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Isolation and Identification of Indigeneous Microbial Bioagents Strains from Meghalaya and *In Vitro* Evaluation of the Antagonistic Properties against Common Fungal Phytopathogens

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Abstract A study was conducted to evaluate the antagonistic properties of known microbial bio-agent strains isolated from Meghalaya. Strains of known bio-agents such as *Bacillus subtilis*, *Pseudomonas fluorescens*, *Trichoderma harzianum* and *Trichoderma viride* were isolated from different agricultural practices. These isolates were tested for antagonism against several fungal plant pathogens such as *Alternaria brassicicola*, *Aspergillus japonicus*, *Colletrotrichum gloeosporiodes*, *Fusarium solani*, *Fusarium sporotrichioides*, and *Phytophthora infestans*. Dual culture experiment shows that *T. harzianum* and *T. viride* has comparable maximum percentage inhibition of mycelia growth whereas from the bacterial bio-agents, *P. fluorescens* has maximum percentage growth of inhibition against all the pathogens evaluated in the study. In dual culture a clear zone of inhibition was observed exhibiting antibiosis between pathogen and antagonist. It was observed that *T. harzianum* and *T. viride* reduced the growth of *Alternaria brassicicola* by 68.9% and 66.7 % , *Aspergillus japonicus* by 61.1% and 64%, *Colletrotrichum gloeosporiodes* by 66.7% and 72.2 %, *Fusarium solani* by 73.3% and 67.7%, *Fusarium sporotrichioides* by 61.1% and 61.2%, and *Phytophthora infestan*s by 55.5% and 62.2% respectively. From the bacterial bioagents evaluated in the study *P. fluorescens* exhibited maximum percentage inhibition of mycelia growth against all the tested phytopathogens.

Keywords: Antagonistic effects, bioagents, phytopathogens, *in vitro*

Introduction

Fungi are known to cause diseases in various agricultural crops thereby causing significant loss both in quality and quantity. Modern method of crop

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production has adverse effect on the environment and therefore a more approachable way of controlling the phytopathogens is the incorporations of effective microbial bioagents strains into successive disease management. Members of the genus *Pseudomonas* and *Trichoderma* have long been known for their potential to reduce the plant disease caused by fungal pathogens and they have gained considerable importance as potential antagonistic microorganisms (Pant and Mukhopadhyay, 2001). Isolation of local strains of *Trichoderma* is essential for successful identification of potential biocontrol agents (Williams and Asher, 1996). In many instances, *Pseudomonas* spp. and *Bacillus* spp. have been applied as biocontrol agents to suppress plant-pathogenic organisms (Joseph *et al*., 2008). *Bacillus* spp. in particular are gaining recognition as safe biocontrol agents in a variety of crops, specifically as seed protectants and antifungal agents (Stein, 2005). Also several strains of the *Trichoderma* spp. are found to be effective biocontrol agents for the various plant pathogens (Amin *et al.*, 2010). Research on exploitations of biocontrol agents in managing plant diseases have advanced in recent decade and many formulations are available commercially using fungal and bacterial bioagents and they have gain much more importance due to their wide applications and environmentally safe aspects . The present study was undertaken to investigate the antagonistic effects of bacterial and fungal biocontrol agents against several known phytopathogens infecting various agricultural crops.

Materials and methods

Isolation and Identification of microbial bioagents

Two bacterial bioagents namely *Bacillus subtilis* and *Pseudomonas fluorescens* were isolated and identified according to morphological, biochemical and physiological tests following Bergeys Manual of Systemic Bacteriology. Two species of *Trichoderma* were isolated and identified following the monographs of Subramaniam (1971), Barnett and Hunter (1972) and Domsch *et al*. (1980).

