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Antagonistic activity of bacteria inhabiting composts against soil-borne plant pathogenic fungi

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Two hundred and seven bacteria were isolated from four different sources viz. farm waste compost (FWC), rice straw compost (RSC), *Gliricidia* vermicompost (GVC) and macrofauna (earthworms, centipedes, slugs and snails). All the isolates were tested for antagonistic activity on four soil borne plant pathogenic fungi, *Sclerotium rolfsii, Macrophomina phaseolina, Fusarium solani* and *Fusarium oxysporum*. Percentage of antagonistic bacteria varied from 19 in FWC to 39 in GVC. Of the 207 isolates, eighteen were antagonistic to all four soil borne plant pathogenic fungi. Inhibition was maximum on Kings B medium (63%), followed by glycerol casamino yeast extract (GCY) (49%) and potato dextrose agar (PDA) (46%). Identification of the 18 antagonistic isolates revealed that twelve belonged to genus *Bacillus* and six were *Pseudomonas*. *B. licheniformis* (EB 13) and *Pseudomonas* sp. (CDB 35) showed antifungal effect higher than other bacteria in plate culture conditions. These strains also showed significant reduction in fungal biomass when grown in broth culture. PDA amended with zinc sulphate further improved antifungal activity of EB 13 and CDB 35. In glass house conditions, soil amended with glucose and/or zinc in combination with EB 13 or CDB 35 reduced *M. phaseolina* infection in sorghum roots and improved shoot weight.

Key words: antagonistic bacteria, composts, pathogenic fungi, soil borne, sorghum

Microbes present in composted agricultural wastes have been reported to suppress soil borne plant diseases and improve plant growth[1]. Biocontrol of plant pathogens is an attractive alternative to chemical fungicides, which cause environmental pollution and development of resistant strains. Species of *Pseudomonas* and *Bacillus* are known for their ability to reduce plant diseases caused by fungal pathogens in glass house and field trials[2,3]. *Bacillus* spp. show antagonistic effect due to antibiosis or production of extracellular chitinolytic enzymes[3]. Species of *Pseudomonas* have received attention as biocontrol agents due to their catabolic versatility, excellent root colonizing ability and production of a wide range of antifungal metabolites[4]. In addition, a few *Pseudomonas* spp. have shown to elicit induced systemic resistance in crop plants[5].

Several species of soil borne plant pathogenic fungi such as *Sclerotium*, *Macrophomina*, *Fusarium* and *Pythium* are distributed globally and are known to cause economic losses in crop yields[6]. *Macrophomina phaseolina* (Tassi) Goid, is one of the most destructive plant pathogens causing charcoal rot, dry root rot, wilt, leaf blight, stem blight and damping off diseases in wide range of host plants and its biocontrol gained importance[7]. Aim of the present study was to examine the ability of bacterial strains isolated from composts on soil borne fungal pathogens. Selected bacterial strains were tested for their antifungal activity with sorghum [*Sorghum bicolor* (L.) Moench] under glass house conditions.

Materials and Methods

Preparation of composts: Farm waste compost (FWC) was prepared in brick chamber using backyard and kitchen waste. Rice straw compost (RSC) was prepared in heaps and *Gliricidia* vermicompost (GVC) was prepared using earthworms (*Eisenia fetida*) in cement cylinders. Four to eight weeks after incubation, ten grams of the compost sample was taken and appropriate dilutions were plated on nutrient agar for isolating bacteria. Bacteria were also isolated from body surface and excreta of macrofauna (earthworms, centipedes, slugs and snails) that were present in FWC.

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Isolation and screening of bacteria for antagonistic activity: Two hundred and seven bacteria were isolated from different composts and macrofauna used in the study. Isolates from FWC and/or the macrofauna associated, RSC and GVC were designated as EB, CDB and BWB, respectively. All the 207 isolates were screened for antagonistic activity against soil-borne plant pathogenic fungi, *S. rolfsii, M. phaseolina, F. solani* and *F. oxysporum* on PDA using dual culture technique. An agar block (6 mm dia) was cut from an actively growing (96 h) fungal culture on agar plate and placed on the surface of fresh agar medium at the center of Petri plate. A loopful growth of 24 h broth culture of each bacterium was tested against soil-borne plant pathogenic fungi by streaking in a straight line on one edge of medium in Petri plate (90 mm dia). Plates inoculated without bacteria were used as control. For each treatment three replicates were maintained and repeated twice. Plates were incubated at 30±1°C and radial growth of fungus was measured after 5 d of inoculation. Per cent inhibition of fungi (growth reduction over control) was calculated by the following equation:

$$I = \frac{100\ (C-T)}{C}$$

where: I = % inhibition of mycelial growth; C = radial growth of fungus in control plate (mm); T = radial growth of fungus on the plate inoculated with bacteria (mm).

