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Plant Growth Promoting Microbes, their Compatibility Analysis and Utility in Biointensive Management of Bacterial Wilt of Tomato

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ABSTRACT

Keywords

Biointensive management, bacterial wilt, tomato, plant growth promoting microbes, *Ralstonia solanacearum*

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A biointensive strategy was developed for management of bacterial wilt of tomato (*Solanum esculentum* Mill) caused by *Ralstonia solanacearum* using four compatible plant growth promoting microorganisms (PGPM), viz., *Trichoderma parareesei*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Azotobacter chroococcum*. Compatibility analysis among the PGPM showed positive interaction among them *in vitro*. The antagonistic potential of these PGPM was tested *in vitro* singly or in consortia and per cent inhibitions of target pathogen *R. solanacearum* were recorded and analyzed. The highest inhibition (91.10 %) of *R. solanacearum* was recorded against consortia of *T. parareesei* + *P. fluorescens* + *B. subtilis* + *A. chroococcum* followed by *T. parareesei* + *P. fluorescens* + *B. subtilis* (81.10 %) and *P. fluorescens* + *B. subtilis* + *A. chroococcum* (68.14%). Talc based bioformulations were prepared using the best three PGPM and their consortia for management of bacterial wilt by applying these as seed treatment, root treatment and soil application under pot condition during 2013-2015 crop seasons. Significantly highest reduction of bacterial wilt incidence (95.09 %) and highest yield (1.692 kg/plant) of tomato was recorded in treatment comprising of *T. parareesei* + *P. fluorescens* + *B. subtilis* + *A. chroococcum*. Correlation studies revealed negative correlation (-0.993) between bacterial wilt incidence and yield of tomato.

Introduction

Assam ranks 11th in all India level of tomato production with 402.4 thousand MT in an area of 17.2 thousand ha and productivity of 23.2 MT/ha (Annon, 2013). Bacterial wilt disease is a serious limiting factor of tomato production in the state which is wide spread throughout all vegetable growing areas. The disease assumes serious proportion particularly in the years when environmental conditions favours the outbreak of the disease.

Different management practices like, use of chemicals, antibiotics, soil amendments, soil solarization etc., has been suggested by many workers to control the disease. However, management of the disease by a single control measure seems to be a difficult proposition due its wide host range and its long survival ability in soil. Therefore, there has been a growing concern to find alternative methods by exploiting potential of bioactive antagonists (Weller

and Cook, 1983). Earlier, various biological agents have been employed for management of different crop diseases. Das and Bora (2000) used *P. fluorescens*, *B. subtilis*, *T. harzianum*, *T. viride*, *T. koningii*, *Aspergillus terreus* and *G. virens* for management of bacterial wilt in tomato. Similarly, Kumar and Sood (2001) found significant reduction in bacterial wilt incidence in tomato by application of *P. fluorescens*. Similarly, *Azotobacter*, which have ability to fix atmospheric nitrogen promotes root hair formation in seedling roots, increases plant weight during early crop growth stage (Rai and Gaur, 1988), produces phytohormones, antibiotics and enhances mineral uptake (Neumann and Romheld, 2001). Srivastava *et al.* (2010) studied the use of consortium of arbuscular mycorrhizal fungus, *T. harzianum* and fluorescent *Pseudomonas* formulation for management of tomato wilt.

In the present study attempts were made to explore some fungal and bacterial biocontrol agents along with few phosphate solubilising and nitrogen fixing microbes in developing a consortium package for developing a biointensive management strategy against bacterial wilt of tomato along with yield enhancement.

Materials and Methods

Evaluation of Compatibility among Different Bioactive Microorganisms and development of Microbial Consortia

The interaction study amongst different bioactive microorganisms, *viz.*, *T. parareesei*, *P. fluorescens*, *B. subtilis* and *A. chroococcum* was tested for their compatibility using PDA and NA as basal media following dual culture techniques (Aspiras and Cruz, 1985) with few modifications. The test plates were incubated at $28 \pm 1^\circ\text{C}$ for 48 to 96 hrs.