Isolation and Identification of Fungal Pathogens

The test pathogens namely *Alternaria brassicicola*, *Phytophthora infestans, Colletrotrichum gloeosporiodes, Fusarium solani*, *Fusarium sporotrichiodes, Aspergillus japonicus* were isolated from infected crops associated with the pathogens using Potato Dextrose Agar medium (PDA) and identification is done based on structural and morphological characteristics

following the monographs of Subramaniam (1971), Barnett and Hunter (1972) and Domsch *et al*. (1980).

In vitro screening of antagonistic activity

The strains of bio-agents such as *B. subtilis, P. fluorescens, T. harzianum* and *T. viride* isolated from Meghalaya were evaluated for their antagonistic properties against fungal pathogens by direct confrontation and culture filtrate.

Dual culture

Out of 3 isolates of *Trichoderma* spp. one isolate was identified as *T. viride* and one isolate was identified as *T. harziarum* from different soil samples. These isolates were tested for antagonism against fungal plant pathogens such as *Alternaria brassicicola, Aspergillus japonicus, Colletrotrichum gloeosporiodes, Fusarium solani, Fusarium sporotrichioides,*and *Phytophthora infestans* by dual culture method (Morton and Stroube, 1955). 5 mm disc cut from the actively growing margins of 72h old culture was placed at the margin of the 90mm petri plates containing 20 ml Potato Dextrose Agar (PDA). Disc of 5 mm size of 72hr old culture of tested phytopathogens was placed opposite to the antagonist. The plates were incubated at 25° C for five days. Each treatment was replicated thrice. A petri plate inoculated with pathogen alone served as the control. The plates were observed regularly for their action over pathogen. Percentage of mycelial growth inhibition was calculated according to the formula:

$$MGI\% = (dc - dt) \times 100/dc$$

Where, dc= fungal colony diameter in control sets, dt= fungal colony diameter in treatment sets.

For bacterial isolates *in vitro* sensitivity antagonism was performed following the method of Toure *et al*. (2004). Two days old culture of bacterial isolates were streaked as a streak line in potato dextrose agar plates and incubated for 48hrs prior inoculation by the tested fungi. 5mm mycelia disc of an actively growing culture was introduce opposite to the other edge of the petriplates and incubated for 5-7days at 30°C. Inhibition zone between the edge of the bacterial isolates and the tested fungal pathogens were measured.

Culture filtrate assay

The effect of cell free culture filtrate on pathogen was studied following the method of Dennis and Webster (1971a). The culture filtrate of the different antagonists was collected by centrifugation at 3000 rpm for 10 min and sterilized by passing it through the Millipore membrane filter paper (0.4 µm pore size). The different volumes of filtrate were added to molten PDA media for fungi and Nutrient Agar media for bacteria to obtain final concentration of 5, 10 and 20% (v/v). The medium was poured into petriplate and inoculated with fresh pathogen mycelia plug. The petriplates were incubated at 28±1°C for 5 days. Control plates were maintained without culture filtrate. The inhibitory effect of culture filtrate were conducted in triplicates in randomized block design and repeated twice. The per cent inhibition of mycelial growth of the pathogens was calculated using following formula (Singh *et al*., 2002)

I = (C-T/C) x 100

where, I = Inhibition (%), C = Colony diameter in control plate and T = Colony diameter in treated plate.

Results

Growth inhibition of phytopathogens by microbial bioagents

Dual culture

In the dual culture experiment both bacterial and fungal bioagents have considerable effect in the reduction of the growth of different test phytopathogens compared to their respective control.

Both species of *Trichoderma* have been found to have a comparable maximum percentage inhibition of mycelial growth against the tested phytopathogens. (Table 1). It was observed that *T. harzianum and T. viride* reduced the growth of *Alternaria brassicicola* by 68.9% and 66.7 % , *Aspergillus japonicus* by 61.1% and 64%, *Colletrotrichum gloeosporiodes* by 66.7% and 72.2 %, *Fusarium solani* by 73.3% and 67.7%, *Fusarium sporotrichioides* by 61.1% and 62.2%, and *Phytophthora infestan*s by 55.5% and 62.2% respectively.

