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RESEARCH ARTICLE

Efficacy of *Pseudomonas fluorescens* and *Trichoderma viride* based bioformulation for management of bacterial wilt disease of ginger

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SUMMARY

An experiment was conducted to evaluate the efficacy of a consortia formulation of *Pseudomonas fluorescens* and *Trichoderma harzianum* for management of bacterial wilt disease of ginger in Assam. Inhibitory effect of the biocontrol agents was evaluated *in vitro* following dual culture assay method for their efficacy against *Ralstonia solanacearum*, the ginger wilt pathogen. Quantitative assay of population dynamics of the two antagonists, mass cultured in organic substrates *viz.*, vermicompost (VC) and mustard oil cake (MOC) revealed that the antagonists maintained a high population count up to 120 days of storage at room temperature. *Pseudomonas fluorescens* recorded highest average population (45.47 x 10^7cfu/g) when mass cultured in the mixture of VC and MOC, while *T. harzianum* recorded maximum average population (34.14 x 10^7cfu/g) when mass cultured in MOC. Bioformulations were further evaluated for their efficacy in ginger wilt management under field condition. Efficacy of one fungicide (Copper oxychloride) and an antibiotic (Streptocycline) was also tested for comparison. Lowest disease incidence (15.63%) was recorded in the treatment of seed treatment (ST) and soil application (SA) of *P. fluorescens* and *T. harzianum* consortia mass cultured in the mixture of VC and MOC. It was followed by ST and SA of *T. harzianum* mass cultured in MOC (21.88%), which was statistically *at par* with the application of copper oxychloride (26.25%).

Key Words : Antagonists, Bacterial wilt, Biological management, Ginger, Substrates

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Ginger (*Zingiber officinale* Rosc.) is one of the most important cash crop in North-East region of India accounting 49 per cent of the country's total ginger area and 72 per cent of total ginger production (Rahman *et al.*, 2009). In Assam, it is grown in an area of 26600 ha with a productivity of 19.8 t/ha (Anonymous, 2005). The bacterial wilt incited by *Ralstonia solanacearum* is the major limiting factors in ginger production in Assam when environmental conditions become favourable for pathogen manifestations (Dev Nath *et al.*, 2002). Different management strategies adopted so far fails to reach the expectations of the growers due to many limitations. Tactics like crop rotation is not a viable as the bacterium is soil inhabitant and persist indefinitely in infested fields, use of antibiotics frequently leads to development of resistance races of the pathogen. In light of these factors exploring plant beneficial microbes for biological management of the dreaded disease could be a better alternative as it is environmentally safe and economically feasible.

Plant beneficial microbes (PBM), a component of extensive microbial biodiversity, affect plant health and development and the importance of these microbes in agriculture is continuously growing. Some PBM like, strains of fluorescent pseudomonads, *Trichoderma* spp. are known antagonists against soil borne plant pathogens including *R. solanacearum* and attempts have been made throughout the world to explore the possibilities of using these saprophytic antagonists for crop disease management (Papavizas, 1985; Nautyal, 2000; Bora and Bora, 2008 and Bora *et al*., 2013).

In light of these factors the present study was undertaken to explore the potential of an environment friendly strategy with bioformulation of *Pseudomonas fluorescens,* and *Trichoderma viride* to combat against the bacterial wilt disease of ginger crop.

MATERIAL AND METHODS

Different aspects of the experiments were carried out during 2010-2013 crop seasons in the laboratory of Department of Plant Pathology, Assam Agricultural University, Jorhat and farmers field of Sonitpur district under supervision of Krishi Vigyan Kendra, Sonitpur. Cultures of *R. solanacearum* were isolated in triphenyl tetrazolium chloride medium (TTC). Biocontrol agents, *viz., P. fluorescens* and *T. harzianum* were obtained from the stock cultures in the Department of Plant Pathology, AAU, Jorhat. The King'B (KB) medium for *P. fluorescens* and potato dextrose Agar (PDA) medium for *T. harzianum,* respectively were used for their multiplication and preservation. In *vitro* tests of the antagonists for their inhibitory property against *R. solanacearum* was done following dual culture method (Dennis and Webster, 1971). The antagonists were further evaluated to suppress wilt incidence in susceptible ginger cultivar Nadia.