Altogether fifteen treatment combinations were tested and compared with growth of *T. viride* alone (Control-1), growth of *P. fluorescens* alone (Control-2), growth of *B. subtilis* alone (Control-3), and growth of *A. chroococcum* alone (Control-4). The microorganisms showing positive compatibility among themselves was recorded, tabulated and selected for further study.

The inhibitory effects of PGPM *T. parareesei*, *P. fluorescens*, *B. subtilis*, *A. chroococcum* and their consortia were tested *in vitro* against *R. solanacearum* adopting dual culture assay plate technique in pathogen favourable media (Triphenyl tetrazolium chloride). A total of sixteen treatment combinations were compared. Assay plates of *R. solanacearum* were prepared by seeding the 24hr growth of the bacterium in TTC. Following this, 0.5 cm bit of fungal antagonist *T. parareesei* grown in PDA was scooped out and transferred to the center of TTC plates seeded with *R. solanacearum*. Following the same procedure, 0.5 cm diameter lawn of *P. fluorescens*, *B. subtilis* and *A. chroococcum* grown in NA was scooped out separately and transferred to the center of TTC plates. Test plates seeded only with *R. solanacearum* served as control. The plates were then incubated at $28 \pm 1^\circ\text{C}$. The inhibitions produced by the bioactive microorganisms and their consortia were measured after 48-96 hrs of dual inoculation. Based on the percent of inhibitions shown by the bioactive microorganisms or their combinations *in vitro*, three best treatments were selected for their further study.

Preparation of Bioformulations of Bioactive Microorganisms

Polypropylene (PP) bags were filled with 1kg finely sieved talcum powder (substrate),

sterilized at 121°C for 15 minutes. Pure culture of the fungal antagonist *T. parareesei* was grown in Potato Dextrose (PD) broth and incubated at 28 ± 1°C for 72hr to obtain a concentration of 1 × 10⁷ colony forming units (cfu/ml). Pure cultures of *P. fluorescens*, *B. subtilis*, *A.chroococcum* were grown in KB and NA slants and incubated at 28 ± 1°C for 24hr. To these slants, sterile water added and bacterial suspensions (15 ml) of the three bacteria was aseptically taken separately and added to 1 lit of Nutrient broth contained in conical flasks. This flask after thorough stirring was incubated at 28 ± 1°C for 72 hr to obtain a population of 1 × 10⁷cfu/ml. Then 10 ml of the each fungal and bacterial cells was inoculated in the above sterilized PP bags containing 1 kg talcum powder. To facilitate greater adherence property of the substrate, 10 ml of sticker, carboxy-methyl cellulose (CMC @1%) was added aseptically. Similarly, 10 ml of an osmoticant (mannitol @ 3%) was added to impart the substrate a higher moisture retaining property. The PP bags after thorough mixing were incubated at 28±1°C for 7 days to get desired concentration of each bioformulation.

Efficacy of Bioformulation Based on Bioactive Microorganisms and their Consortia in Management of Bacterial Wilt of Tomato

Three best bioactive microorganisms showing inhibitory property against *R. Solanacearum* *in vitro* were selected for further evaluation of their ability to suppress bacterial wilt of tomato using cultivar Pusa Ruby, during crop seasons 2013-5. Biofor-Pf-2, organic biopesticide produced in Assam Agricultural University, Jorhat, was also used as a control treatment. The treatment combinations arrange in CRD with 5 replications were: Compatible bioactive microbe -1 (CBM) as seed + root

+ soil treatment; Compatible bioactive microbe - 2 (CBM) as seed + root + soil treatment; Compatible bioactive microbe -3 (CBM) as seed + root + soil treatment; Biofor-Pf-2 as seed + root + soil treatment; Control.

The substrate based bioformulation of *T. parareesei*; *P. fluorescens*, *B. subtilis* and *A.chroococcum* were applied as seed treatment, root dip treatment and soil application methods. For seed treatment, paste slurry of each substrate based formulation was prepared and to these paste slurry, tomato seeds were dipped @ 1000 seed/100 ml of paste solution for 1 hr. The treated seeds were then sown in the tomato nursery. For root treatment, each of the substrate based formulations was mixed with water @ 20 gm in 1000 ml to prepare 2 per cent bio-formulation solution. At the time of transplanting uprooted plants were root dipped (@1000 seedlings/1000ml) in the solution for 1 hr. Treated seedlings were dried under shade for 1 hr before transplanting in to the earthen pots. For soil application, each of bioformulation was mixed with soil @ 100 g/plant after 15 days of sowing, at the base of the plants. The number of wilted plants was recorded throughout the experiment. The data were tabulated and disease incidence (DI %) was calculated. The yield of each treatment (kg) was recorded at harvest.