Selection and identification of bacteria: Eighteen bacterial isolates, selected based on antagonism on PDA, were further tested on two media, glycerol casamino acid yeast extract (GCY) and Kings B (KB) agar, against four soil borne plant pathogenic fungi. They were tested for siderophore production on CAS agar[8] and chitinase production on minimal medium amended with chitin[9]. Isolates were identified following the standard methods[10].

Antagonistic activity of bacteria: Based on antagonistic activity in plate culture, two bacterial isolates, *B. licheniformis* EB 13 and *Pseudomonas* sp. CDB 35 were selected for inhibition of growth of *S. rolfsii, M. phaseolina, F. solani* and *F. oxysporum* in broth culture. Actively growing fungal culture in GCY medium was homogenized and 1 mL of suspension was inoculated to 100 mL GCY broth in 250 mL conical flasks and incubated on a rotary shaker at 30±1°C. After 24 h of fungal growth, 1 mL of actively growing bacterial broth culture was inoculated into each flask and incubated on shaker. Flasks without bacterial culture served as controls. For each treatment three replicates were maintained and repeated twice. Fungal mycelium was harvested after 96 h by filtering through pre-weighed Whatman No. 1 filter paper, dried for 24 h at 65°C and dry wt was recorded. A drop of culture from the flasks inoculated with each pathogenic fungus and EB 13 or CDB 35 was taken on a clean glass slide with lactophenol cotton blue and observed under light microscope for morphological changes of inhibited fungi.

Influence of mineral nutrients: In order to find out the effect of various mineral nutrients viz. ferrous sulphate, ($FeSO_4.H_2O$), magnesium sulphate ($MgSO_4.4H_2O$), sodium chloride (NaCl), zinc sulphate ($ZnSO_4.7H_2O$), two bacterial strains (EB 13 and CDB 35) were tested for anti-fungal activity on *M. phaseolina* on PDA amended with four mineral nutrients individually as well as in combination of all and were aseptically transferred to give 1mM concentration. Anti-fungal activity was tested by dual culture technique and it was repeated twice with three replications each time. The experiment was evaluated in terms of per cent inhibition of fungi.

Glasshouse studies: Inoculum of *M. phaseolina* was prepared in sorghum meal. Three hundred g seeds of sorghum were soaked overnight in 500 mL distilled water and autoclaved at 121°C for 15 min in 1 L conical flask, cooled and mixed with 20 mL of 5% glucose solution. Seeds were inoculated with *M. phaseolina*, grown on PDA (8 blocks of 6 mm dia) and incubated for 7 d at 30±1°C and intermittently shaked to get homogeneous fungal growth. Ten grams of *M. phaseolina* inoculum was mixed to 1 kg unsterilized vertisol soil and added as 5 cm top soil in plastic pots of 10 cm dia. *M. phaseolina* inoculated soil was amended with glucose (0.1%) and /or $ZnSO_4.7H_2O$ (1 mg kg^{-1} soil) in different combinations, as per the treatments. Pots were irrigated with tap water and incubated for 3 d at room temperature before sowing. Peat based inoculum of *B. licheniformis* (EB 13) and *Pseudomonas* sp. (CDB 35) was prepared separately by mixing 40 mL of overnight

grown LB broth culture to 30 g of sterilized Australian peat in HDPE bags and incubated for a week at 30°C. Seed coating was done by mixing 1 g of peat culture and 10 g of seed with 1 mL of 1% carboxymethyl cellulose as adhesive. The experiment included 13 treatments (Table 2). Ten seeds of sorghum CSV15 were sown per pot and allowed to grow in glass house at 30-32°C. After 30 d, shoot dry wt and per cent root infection were recorded. Root infection was calculated by taking roots of five randomly chosen plants from each pot. Roots were thoroughly washed under running tap water and cut into 5 mm segments. Root segments were surface sterilized using 3% sodium hypochlorite for 3 min, rinsed 6-8 times in sterile distilled water and five segments were plated onto chloroneb-mercuric chloride-Rose Bengal (CMR) agar containing (in mg L^{-1}) chloroneb 312, mercuric chloride 8.5, Rose Bengal 112 and streptomycin sulphate 500. Per cent infection of sorghum roots by *M. phaseolina* was calculated as:

$$\% \text{ Infection} = \frac{\text{Number of plants infected by fungus x 100}}{\text{Total number of plants}}$$