Development of bioformulation :

Two organic substrates *viz.,* vermicompost (VC) and mustard oil cake (MOC) were used for mass multiplication of *P. fluorerscens* and *T. harzianum.* Substrates were first air dried and passed through 350 mesh sieves to obtain fine powders. These were filled into polypropylene bags (@1kg/bag) separately and plugged with non-absorbent cotton and sterilized at 121^0C for 30 min. Mass culture of fluorescent pseudomonad *P. fluorescens* was prepared by transferring aseptically its 24 h old culture in KB agar into 1000 ml KB broth and incubated at 28^0 C for 24 h. Similarly, mass cultures of *T. harzianum* was prepared by transferring aseptically their 72 h old growth in PDA to 1000 ml PD broth and incubated at 28^0 C for 120 h. From these, 10 ml of the *P. fluorescens* cells (10^7colony forming units/ml) and 10 ml of *T. harzianum* cells (10^7 cfu/ml), respectively were added to the sterilized substrates. A standard sticker, carboxy-methyl cellulose (CMC @ 1% w/w) was added for adherence property and a standard osmoticant (mannitol @ 3%) was also to retain moisture in the substrates. The inoculated substrates were incubated at 28^0 C for 72 h. The bags were stored at room temperature (26 ± 2^0 C) after incubation.

The population density of *P. fluorescens* and *T. harzianum* in different substrate formulation was assessed in seven different treatment combinations, *viz.,* VC + *P. fluorescens,* VC + *T. harzianum,* MOC + *P. fluorescens,* MOC + *T .harzianum*, VC + MOC + *T. harzianum,* , VC + MOC + *P. fluorescens* , VC + MOC + *P. fluorescens* + *T .harzianum*. The viable colonies of *P. fluorescens* and *T. harzianum* in different substrates were counted after 60, 120, 180 and 240days of inoculation by following the dilution plate technique (Waksman 1922).

Field experiment :

Based on the population density of *P. fluorescen,* and *T. harzianum* the best formulations were further

evaluated under field condition. Field experiments were carried to evaluate the bioformulations applied as seed treatment(ST) and soil application(SA) for management of bacterial wilt of ginger following RBD with 4 replications. . The treatment combinations were as follow:

T_1 ST+ SA of MOC+ *T. harzianum*

T_2 ST+ SA of VC+ MOC+ *P.fluorescens* + *T. harzianum;*

T_3 ST+ spray application with Streptocycline;

T_4 ST + foliar spray with copper oxychloride (Blitox 50)

T_5 Control.

For seed treatment, a paste of bioformulation with water and rice gruel (1 kg in 2 lit) was prepared. Ginger rhizomes (cv. NADIA) were first cleaned with water and treated (1 kg /10 kg of rhizomes) with the paste for 1 hr and the coated rhizomes were spread on a clean plastic sheet and dried overnight. For soil treatment, the formulations were individually mixed with dry cowdung (1 kg/10 kg) and each mixture of was applied at the base of the plant (100 g / plant) 90 d after planting. One fungicide, copper oxychloride (commercial formulation Blitox-50) spray drenched @ 0.25% and an antibiotic streptocycline was sprayed @ 100 ppm for comparison. All plants were inoculated with *R. solanacearum* (10^8cfu/ml) 30 days after planting by rhizome inoculation technique. The control plants were treated with sterile distilled water before inoculation with the pathogen. Observations were made to record disease incidence (%) and yield of ginger was recorded at harvest. During the crop period all the recommended agronomic practices were followed. The data were analyzed using Fisher's method of analysis of variance (Fisher, 1937).

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Efficacy of antagonists in inhibiting the growth of pathogen in dual culture assay :

The antagonists could produce varying level of inhibition zones against *R. solanacearum* in dual culture assays *in vitro* (Table 1). The inhibition zone due to *P. fluorescens* was significantly higher (38.7 mm) followed by combination of *P. fluorescens* and *T. harzianum* (31.8mm). The fluorescent pseudomonads could inhibit the growth of plant pathogens by producing a range of metabolites like antibiotics, siderophores and other

Table 1 : Inhibition zones produced by antagonists against *R. solanacearum* dual cultured in different media

Treatments	Inhibition zone (mm)*
R. solanacearum + *P. fluorescens* in TTC	28.5
R. solanacearum + *P. fluorescens* in Kings' B	38.7
R. solanacearum + *T. harzianum* in TTC	13.5
R. solanacearum + *T. harzianum* in PDA	20.3
R. solanacearum + *P. fluorescens* + *T. harzianum* in PDA	31.8
Control = *R. solanacearum* alone	0.0

*Data are mean of 5 replications

Table 2 : Population dynamics of *T. harzianum* in organic substrate based formulations after different days of storage