Results and Discussion

Interactive Action of bioactive microorganisms

Interactive action among *T. parareesei*, *P. fluorescens*, *B. subtilis* and *A. chroococcum* were tested *in vitro* adopting dual culture assay plate technique in PDA and NA as basal media. The data showed compatibility among all the treatment combinations of the

four bioactive microorganisms *in vitro* (Table 1).

Four bioactive microorganisms produced varying inhibitions (%) *in vitro* against *R. solanacearum* (Table 2). The inhibition (%) produced by the consortia of four bioactive microorganisms *T.parareesei*+ *P. fluorescens* + *B. subtilis* + *A.chroococcum* was significantly highest (91.10 %) followed by *T.parareesei*+ *P. fluorescens* + *B. subtilis* (78.33%). Among individual bioactive microorganisms highest inhibition was shown by *T.parareesei* (53.94%) followed by *P.fluorescens* (50.00%). Among individual bioagents, the lowest inhibition per cent was showed by *A.chroococcum* (27.77%).

Efficacy of Different Bioactive Microorganisms and their Consortia based Formulations in Management of bacterial wilt of tomato

Bioformulations of different combinations of *T. parareesei*, *P. fluorescens*, *B. subtilis* and *A. chroococcum* were applied as seed, root and soil treatment for management of bacterial wilt of tomato. The effect of these bioformulations of was also compared with efficacy of Biofor-Pf 2 (a popular bioformulation of Assam) applied as seed, root and soil treatment. The disease incidence of tomatodecreased significantly accompanied by significant increase of yield (kg/plant) in plants treated with consortia of different bioactive microorganisms (Table 4). Lowest disease incidence (4.04%) was exhibited by the consotia of *T. parareesei* + *P. fluorescens* + *B. subtilis* + *A. chroococcum* followed by *T.viride* + *P. fluorescens* + *B. subtilis* (10.27%)applied asseed treatment, root treatment and soil treatment. Plants untreated with bioformulation showed highest disease incidence (81.75%). The yield data of

tomato (Table 3) showed that significantly highest yield (kg/plant) was recovered from plants treated with bioformulation consortia of *T. parareesei* + *P. fluorescens* + *B. subtilis*+ *A. chroococcum* (1.692 kg/plant) followed by *T. parareesei* + *P. fluorescens* + *B. subtilis* (1.516 kg/plant)applied asseed treatment, root treatment and soil treatment. Plants untreated with bioformulation showed lowest yield (0.369 kg/plant).

Correlation Studies

A correlation was drawn between the disease incidence and the yield of different treatments. From the correlation studies, it was evident that there was a negative correlation(-0.993) between disease incidence (%) and yield (kg/plant) of the different treatments. From the data presented in the Table 3, simple linear regression line (Fig. 1) was drawn taking disease incidence as independent value and yield as dependent value. From the slope of the line it is evident that the disease incidence had negative effect on yield.The negative correlation between yield and PWI, might be due to the fact that higher population of bioagents resulted either in suppression of the pathogen population or exclusion of the pathogen from rhizosphere niches by their enhanced antagonists activity (Mulyaet al., 1996).

Earlier, Deuri (2013), reported positive compatibility amongst saprophytic antagonists like *P. fluorescens*, *T.viride*, *M.anisopliae*. Similar compatible observations amongst bioagents like *P. fluorescens*, *T. viride*, *T.harzianum*, *M. anisopliae* and *Beauveria bassiana* was made by Bora (2012) and Bora et al. (2013).