Glasshouse experiments were repeated twice and arranged in completely randomized block design with five replications of each treatment. Data was subjected to analysis of variance (ANOVA) using Genstat 6.1 statistical package (Lawes Agricultural Trust, Rothamsted, UK).

Results and Discussion

Five bacterial isolates from FWC, 18 from macrofauna, 19 from GVC and 21 from RSC showed antagonistic activity against one or more plant pathogenic fungi and 18 bacterial isolates inhibited all the four fungi (*S. rolfsii*, *M. phaseolina*, *F. solani* and *F. oxysporum*) used in the study. Inhibition of these soil-borne plant pathogenic fungi was maximum on KB agar (63%) followed by GCY (49%) and PDA (46%) (Fig. 1). Variation in the spectrum of antifungal activity of microorganisms or their metabolites is common[11]. It is known that growth of bacteria on PDA is normally less compared to other media used. Suppression of fungal mycelium was also less on PDA when compared to GCY and KB media, which may be attributed to lack of nitrogen or nutrients for bacterial growth and production of anti-fungal compounds. King's B medium enhances pigment production by *Pseudomonas* sp., favours siderophore production and GCY medium enhances antibiotic production[12,13].

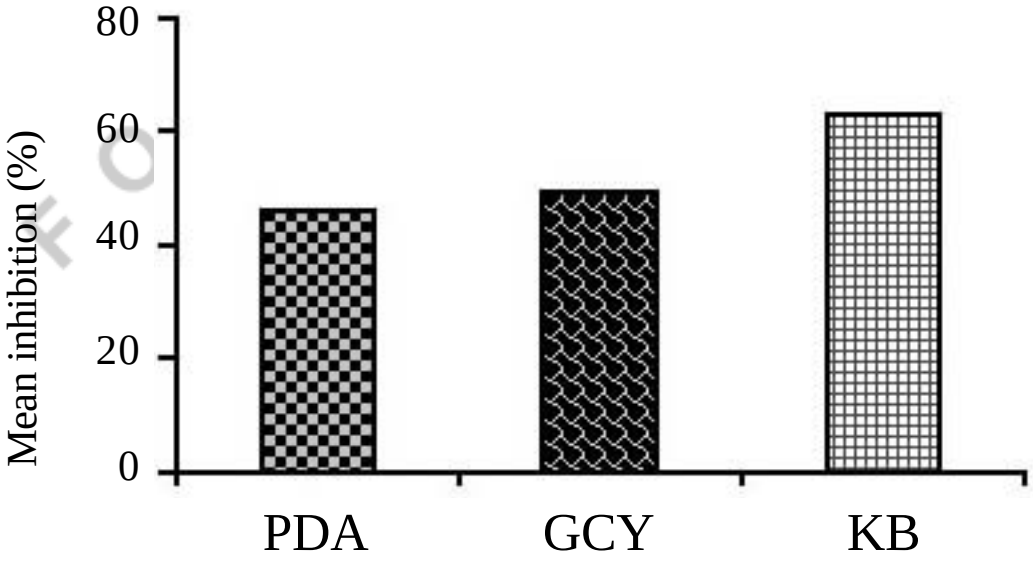

Fig. 1. Mean inhibition % of four plant pathogenic fungi by eighteen bacterial isolates on potato dextrose agar (PDA), glycerol casamino yeast extract (GCY) and Kings B (KB) media.

All the 18 bacterial strains showed significant (P=0.05) inhibition of all the four fungal pathogens tested in all the three media used. Maximum inhibition of *S. rolfsii* was with *B. licheniformis* EB 13 and of *F. oxysporum*, F. *solani* and *M. phaseolina* with *Pseudomonas* sp. CDB 35. Overall on PDA, GCY and KB media, mean inhibition by *B. licheniformis* EB 13 and *Pseudomonas* sp. CDB 35 against *S. rolfsii* was 80 and 66%, *M. phaseolina* 60 and 62%, *F. solani* 63 and 70% and *F. oxysporum* 66 and 76% (Table 1).