Treatments	Population ($x10^7$ cfu/g of substance)				
	60 Days	120 Days	180 days	240 Days	Mean
MOC + *T. harzianum*	35.94 (8.45)*	43.85 (8.62)	44.15 (8.64)	12.62 (8.04)	34.14 (8.44)
VC + *T. harzianum*	20.71 (8.30)	26.00 (8.41)	17.48 (8.23)	4.98 (7.55)	17.29 (8.12)
VC + MOC + *T. harzianum*	31.46 (8.47)	36.96 (8.56)	35.95 (8.53)	6.14 (7.69)	27.63 (8.31)
VC + MOC + *T. harzianum* + *P. fluorescens*	33.06 (8.51)	40.46 (8.60)	33.26 (8.49)	2.51 (7.25)	27.32 (8.21)
Mean	30.29 (8.43)	36.82 (8.55)	37.21 (8.47)	6.36 (7.63)	
	S.E. ±	C.D. (P=0.05)			
Treatment (T)	0.09	0.18			
Days (D)	0.09	0.18			
TxD	0.18	0.36			

*Data are the logarithmic transformed values of original mean population

substances such as cyanide (Loper and Buyer, 1991). Karuna and Khan (1994), recorded that *P. fluorescens* was very effective in inhibiting the growth of wilt pathogen *in vitro*. Ciampi-Panno *et al*. (1996) reported *P. fluorescens* to be the primary candidates for biological control of bacterial wilt pathogen because of their own ability to synthesize a wide range of secondary metabolites, many of which possess antibacterial and fungal activity.

The fungal antagonist *T. harzianum* also showed better effect in suppression of *R. solanacearum.* The general mechanism of antagonistic activity of *Trichoderma* sp. has been reported to be antibiosis, lysis, competition and mycoparasitism (Papavizas and Lumsden, 1980). Ayer and Adam (1981) showed that *T. viride* produced diffusible substances toxic to the pathogens and also they could effectively act as ectoparasite by overcrowding the other organisms.

Population of antagonists (x 10^7 cfu/g) in various substrate based formulations after different days of storage :

The mean population of both the antagonists in formulations of three substrates significantly increased up to 180 days of storage after which it showed declining trend (Table 2 and 3). The highest mean population of *P. fluorescens* (45.47 × 10^7 cfu/g) recovered from formulation with vermicompost as substrate. While, the least population was recovered from the formulation with mustard oil cake (MOC) as substrate. In contrast, highest mean population of *T. harzianum* (34.14 × 10^7 cfu/g) was recovered from formulation with MOC as substrate. The lowest population of *T. harzianum* was recovered from the formulation with mixture of VC and MOC as substrate. Earlier, Suslow and Schroth (1982) reported that fluorescent pseudomonad (*P. fluorescens*) could survive up to 1 year with a higher population level when it was incorporated into carrier materials *viz*., talc or peat along with CMC. The higher population of antagonists might be due to high nutrient content of vermicompost, which is a good source of humus, Vitamin-B, auxin and antibiotics. Moreover, it contains 2.5-3.5 per cent nitrogen, 1.5-2.0 per cent phosphorus and 2.0-3.5 per cent potassium. Vermicompost causes a shift of pH towards neutral, a reduction in electrical conductivity, and, therefore, fluorescent pseudomonad *P. fluorescens*, which prefers neutral to alkaline pH tends to exhibit higher population shift in vermicompost (Alexander, 1997). Carboxy methyl cellulose (CMC) was used in the formulation as an adhesive, which might have also played

Table 3 : Population dynamics of *P .fluorescens* in organic substrate based formulations after different days of storage

Treatments	Population (x10^7 cfu/g of substance)				
	60 Days	120 Days	180 days	240 Days	Mean
Vermicompost + *P .fluorescens*	35.50 (8.55)*	44.03 (8.64)	46.36 (8.65)	17.54 (8.23)	35.86 (8.52)
MOC + *P. fluorescens*	5.61 (7.70)	17.48 (8.23)	16.56 (8.12)	8.71 (7.92)	12.09 (7.99)
VC + MOC + *P. fluorescens*	54.29 (8.73)	55.56 (8.74)	37.58 (8.54)	634.45 (8.53)	45.47 (8.63)
VC + MOC + *P. fluorescens* + *T. harzianum*	25.81 (8.41)	31.21 (8.48)	28.26 (8.42)	4.76 (7.56)	22.51 (8.22)
Mean	30.62 (8.35)	36.75 (8.52)	32.19 (8.43)	16.36 (8.06)	
	S.E. ±	C.D. (P=0.05)			
Treatment (T)	0.07	0.13			
Days (D)	0.07	0.13			
TxD	0.13	0.26			

*Data are the logarithmic transformed values of original mean population

Table 4: Effect of different treatments on disease incidence and yield of ginger

Treatments	Wilt (%)	Yield (q/ha)
ST+ SA of MOC+ *T. harzianum*	21.88 (27.59)	242.00
ST+ SA of VC+ MOC+ *P.fluorescens* + *T. harzianum*	15.63 (23.24)	265.50
ST+ Spray application with Streptocycline	26.25 (30.66)	234.25
ST+ Foliar spray with Copper oxychloride (Blitox 50)	22.50 (27.59)	179.50
Control	81.98 (65.63)	54.56
S.E. ±	4.39	14.56
C.D. (P=0.05)	9.36	31.03