Table 1. Antagonistic activity of *T. harzianum* and *T. viride* against different fungal plant pathogens by dual culture method.

Sl. no	Plant Pathogens	% Growth inhibition of various fungal pathogens by dual culture method	
		T. harzianum	***T. viride***
1	*Alternaria brassicicola*	68.88	66.66
2	*Aspergillus japonicus*	61.10	64.44
3	*Colletrotrichum gloeosporiodes*	66.66	72.22
4	*Fusarium solani*	73.33	67.77
5	*Fusarium sporotrichioides*	61.11	62.22
6	*Phytophthora infestans*	55.55	62.22

*Each value is mean of three replicates

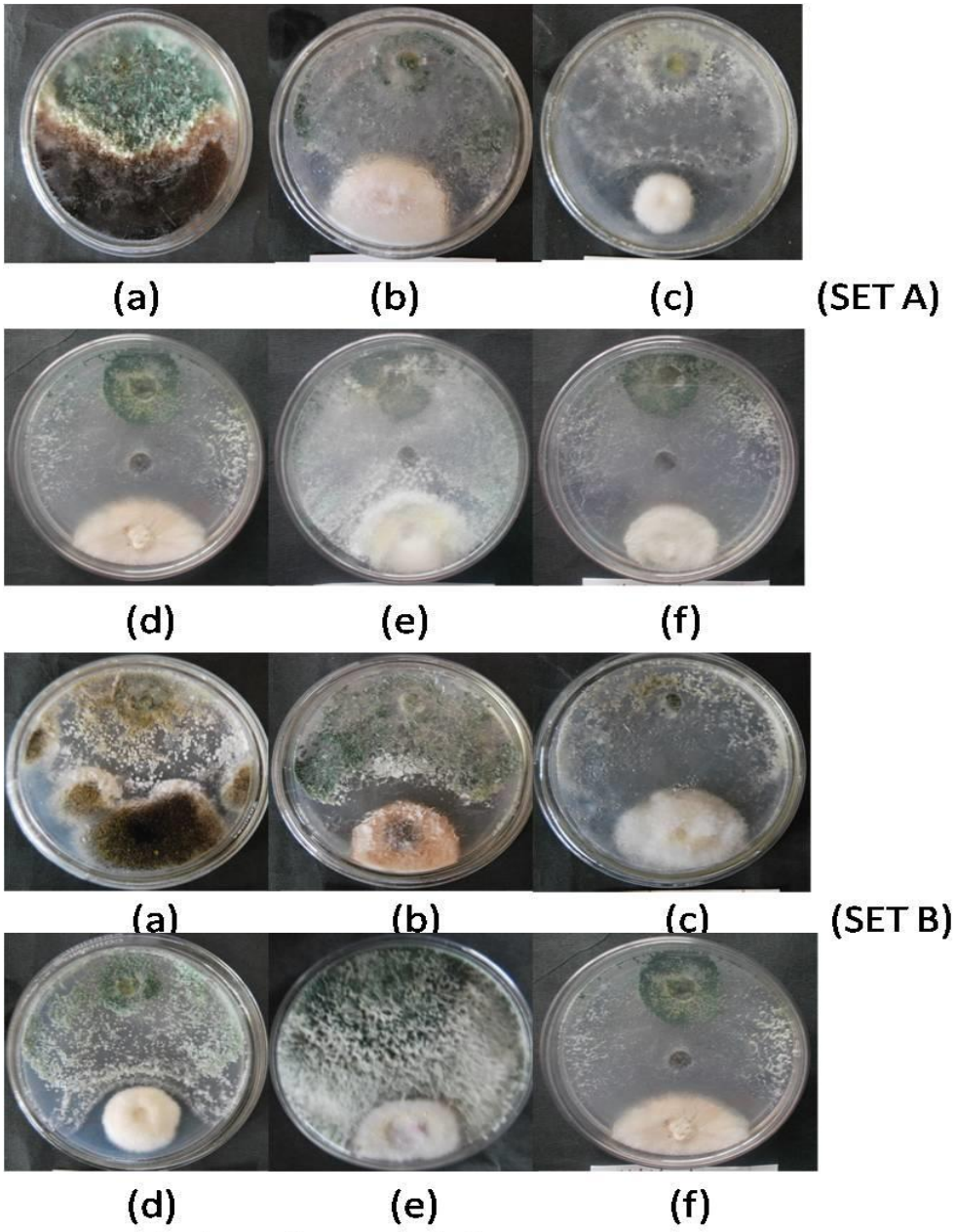

Fig.1. Dual culture culture method showing antagonistic activity of *T.viride* Set (A) and *T.harzianum* Set (B) against (a) *Aspergillus japonicus* (b) *Alternaria brassicola* (c) *Phytophthora infestans* (d) *Fusarium solani* (e) *F. sporotrichioides* (f) *Colletotrichium gloeosporioides*.

The antagonistic effect different bacterial strains were measured by percentage inhibition zone (Table 2 and Fig. 4). Results from the dual culture assay showed that the antagonistic microorganism *Pseudomonas fluorescens* significantly inhibited the growth of all pathogenic fungi tested. The maximum inhibition zones were reported with *Fusarium solani* (77.78%) and *Alternaria brassicicola* (76.67%) followed by *Phytophthora infestans* (75.55%) and *Colletotrichum gleosporioides* (73.33%), while the lowest inhibition zones of 67.78% and 57.78% were detected in *Fusarium sporotrichioides* and *Aspergillus japonicus* respectively.

Table 2. Antagonistic activity of *B. subtilis* and *P. fluorescens* against different fungal plant pathogens by dual culture method

Sl. no	Plant Pathogens	% Growth inhibition of various fungal pathogens by dual culture method	
		B. subtilis	*P. fluorescens*