The bacteria inhabiting composts showed antagonistic activity with the aforesaid soil-borne plant pathogenic fungi. Antagonistic effect of bacteria towards the pathogenic fungi may be due to production of lytic enzymes, antibiotics, siderophores and other secondary metabolites[14]. Of the 18 isolates, 6 produced siderophores and 2 showed chitinase activity in our observations (Table 1). Two major genera *Bacillus* and *Pseudomonas* were identified to be involved in the antagonistic activity. Biocontrol agents, especially of *Pseudomonas* sp. and *Bacillus* sp., may be ecologically sound alternative to chemical seed treatment[15,16]. All the *Pseudomonas* spp. used in the study showed siderophore production (Table 1). The use of *Pseudomonas* as a biocontrol agent has been reported for its ability to grow on various carbon substrates. Similarly *Bacillus* being a spore former is known to survive in soil for a longer time and serve as a potential biocontrol agent[17]. The antagonistic nature of bacterial strains was studied using PDA, GCY and KB media in order to avoid the unpredictability due to competition for nutrients. Variability of inhibition on different media explains the importance of nutrition on the efficacy of biocontrol microorganisms. Similar results were observed when *Burkholderia cepacia* strains were tested against selected soil borne fungal pathogens[18].

In broth culture conditions, both strains significantly reduced the fungal biomass. Per cent inhibition by *Pseudomonas* sp. CDB 35 and *B. licheniformis* EB 13 against *F. solani* was 85 and 68, followed by *F. oxysporum* 80 and 65, *S. rolfsii* 72 and 64 and *M. phaseolina* 65 and 62 respectively (Fig. 2). Microscopic observations of inhibited fungi indicated mycelial deformation, cytoplasmic granulation and inhibition of sclerotia formation in presence of EB 13 or CDB 35. Inhibition of fungi by studied bacteria might be due to production of antifungal metabolites such as antibiotics and/or siderophores. *Bacillus* spp. and *Pseudomonas* spp. are reported to produce anti-fungal metabolites[19,20].

Enhancement of antagonistic activity of bacterial strains EB 13 and CDB 35 in plate culture on PDA, amended with different nutrients showed that zinc

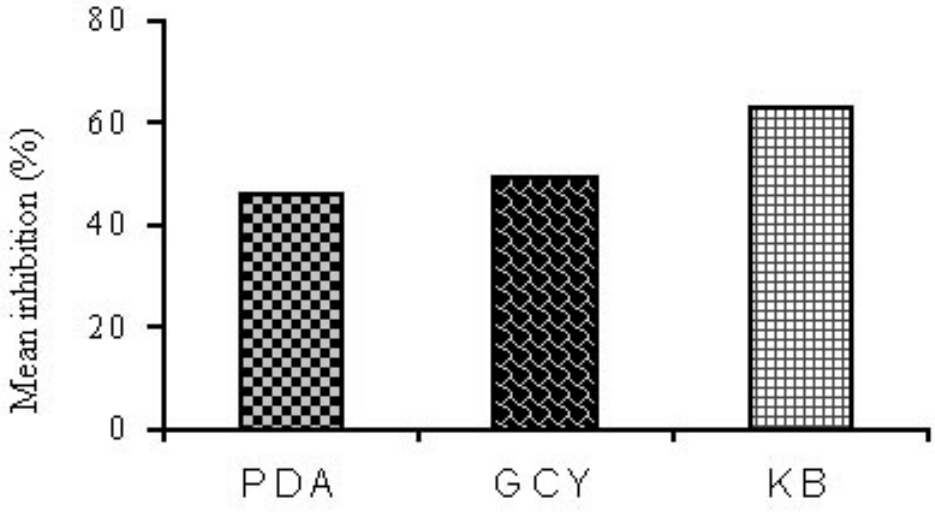

Fig. 2. Biomass reduction (%) of four soil-borne pathogenic fungi by antagonistic bacterial strains (■) *Pseudomonas* sp. CDB 35 and (□) *B. licheniformis* EB 13.

