B. amyloliquifaciens* and *M. anisoplae* (Fig. 1).

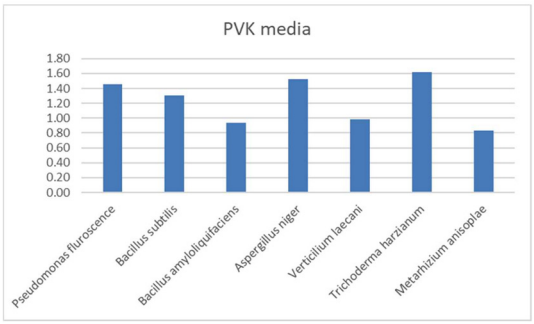

Fig. 1. P-solubilizing efficiency of microbes on chloride (M_1) and sulphate (M_2) media.

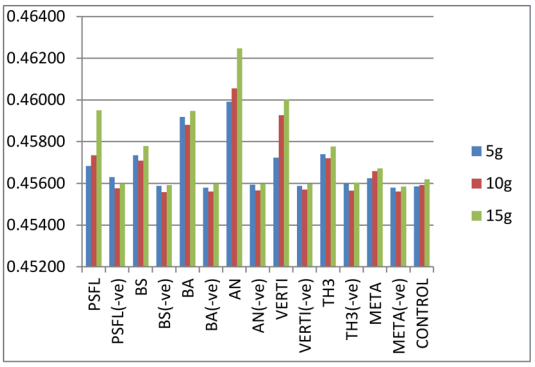

Fig. 3. P-solubilization efficiency of microbes in PVK broth containing sulphate with three doses of bonemeal.

P- solubilization efficiency of microbes and seed germination and seedling growth

All seven microbes showed P-solubilization ability in liquid media amended with bone meal as a P source and they also confirmed good results in the P-solubilization in solid medium using TCP as a P source. It was discovered that the outcomes varied significantly. Efficacy of P-solubilization by all the micro-organisms was presented in Figs. 2-3 in both Cl^- and SO_4^{2-} containing medium. The observation was recorded as maximum P-solubilization efficiency was shown by *A. niger* followed by *B. subtillis, B. amyloliquifaciens, V. laecanii* and *T. harzianum* in broth. As the doses of bone-meal increases the P-solubilization efficiency of microbes also increases gradually (Figs. 2-3). It is also found that the seed treated with culture filtrate obtained from

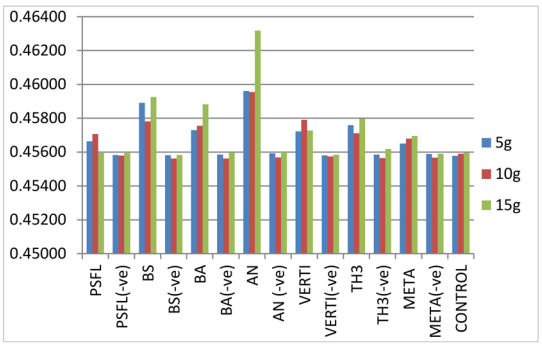

Fig. 2. P-solubilization efficiency of microbes in PVK broth containing chloride with three doses of bonemeal.

Cl- and SO_4^{2-} media containing 5g and 15g of bone meal which were inoculated with organisms shown very reliable progress which was recorded on germination percentage, seedling vigour, seedling length and root length. The degree of solubilization varied depending on the medium composition and bone dosages (Wyciszkiewicz *et al.* 2015). Aspergillus niger in Cl- based culture filtrate with bonemeal (5 and 15 g) had the induced highest seed germination (100% and 56%) and seed vigour index (1654.13 and 956.67), respectively (Table 1 and 3), however in SO_4^{2-} based culture filtrate of *A. niger* with bonemeal (5g and 15g) as seed germination (90% and 40%) and seed vigour index (1151.36 and 580.0), respectively (Tables 2-4). Although, in case of without bone-meal of Cl^- based culture filtrate without bonemeal as seed germination (73%) and seed vigour index (1024.92) which was lower in SO_4^{2-} based culture filtrate seed germination was (65%) and seed vigour index (479.91).

Almost same trend on effect of germination and seedling vigour in all the organisms were recorded in two doses (5 and 15g) with both media (Cl^- and SO_4) alongwith bone-meal and without bone-meal (Tables 1- 4).