* Figures within the parenthesis are angular transformed values

** Data are the logarithmic (log x+1) transformed values of original mean population

a role of preserving the bacteria with viability for long-term. Moreover, mannitol used as osmoticant, has the ability to protect the antagonist from desiccation and thereby increases their survivality (Vidhyasekaran and Muthamilan, 1995). However, fungal antagonist *T. harzianum* showed high population when MOC was used as its substrate. MOC might have helped *T. harzianum* in better sporulation and production of colony forming units as it could release enzymes like b-1-3 glucanase and chitinase for the utilization of cellulose and chitin present in different substrates. *Trichoderma* spp. multiply faster at higher concentration of $CO_{2,}$ a condition favoured by MOC as substrate. Similarly, it might also have been favoured by humic acid present in the MOC (Ushasree *et al.,* 1989).

Efficacy of bioformulation on reduction of bacterial wilt in ginger :

The efficacy of the substrate based formulations of *P. fluorescens* and *T. harzianum* for management of bacterial wilt of ginger revealed that all the treatment combinations, irrespective of antagonist and substrate used, were significantly effective in reducing the wilt incidence and enhancing yield of ginger (Table 4). However, *P. fluorescens* and *T. harzianum* formulated in vermicompost and MOC was significantly effective showing minimum diseases incidence (15.63%) as well enhancement of ginger yield (265.5q/ha). It was followed by the treatment of *T. harzianum* cultured in MOC as substrate, (wilt incidence of 21.88% and yield of 242.0q/ha), which was statistically at par with application of copper oxy chloride spray-drenched @ 0.25% (wilt incidence of 22.5% and 179.5q/ha) and spray application of streptocycline @ 100ppm (wilt incidence 26.25% and 234.25q/ha). Earlier, Aspiras and Dela Cruz (1985) reported similar result in tomato (*R. solanacearum*) by using *P. fluorescens* as seed and seedling inoculation. Fluorescent pigments produced by the pseudomonads sequester Fe^{3+} and are considered siderophores, which inhibits large number of phytopathogenic bacteria and fungi in soil. Moreover, fluorescent pseudomonad could rapidly colonize and inhibit certain components of the root zone microflora and along with rich substrate. The decrease of bacterial wilt incidence and increase in ginger yield in these two treatments might be due to direct effect of the antagonists on reduction of the pathogen population present in the plant rhizosphere. In addition, antagonists like *P. fluorescens* induces systemic resistance (Meena *et al*., 2002) in plants and enhances overall physio-biochemical activities culminating to the increase in yield. Some rhizospheric bacteria have a beneficial agronomic effect on plant growth. These bacteria are commonly referred to as plant growth-promoting rhizobacteria (PGPR) (Compant *et al*., 2005). PGPR can directly stimulate plant growth by synthesizing hormones (phytostimulators) or by supplying the plants with nutrients. In present study, *P. fluorescens* and *T. harzianum* in vermicompost and MOC as substrate was more effective in reduction of diseases incidence in ginger. MOC influences *T. harzianum* in better sporulation and production of colony forming units. Danielson and Davey (1973) also reported that *Trichoderma* spp. multiply faster at higher concentration of CO_2 and when substrates like MOC containing carbon are degraded, CO_2 is evolved. It is evident that substrates like vermicompost and MOC can enhance the activity of antagonists in soil, which compete with the soil borne plant pathogens. Similarly, an increase in the available phosphorus content of soil has been reported to be effective in suppression of the diseases incidence. The vermicompost increases available phosphorus content in the soil, which could provide maximum protection to the plants. The increased availability of phosphorus and potash in soil might have contributed towards the resistance of the plants against the pathogen (Radhakrishnan and Narayanaswami, 1994). From above discussion, it is evident that substrates like vermicompost and MOC enhances the activity of antagonists, which compete with the soil borne plant pathogen for nutrient and space.

The addition of antagonists along with different substrates might have influenced of soil organic carbon. Earlier, Hoitink and Fahy (1986) tried to establish positive correlation between C : N ratio of residues of organic carbon and disease severity. Similarly, an increase in the available phosphorus content of soil has been reported to be effective in suppressing the disease incidence. The substrates particularly vermicompost increases available phosphorus content in the soil in contrast to the other treatment, which could provide maximum protection to the plants from the disease. The increased availability of phosphorus and potash in soil might have contributed towards the resistance of the plants to the diseases as have been recorded in many other pathogens (Sharif *et al.,* 2003).

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