Table 1. Antagonistic activity of selected bacterial strains against soil borne plant pathogenic fungi in three growth media and production of siderophores/chitinase.

Bacterial strain	Per cent inhibition of soil borne plant pathogenic fungi in three growth media																Production of	
	S. rolfsii				*M. phaseolina*				*F. solani*				*F. oxysporum*					
	PDA	GCY	KBM	Mean	PDA	GCY	KBM	Mean	PDA	GCY	KBM	Mean	PDA	GCY	KBM	Mean	Siderophore	Chitinase
B. pumilus EB 3	53	65	74	64	45	51	56	51	42	48	49	46	58	63	65	62	-	-
B. cereus EB 10	62	70	75	69	51	62	61	58	48	50	58	52	38	52	58	49	-	-
B. licheniformis EB 13	86	73	81	80	59	58	62	60	62	58	70	63	70	63	79	66	-	-
B. coagulans EB 15	47	38	75	53	29	53	35	39	20	50	35	35	14	55	30	33	-	-
B. subtilis EB 48	76	50	75	67	41	47	53	47	29	65	38	44	66	53	75	65	-	-
B. cereus CDB 6	38	41	58	46	45	55	58	53	52	51	60	54	49	50	65	55	-	-
B. alvei CDB 15	53	62	71	62	51	53	56	53	50	65	57	57	57	55	58	57	-	-
B. pumilus CDB 22	51	58	66	58	51	57	62	57	50	53	57	53	47	57	58	54	-	-
B. coagulans CDB 31	44	37	45	42	48	47	41	45	29	50	45	41	37	50	65	51	-	-
B. licheniformis CDB 47	47	38	72	52	42	41	52	45	40	50	53	48	57	50	70	59	-	-
B. coagulans BWB 34	62	38	81	60	35	16	47	33	29	50	75	51	20	45	70	45	-	+
Bacillus sp. BWB 41	53	51	75	60	35	39	41	38	50	43	38	44	50	52	57	53	-	+
Pseudomonas sp. CDB 35	54	61	84	66	60	62	65	62	66	65	79	70	71	70	88	76	+	-
Pseudomonas sp. CDB 36	36	42	81	53	33	41	49	41	52	50	74	59	54	45	81	60	+	-
Pseudomonas sp. BWB 21	22	36	76	45	38	27	47	37	44	45	79	56	46	48	54	49	+	-
Pseudomonas sp. BWB 32	54	59	74	62	35	38	58	44	33	40	75	49	33	34	80	49	+	-
Pseudomonas sp. BWB 36	37	31	78	49	46	18	50	38	46	48	73	56	45	43	55	48	+	-
Pseudomonas sp. BWB 40	38	38	61	46	50	24	43	39	39	50	63	51	34	48	78	53	+	-
Mean	51	49	72		44	44	52		43	52	60		46	52	66			
LSD (P=0.05)	Isolate (1.2)				Fungi (0.5)				Media (0.6)									
LSD (P=0.05)	Isolate*fungi (2.4)				Isolate*media (2.1)				Media*fungi (0.9)				Isolate*fungi* media (4.1)					
CV %	5																	

PDA=potato dextrose agar, GCY=glycerol yeast extract, KBM=kings B medium.

sulphate and mixture of salts inhibited *M. phaseolina* (Fig. 3). Not much difference when different concentrations of Fe were used towards inhibition of *S. rolfsii*, *S. sclerotiorum* and *F. oxysporum* by *Pseudomonas* spp[21]. Zinc stimulated the production of 2,4-diacetylphloroglucinol, pyoluteorin[22] and phenazine type antibiotics[23] in *Pseudomonas fluorescence*.

In glasshouse conditions, infection of sorghum roots in soil infested with *M. phaseolina* and inoculated with *B. licheniformis* EB 13 and *Pseudomonas* sp. CDB 35 was 45 and 34% respectively. Soil amendment with zinc or glucose separately without the bacterial inoculants did not affect root infection caused by *M. phaseolina* but when amended with bacteria did reduce the pathogen infection (Table 2). *Pseudomonas* sp. CDB 35 applied along with zinc proved best to control infection of sorghum roots by *M. phaseolina*. When the efficacy of two antagonistic bacteria was compared, *Pseudomonas* sp. CDB 35 was similar to the reference strain, *Trichoderma viridae*. Shoot wt of sorghum was significantly higher when either of the isolates was applied along with glucose and/or zinc. Positive correlation (r) was obtained between laboratory and glasshouse conditions for inhibition of *M. phaseolina* in presence of EB 13 (r=0.88) and CDB 35 (r=0.99) with zinc. *M. phaseolina* in sorghum roots was significantly inhibited in soil amended with antagonistic bacterial strains than zinc alone. Amendments of soil with nutrients and carbon sources reduced the infection of tomato roots by *M. phaseloina*[24]. The present investigation provides information on the use of *Pseudomonas* and *Bacillus* isolated from composts as biocontrol agents against