From the results (Tables 3-4) it was clear that *T. harzianum* perfomes better in media supplied with sulphates with 100 % seed germination and significantly better seedling vigour. P solubilization directly have negative effect on seed germination while have indirect effects on length of shoot and root in both (Cl^-

Table 1. Effect of microbial culture filtrates with valorised bones (5g) rich Cl based media on mustard.

Treatment	Germination %		Shoot length		Root length		SVI	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Pseudomonas fluroscence	86	46	6.30 ±1.52	3.36±1.90	7.29±1.96	6.70±2.86	1177.20	654.73
Bacillus subtilis	38	67	4.02 ±2.20	7.16±1.03	8.39±2.08	6.83±2.16	544.16	1002.09
Bacillus amyloliquifaciens	52	50	6.31±1.88	6.85±9.61	7.79±2.26	7.96±2.69	738.22	772.66
Aspergillus niger	100	73	6.67±1.26	6.86±1.11	9.77±2.35	8.67±1.55	1654.13	1024.92
Verticilium laecani	54	73	6.47±1.87	5.81±1.58	8.20±2.35	8.02±2.36	815.40	1100.21
Trichoderma harzianum	40	63	4.93±1.84	6.89±0.94	8.98±1.54	7.27±1.40	647.14	984.97
Metarhizium anisoplae	30	72	3.71±1.77	4.51±1.45	7.10±2.49	6.86±1.75	449.31	1235.57
Control	79		6.40±1.10		10.71±11.22		1716.07	

(+ve = with bonemeal, -ve = without bonemeal).

and SO_4^-) culture filtrate having bone-meal. However, sulphate based culture filtrate had better effect on seed and seedling on germination and vigour, respectively.

Mustard production and quality are affected by soil nutrient availability, as well as biotic and abiotic aspects. Because of the phosphorus is stationary nature in soil, bioavailable phosphoric nutrient to plants is not particularly prevalent. Several ecosystems with phosphate-solubilizing microorganisms such as fungi (*Aspergillus, Penicillium, Mucor, Trichoderma*), bacteria (*Bacillus, Pseudomonas, Micrococcus, Flavobacterium*), and actinomycetes (*Streptomyces*) have been reported for their ability to solubilize insoluble phosphorus from rock phosphate (Gaur and Pareek 1974). These bacteria stimulate seed germination and plant vigour by generating growth-promoting compounds in addition to phosphate solubilizing chemicals (Kumar *et al.* 2020). Bio-priming aids in the consistent germination of seeds, even in severe conditions. One possible use of phosphate-solubilizing bacteria is seed biopriming, which aids in seedling and root framework growth (Comejo *et al.* 2014. Prasad *et al.* 2022).

Results indicates that substituting the chlorides by sulphates in growth medium which influenced the growth of micro-organisms which helps to increase seed germination percentage as well as seed vigour index (Wyciszkiewicz *et al.* 2015). However, high phosphate rich medium has initially negative effects on seed germination as well as seedling vigour which was also observed in our experiments as impregnated valorised biogenic apatite induce such effects and the reports of Follmer *et al.* (2021) supporting as phosphorus alone inhibited seed germination. This result is in accordance with the report of Sharma *et al.* (2007) and Aipova *et al.* (2010) in which the PSB inoculants increase shoot and root length. This could be because of higher amount of growth promoting

Table 2. Effect of microbial culture filtrates with valorised bones (5g) rich SO_4 based media on mustard.

Treatment	Germination %		Shoot length		Root length		SVI	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Pseudomonas fluroscence	78	66	6.26±1.43	5.81±1.08	7.48±1.32	7.75±1.88	1084.77	505.56
Bacillus subtilis	92	73	6.29±1.10	5.83±1.65	6.43±1.74	8.24±1.26	1170.59	600.30
Bacillus amyloliquifaciens	34	41	4.37±1.56	4.20±1.31	5.02±1.53	7.56±1.25	485.07	312.79
Aspergillus niger	90	65	6.15±1.34	6.77±0.97	6.63±1.24	7.41±1.10	1151.36	479.91
Verticilium laecani	88	53	6.32±1.18	5.99±1.24	6.98±1.23	5.72±1.54	1172.96	303.58
Trichoderma harzianum	42	100	5.04±1.46	5.78±1.07	7.84±2.31	5.91±2.04	606.45	574.58
Metarhizium anisoplae	86	92	6.82±1.15	7.43±1.25	7.54±1.61	10.01±1.89	1214.24	922.14
Control	57		6.31±1.03		7.97±1.99		1190.22	

(+ve = with bonemeal, -ve = without bonemeal).