1	*Alternaria brassicicola*	62.22	76.67
2	*Aspergillus japonicus*	46.67	57.78
3	*Colletrotrichum gloeosporiodes*	66.67	73.33
4	*Fusarium solani*	65.87	77.78
5	*Fusarium sporotrichioides*	58.89	67.78
6	*Phytophthora infestans*	45.55	75.55

*Each value is mean of three replicates

The antagonistic effect of *Bacillus subtilis* on fungal pathogens was presented in Table 4 and Fig.1. The highest inhibition zones were found in *Colletotrichum gleosporioides* (66.67%), *Fusarium solani* (65.87%) and *Alternaria brassicicola* (62.22%), followed by *Fusarium sporotrichioides* (58.89%), while the lowest percentage inhibition zones of 46.67% and 45.55% was reported with *Aspergillus japonicus* and *Phytophthora infestans* respectively. The highest inhibition percentage was reported in *Psuedomonas fluorescens* as compared to *Bacillus subtilis*. The inhibition effect of the bacteria on the tested pathogenic fungi differed with different bacterial strains.

Culture filtrate assay

The inhibitory effect of cell free culture filtrate of the antagonists against the pathogens is represented in Tables 3-5. The culture filtrate of the fungal antagonists restricted the growth of all the tested pathogens with better efficacy at higher concentration (20%v/v). Whereas the bacterial antagonists *Pseudomonas fluorescens* exhibited maximum % growth inhibition against all the tested pathogens evaluated in the study. The highest % growth inhibition

was recorded against *Colletotrichum gloeosporiodes* (76.67), *Phytophthora infestans* (72.22) followed by *Aspergillus japonicus* (66.67) and *Alternaria brassicicola* (63.33) and the lowest % growth inhibition was recorded against *Fusarium solani* (62.22) and *Fusarium sporotrichioides* (58.89).