Table 2. Effect of nutrients and seed bacterization with *Bacillus licheniformis* EB 13 or *Pseudomonas* sp. CDB 35 on shoot wt and root infection of sorghum in *M. phaseolina*-infested soil.

Treatments	Shoot weight (g)	*M. phaseolina* infection %
Control	0.5	80 (64)
Trichoderma viridae (+ve control)	1.8	34 (35)
Glucose	0.6	72 (58)
Zinc	1.8	62 (46)
Glucose+Zinc	1.5	61 (46)
B. licheniformis EB 13 (control)	1.7	45 (42)
EB 13 + Glucose	1.1	42 (40)
EB13 + zinc	2.1	36 (37)
EB 13 + Glucose + zinc	1.9	42 (40)
Pseudomonas sp. CDB 35 (control)	1.6	34 (35)
CDB 35 + Glucose	1.6	46 (43)
CDB 35 + zinc	2.3	20 (27)
CDB 35 + Glucose + zinc	1.7	27 (31)
Mean	1.6	45 (43)
LSD (P=0.05)	0.4	14.2 (8.7)
CV %	22	25 (16)

Figures in parentheses are angular transformed values.

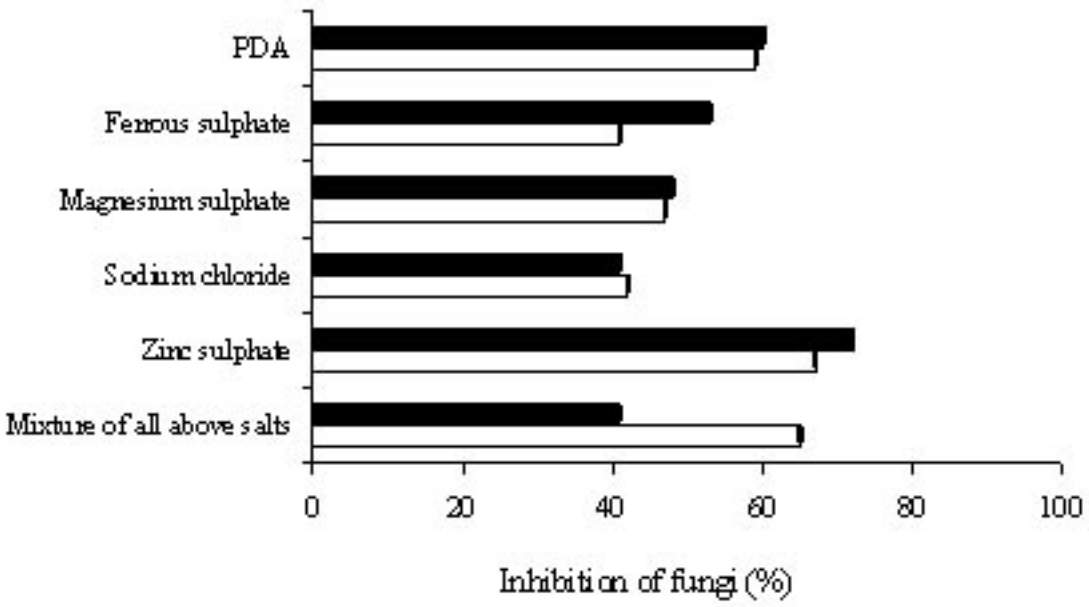

Fig. 3. Influence of mineral nutrients on suppression of *M. phaseolina* by antagonistic bacterial strains, (■) *Pseudomonas* sp. CDB 35 and (□) *B. licheniformis* EB 13.

soil-borne plant pathogenic fungi. Further, the study also revealed the fortification of mineral nutrients enhances the biocontrol ability of these bacteria. However, the mechanism behind the inhibition needs further investigation.

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