Table 3. Effect of microbial culture filtrates with valorised bones (15g) rich Cl based media on mustard.

Treatment	Germination %		Shoot length		Root length		SVI	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Pseudomonas fluroscence	28	46	3.70±1.55	3.36±1.90	5.57±1.77	6.70±2.86	393.80	654.73
Bacillus subtilis	58	67	6.58±1.83	7.16±1.03	9.36±3.21	6.83±2.16	926.24	1002.09
Bacillus amyloliquifaciens	34	50	3.79±1.91	6.85±9.61	6.03±3.18	7.96±2.69	502.51	772.66
Aspergillus niger	56	73	4.79±1.40	6.86±1.11	6.60±2.28	8.67±1.55	956.67	1024.92
Verticilium laecani	50	73	5.08±1.92	5.81±1.58	9.35±2.34	8.02±2.36	731.69	1100.21
Trichoderma harzianum	58	63	6.15±1.50	6.89±0.94	9.24±2.06	7.27±1.40	906.87	984.97
Metarhizium anisoplae	42	72	5.70±1.57	4.51±1.45	8.66±2.08	6.86±1.75	604.45	1235.57
Control	79		4.71±1.89		11.11±3.32		0.00	654.73

(+ve = with bonemeal, -ve = without bonemeal).

substances and biocontrol substances released by inoculants. In general, in addition to P-solubilizing microbes have ability to improve seed germination (Demissie *et al.* 2013). Reports indicating about betterment of seed and seedling parameters could be due to the synergistic effect of microbes by releasing of some growth substances as well as mineralization and solubilization of P-sources (Kumari *et al.* 2009). The element solubility (such as phosphorus and potassium) by changing soil fertility was promoted by *Trichoderma* strains (Mweetwa *et al.* 2009). Widawati and Suliasih (2001) reported *B. subtilis* and *Pseudomonas fluorescens* as dominant *P solubilizers* in the rhizosphere of different plants in Gunung Halimun National Park, Indonesia. *Trichoderma, Verticilium* and *Aspergillus* are working as agents for biocontrol and *P. solubilizers* (Cairns *et al.* 2007). Whitelaw (2000) was found the filamentous fungi can secrete hydrolytic enzymes, organic acids and also confer several functions, including P solubilization. Shukla and Vyas (2014) reported that P solubilizing efficacy of *Trichoderma* spp. proved to be the best. These experiments should be long term to have a possibility to observe potential effect of toxicity of chloride and the effect of a lower amount of phosphorus available to plants, as a result of a lower solubilization (an effect of substituting chlorides with sulphates).

CONCLUSION

It is necessary to evaluate the utilitarian properties of such fertilizer formulations in germination tests to find the influence of the substitution of chlorides with sulphates on the yield parameters and the content of elements in plants. It has been proved that all phosphorus solubilizing microbes did their job of P-solubilization which are observed in TCP medium as well as poor seed germination in bone-meal containing culture filtrates as compare to less content of phosphorus in without bone-meal amended growth media. The second stage for this work will

Table 4. Effect of microbial culture filtrates with valorised bones (15g) rich SO_4 based media on mustard.

Treatment	Germination %		Shoot length		Root length		SVI	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Pseudomonas fluroscence	14	66	1.82±1.65	5.81±1.08	2.87±1.69	7.75±1.88	197.00	505.56
Bacillus subtilis	50	73	6.50±3.07	5.83±1.65	9.92±2.42	8.24±1.26	845.50	600.30
Bacillus amyloliquifaciens	34	41	4.08±2.02	4.20±1.31	4.83±1.37	7.56±1.25	461.09	312.79
Aspergillus niger	40	65	4.06±2.53	6.77±0.97	5.61±1.97	7.41±1.10	580.00	479.91
Verticilium laecani	48	53	5.44±2.98	5.99±1.24	8.66±1.46	5.72±1.54	690.24	303.58
Trichoderma harzianum	48	100	7.09±2.48	5.78±1.07	10.05±1.73	5.91±2.04	838.04	574.58
Metarhizium anisoplae	32	92	4.51±2.66	7.43±1.25	6.89±0.94	10.01±1.89	547.20	922.14
Control	57		4.33±3.10		6.42±1.33		0.00	

(+ve = with bonemeal, -ve = without bonemeal).

allow a comparison of the application results of both formulations.

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