Dagostin et al. (2008) reported that *Trichoderma sp.* have ability to produce a series of antibiotics and fungal cell wall-

degrading enzymes proteinase (Prb1), which play important role in mycoparasitism and mycelial lysis of the target pathogenic microbes. Durell (1968) documented with electron microphotography the haustoria and hyphae of *T. viride* within hyphae of Phycomycetes and digestion of their contents. Papavizas (1985) reported that *T. harzianum* could inhibit the growth of *R. solanacearum* by production of diffusible substances and overcrowding the pathogen. Bora *et al.* (2015) suggested utility of *T. parareesei* combined with other saprophytic antagonists for management of bacterial wilt of bhootjolokia caused by *R. solanacearum*. The present observation of antagonism of *T. parareesei* might be due to similar type of antagonistic mechanisms of parasitism.

Fluorescent pseudomonads could show diverse mechanisms of biocontrol which include antibiosis, cyanide production, siderophore production, competition for space and nutrient and induced systemic resistance. Root colonizing fluorescent pseudomonads could suppress soil born pathogenic fungi by producing antifungal metabolites such as 2, 4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT), pyrrolnitrin (PRN), phenazines, and hydrogen cyanide (Haas and Defago 2005). Moreover, fluorescent pseudomonads produce yellow green fluorescent siderophores (pyoverdine type) under low iron condition, which is an important mechanism for the biocontrol activity of PGPR (Neilands and Leong, 1986; Bakker *et al.*, 1993).

Fluorescent pseudomonads also activate ISR against various fungal, bacterial and viral diseases (Liu *et al.*, 1995). The present observation of antagonism of *P. fluorescens* might be due to similar type of antibiosis, parasitism and ISR induced in tomato plant by *P. fluorescens*. Venkateswarlu *et al.* (2007) described that phosphate solubilizing

bacteria (PSB) could solubilize the unavailable inorganic-P like tricalcium, iron, aluminium and rock phosphates into soluble forms by release of a variety of organic acids like succinic, citric, malic, fumaric, glyoxalic and gluconic acids. Besides plant growth promoting *Bacillus* sp. have been found to be very good antagonists against various fungal and bacterial pathogens. Lazzaretti *et al.* (1995) reported that *B. subtilis* could produce antagonistic proteins highly inhibitory to seed borne fungal and bacterial pathogens. Similarly, plant growth promotion, *Azotobacter* sp. could produce various antifungal compounds and suppress pathogenic or toxin producing soil microflora (Jen-Hshuan, 2006). Kamil *et al.* (2008) reported that, *Azotobacter* sp. could produce different growth hormones (IAA and other auxins, such as gibberellins and cytokinins), vitamins and siderophores. The present observation of antagonism of *B. subtilis* and *A. chroococcum* might be as a result of similar type of mechanisms or other growth enhancing mechanisms.

The decrease in disease incidence in tomato and increase in yield might be due to suppression of the pathogen by application of bioformulation of different bioactive microorganisms. This finding is in agreement with Bustamanate *et al.* (1989). Das (1999) recorded that *T. viride* and *T. harzianum* could significantly reduce the bacterial wilt severity caused by *R. solanacearum* in tomato. Similarly, Ranjan *et al.* (2002) reported that *Trichoderma* sp. could control rhizome rot of ginger caused by *Pythium*, *Fusarium* and *R. solanacearum*. Sutunya (1984) reported that *P. fluorescens* could effectively reduce the growth of *R. solanacearum* in rhizosphere of tomato plant. Similarly, Aspiras and Cruz (1985) observed the antagonistic activity of *P. fluorescens* against *R. solanacearum* in tomato and potato.

Table.1 Compatibility amongst different bioactive microorganisms and their consortia

Treatment combinations	Compatible/ Non-compatible
<i>Trichoderma parareesei</i> (Control1)	+ve
<i>Pseudomonas fluorescens</i> (Control2)	+ve
<i>Bacillus subtilis</i> (Control3)	+ve
<i>Azotobacter chroococcum</i> (Control4)	+ve
<i>T.parareesei</i> + <i>P. fluorescens</i>	+ve
<i>T. parareesei</i> + <i>B. subtilis</i>	+ve
<i>T. parareesei</i> + <i>A. chroococcum</i>	+ve
<i>P. fluorescens</i> + <i>B. subtilis</i>	+ve
<i>P. fluorescens</i> + <i>A. chroococcum</i>	+ve
<i>B. subtilis</i> + <i>A. chroococcum</i>	+ve
<i>T. parareesei</i> + <i>P. fluorescens</i> + <i>B. subtilis</i>	+ve
<i>T. parareesei</i> + <i>P. fluorescens</i> + <i>A. chroococcum</i>	+ve
<i>T. parareesei</i> + <i>B. subtilis</i> + <i>A. chroococcum</i>	+ve
<i>P. fluorescens</i> + <i>B. subtilis</i> + <i>A. chroococcum</i>	+ve
<i>T. parareesei</i> + <i>P. fluorescens</i> + <i>B. subtilis</i> + <i>A. chroococcum</i>	+ve