Table 3. Effect of cell free culture filtrate of *T. harzianum* on growth of different common fungal Pathogens.

Sl. no	Conc. of *T. harzianum* metabolites (%)	% Growth inhibition of various fungal pathogens					
		Alternaria brassicicola	***Aspergillus japonicus***	***Colletotrichum gloeosporioides***	***Fusarium solani***	***Fusarium sporotrichioides***	***Phytophthora infestans***
1	5	77.77	44.56	74.44	55.43	66.66	73.4
2	10	85.55	58.40	83.33	62.80	76.66	76.6
3	20	88.88	63.0	90.00	76.69	80.00	89.9

*Each value is a mean of three replicates.

Table 4. Effect of cell free culture filtrate of *T. viride* on growth of different common fungal pathogens.

Sl. no	Conc. of *Trichoderma viride* metabolites(%)	% Growth inhibition of various fungal pathogens					
		Alternaria brassicicola	***Aspergillus japonicus***	***Colletotrichum gloeosporioides***	***Fusarium solani***	***Fusarium sporotrichioides***	***Phytophthora infestans***
1	5	64	48	59	71.11	54	55.55
2	10	72	54	76	83.33	65	61.11
3	20	87	60	87	85.55	81.2	73.33

*Each value is a mean of three replicates

Table 5. Effect of cell free culture filtrate of *B. subtilis* and *P. fluorescens* on growth of different common fungal Pathogens.

Sl. no	Plant Pathogens	% Growth inhibition of various fungal pathogen by dual culture method	
		B. subtilis	***P. fluorescens***
1	*Alternaria brassicicola*	46.66	63.33
2	*Aspergillus japonicus*	50.00	66.67
3	*Colletotrichum gloeosporiodes*	74.44	76.67
4	*Fusarium sporotrichioides*	56.67	58.89
5	*Fusarium solani*	55.55	62.22
6	*Phytophthora infestans*	52.22	72.22

*Each value is a mean of three replicate.