Table.2 Inhibition of *R. solanacearum* growth in vitro by different bioactive microorganisms and their consortia

Treatment Combinations	Radial growth Inhibited (mm diam)	Inhibition (%)
Control	0.00	0.00(4.05)*
<i>Trichoderma parareesei</i>	48.55	53.94 (26.64)
<i>Pseudomonas fluorescens</i>	45.00	50.00 (30.00)
<i>Bacillus subtilis</i>	37.50	41.66 (25.68)
<i>Azotobacterchroococcum</i>	25.00	27.77(16.13)
<i>T.parareesei</i> + <i>P. fluorescens</i>	43.00+31.50	82.77 (32.61)
<i>T.parareesei</i> + <i>B. subtilis</i>	44.00+19.50	70.55 (30.26)
<i>T.parareesei</i> + <i>A. chroococcum</i>	36.55+23.00	66.16 (27.33)
<i>P. fluorescens</i> + <i>B. subtilis</i>	38.33+24.67	70.00(31.69)
<i>P. fluorescens</i> + <i>A. chroococcum</i>	34.66+17.50	57.95 (30.83)
<i>B. subtilis</i> + <i>A. chroococcum</i>	15.00+11.33	29.25 (25.89)
<i>T. parareesei</i> + <i>P. fluorescens</i> + <i>B. subtilis</i>	35.00+25.00+ 10.50	78.33 (39.07)
<i>T.parareesei</i> + <i>P. fluorescens</i> + <i>A. chroococcum</i>	38.50+26.33+ 8.11	81.10 (32.86)
<i>T. parareesei</i> + <i>B. subtilis</i> + <i>A. chroococcum</i>	36.00+16.00+ 7.33	65.92(31.43)
<i>P. fluorescens</i> + <i>B. subtilis</i> + <i>A. chroococcum</i>	33.33+19.50+ 8.50	68.14 (36.66)
<i>T. parareesei</i> + <i>P. fluorescens</i> + <i>B. subtilis</i> + <i>A. chroococcum</i>	33.00+32.33+ 10.00+6.66	91.10 (49.43)
S.Ed .(±)		2.425
CD (0.05)		4.733

*Data in the parentheses are angular transformed values

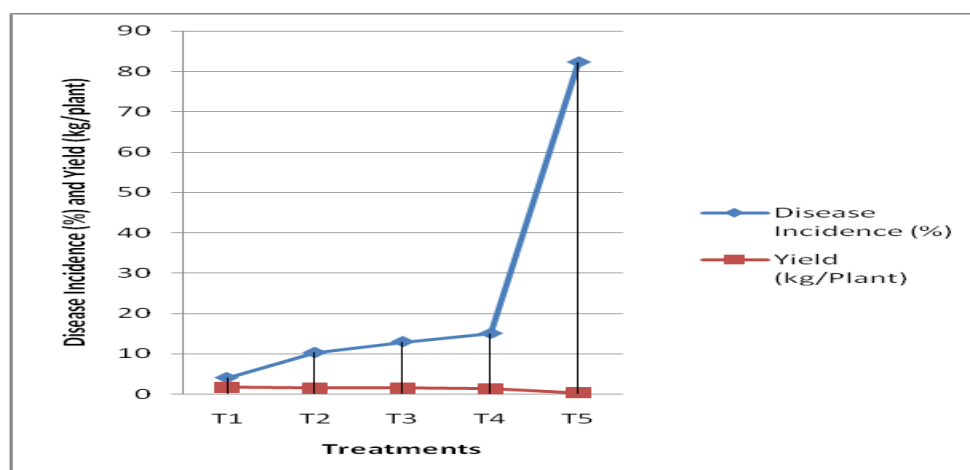
Table.3 Effect of substrate based formulations of bioactive microorganisms on bacterial wilt incidence and yield of tomato

Treatments	Disease incidence (%)		Pooled	Disease reduction (%)	Yield (kg/plant)		Pooled	Yield increase over control (%)
	2013-14	2014-15			2013-14	2014-15		
T ₁ *	4.42 (12.13)**	3.66 (11.01)	4.04 (11.58)	95.09	1.644	1.741	1.692	358.54
T ₂	10.72 (19.10)	9.82 (18.26)	10.27 (18.68)	87.52	1.461	1.571	1.516	310.84
T ₃	13.42 (21.48)	12.50 (20.70)	12.96 (21.09)	84.25	1.378	1.518	1.448	292.41
T ₄	15.46 (23.15)	14.56 (22.43)	15.01 (22.79)	82.26	1.295	1.382	1.340	274.53
T ₅	82.52 (65.29)	82.00 (64.91)	82.26 (65.10)	81.75	0.359	0.379	0.369	
S.Ed.(±)	0.378	0.429	0.401		0.058	0.055	0.055	
CD (0.05)	0.652	0.739	0.689		0.100	0.095	0.095	

*T₁= *T. parareesei*+ *P. fluorescens* + *B. subtilis*+ *A. chroococcum*; T₂= *T. parareesei*+ *P. fluorescens* + *B. subtilis*; T₃= *P. fluorescens* + *B. subtilis*+ *A. chroococcum*; T₄= Biofor-Pf-2; T₅= Control

**Data in the parentheses are angular transformed values

Fig.1 Correlation between disease incidence and yield of tomato treated with different bioformulation consortia



Where, T₁= *T. parareesei*+ *P. fluorescens* + *B. subtilis* + *A. chroococcum* ;T₂= *T. parareesei*+ *P. fluorescens* + *B. subtilis*; T₃= *P. fluorescens* + *B. subtilis*+ *A. chroococcum*;T₄= Biofor-Pf-2 ; T₅= Control

Das and Bora (2000) recorded biological control of bacterial wilt in tomato with *P. fluorescens*, *B. subtilis*, and *Trichoderma sp.* Rodriguez and Fraga (1999) observed

simultaneous increases in P uptake and crop yields after inoculation with *Bacillus firmus*, *B. polymyxa* and *B. cereus* which indicated the advantage of the mixed inoculations of

PGPR strains comprising PSB. Sutunya (1984) recorded slow disease development and reduced *R. solanacearum* when roots of tomato plants were inoculated with *Bacillus cereus*. Aspiras and Cruz (1985) observed reduced wilt incidence in tomato cultivar "Yellow plum" by treating the seedlings with *B. polymyxa* FU6 for 5 hr. Similarly, Karuna and Khan (1994) observed effectiveness of *B. subtilis* and *B. polymyxa* in inhibiting the growth of *R. solanacearum* under field condition.

Azotobacter is one of the potential contributor to plant growth with their ability to fix atmospheric nitrogen, promotes root hair formation in seedling roots, and increase plant weight during early crop growth stage (Rai and Gaur, 1988), produce phytohormones and antibiotic substances and enhance mineral uptake etc. (Suneja and Lakshminarayana, 1998; Neumann and Romheld, 2001).

In addition to nitrogen fixation, siderophore production could be another property, contributing towards the agronomic utility of *Azotobacter* (Suneja and Lakshminarayana, 1998).

With the advent of green revolution in India during 1960s, the level of consumption of agro-chemicals has been held as yardstick of agricultural development. The green revolution boosted up agricultural production in India to an extent that large-scale exports of many agricultural products become a reality. However, agro-chemicals also damaged the eco-system and the delicate balance between various components of interacting eco-system. So, a stage has now reached to review whether promoting the use of these toxic chemicals is appropriate strategy or not. One such new strategy, biological control of pathogens, although subject to numerous ecological

limitations, is expected to become an important part of the control measures employed against many more diseases. In the present study, the application of *T. parareesei* + *P. fluorescens* + *B. subtilis* + *A. chroococcum* based bio-formulations promises as an effective biocontrol option along with better plant growth, yield and soil health management.

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