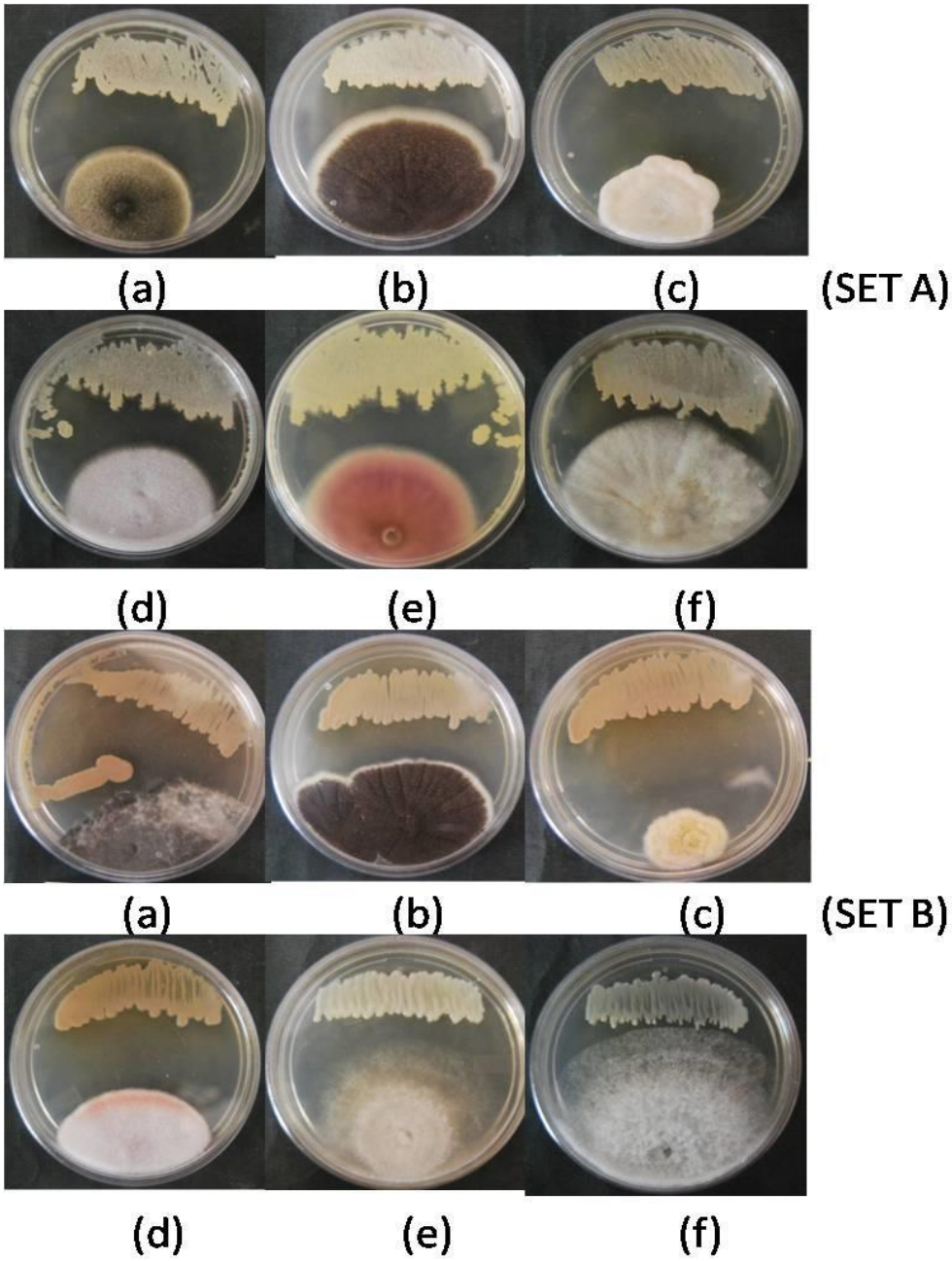

Fig.2. Dual culture culture method showing antagonistic effects of *B. subtilis* (Set A) and *Pseudomonas fluorescens* (Set B) against (a) *Alternaria brassicicola,* (b) *Aspergillus japonicus,* (c) *Colletotrichium gloeosporioides,* (d) *Fusarium solani* (e) *F. sporotrichioides* (f) *Phytophthora infestans.*

Discussion

Both the bacterial and fungal bioagents were effective in inhibiting the growth of a broad spectrum of phytopathogenic fungi *in vitro.* *T. harzianum* and *T. viride* are the most effective biocontrol agents applied to control plant diseases caused by soil-borne plant pathogens (Weller, 2002). *T. viride* may have secreted some antibiotic metabolites like trichodermin, dermadin, trichovirdin, and sesquiterpene, heptalic acid (Nakkeeran *et al.* 2002) which inhibited the growth of pathogens. The antagonistic activity of bacteria is enabled by the production of bacterial allelochemicals that include lytic enzymes, iron-chelating siderophores and antibiotics (Compant *et al*., 2005). The production of extra cellular chitinase in bacteria is considered crucial for their antagonistic activity (Shali *et al.*, 2010). Zehnder *et al.* (2001) reported that the biological control of soil-borne pathogens with antagonistic bacteria, particularly *Pseudomonas* spp. belonging to Plant Growth Promoting Rhizobacteria, has received prominent attention because of the dual role of these bacteria in plant-growth promotion and disease control. Beneficial rhizobacteria are known to colonize rapidly and aggressively the root systems, suppress pathogenic microrganism, and enhance plant growth and development (Weller, 1988). *B. subtilis* can secrete several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin which have an inhibitory effect on fungal pathogens (Alippi and Monaco, 1994).

Conclusion

The present study clearly indicates the high potential of the isolated biocontrol agents both bacterial and fungal isolates for different plant pathogens.

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