BIOCONTROL OF EARLY BLIGHT OF TOMATO USING CONSORTIUM OF *BACILLUS SUBTILI S* AND *PSEUDOMONAS FLUORESCENCE*

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Abstract: Rhizosphere bacteria are one of the most potential biological control agents in the plant disease protection. They are reported to be effective in controlling soil-borne pathogens in the field. In this study, soil samples from tomato rhizosphere were collected from Allahabad area of Uttar Pradesh State, India. Five promising antagonists exhibiting higher zone of inhibition (ZOI) (31.10mm and above) and percent disease control (ranging from 32.57 to 63.00%) were selected. These strains of *Bacillus subtilis* (Bs12 and Bs16) and *Pseudomonas fluorescens* (Pf2, Pf9 and Pf17) were tested individually and in combination for their effectiveness against early blight of tomato incited by *Alternaria solani*. The results revealed that the strains of the two bacteria were compatible. Under *in vitro* conditions the combined application of Bs12+ Pf2 was found to effectively inhibit the mycelial growth of the pathogen and promote the growth of tomato seedlings when compared to application of individual strains of the antagonists. These findings suggest that synergistic consortia of biocontrol agents may be successfully employed as an eco-friendly strategy for the management of early blight disease of tomato.

Introduction: Tomato (*Lycopersicon esculentum* Mill.) has become one of the widely grown vegetables in the world and is regarded as a top priority vegetable. Tomato contributes to a healthy, well-balanced diet. It is rich in minerals, amino acids, sugars, dietary fibers and is considered to be fairly high vitamins of high cash value and with potential for better-quality processing [1]. Recently, there has been more emphasis on tomato production as a source of income and security [2].Today the importance of tomato is increasing and is widely being accepted and use as a variety of dishes as row cooked or processed products more than any other vegetable. Allahabad is one of the most important tomato-growing regions in India. In Uttar Pradesh, tomato is grown in an area of 18,433 ha, with a production of 2, 62,912 tonnes and a productivity of 11,611 kg/ha [3].

Tomato is known to be affected by a number of diseases among which, early blight caused by *Alternaria solani* (Ellis and Martin) causes yield losses up to 80% [4].The disease manifests as leaf spots with dark brown to black concentric rings which later coalesce and results in blighting of leaves; defoliation and shedding of immature fruits. Also, dark spots and sunken lesions appear near the base of stem resulting in stunting and girdling of stem.

Presently, the management of tomato early blight disease has been done with the application of chemical fungicides.

However, it may not be sustainable in the longer run as chemical fungicides are known to cause residual toxicity, toxicity to non-target organisms and other environmental hazards. Therefore, recent efforts have been focused on developing eco-friendly, safe, long lasting and effective management strategy for the management of plant diseases [5] and [6].

The biological control of plant pathogenic fungi has received considerable attention as an alternative strategy. Antagonistic bacteria have been extensively studied as biocontrol agents effective against various soil–borne pathogens. Among 20 genera of bacteria, *Bacillus spp*. and *Pseudomonas spp*. are widely used as biocontrol agents. Several *Bacillus spp.* including *B. subtilis* are antagobistic to plant pathogenic fungi and bacteria. *Bacillus spp.* produced at least 66 different antibiotic compounds [7]. Subtilin, bacillin, bacillomycen, subtenolin, mycosubtilin, toximycin and bacitracin are different names given to antibiotics produced by *Bacillus subtilis* [8] and [9]. The antagonistic effect *Bacillus subtilis* against several fungi *in vitro* and *in vivo* have been reported by [10]; [11]; [12]; and [13]. However, investigation on mechanisms of biological control by *Pseudomonas spp*. revealed that *P. fluorescens* produced antibiotics highly effective which protect plants from various pathogens through inducing systemic resistance [14] and [15].

Materials and Methods:

Isolation of the Fungus in Pure Culture: Infected leaves of tomato plants displaying the characteristic symptoms of early blight disease were collected in winter (2015) from Allahabad area. Each of the diseased leaves was cut into small pieces (5-10 mm), washed with distilled water and surface sterilized with sodium hypochlorite (conc. 0.2%) for two minutes. Then sterilized tissues were rinsed several times in sterilized distilled water and dried. Four pieces were transferred with sterilized needle (flamed on Bunsen burner) and distributed on PDA medium in 9 cm Petri-dishes and then incubated at a temperature of 27°C and left to grow for 7-10 days. After suitable growth of the fungus, pure cultures were made on new media.

Source of Potentially Antagonistic Bacteria: Five soil samples as a good source of antimicrobially active *Bacillus* and *Pseudomonas* species were taken from tomato rhizosphere collected from Allahabad area. All the samples were cooled to 4°C so that any change in the original microflora would be prevented. The weight of individual samples was 100 g.

Isolation of Antagonistic Bacteria: Serial dilution technique was used for isolation of the bacteria from the rhizosphere soil samples. One gram of air-dried soil samples were weighted and suspended in 9 ml of sterilized distilled water and shacked well. Amount of 1 ml of the soil suspension at 10^5, 10^6, 10^7dillution was spread on each Petri plates of NA (Nutrient Agar) and KB(King's B) media and the plates were incubated at 30°C for 48 hrs. *Bacillus* and *Pseudomonas* colonies were picked from the media and sub cultured on freshly prepared slant tubes of NA and KB agar media.

***In Vitro* Screening of Antagonistic Bacteria:** Antagonistic activity of *Bacillus* and *Pseudomonas* isolates on *Alternaria solani* were carried out according to the agar diffusion method as described by [16]. The radius of the inhibition zones was measured. The fungus was tested as a plug of mycelium at the center of a Petri dish (9 cm) of half-strength PDA using sterile cork borer 0.78 mm in diameter. Bacterial isolates were spotted on to the agar near the outer edge of the dish. Sterile toothpicks were used to transfer the test isolates of the two bacteria from 2-days old cultures. Plates were incubated at 27°C and inhibition zones were measured after 7-days. Only those isolates that produced a clear inhibition zone were considered effectively.

Compatibility among Bacterial Strains : The isolates of *Pseudomonas* and *Bacillus* were tested for their compatibility among each other following the method of [17]. The compatibility was determined for *P. fluorescens* and *B. subtilis* strains using NA medium. The bacterial strains were streaked horizontally and vertically to each other. The plates were incubated at room temperature (28 ± 2°C) for 72 h and observed for the inhibition zone. Absence of inhibition zone indicates the compatibility with respective bacterial strains and the presence of inhibition zone indicated the incompatibility.

***In Vitro* Evaluation of Individual and Combined Biocontrol Agents against *Alternaria Solani*:** The mycelial disc (9 mm) of the tomato early blight pathogen *Alternaria solani* was placed in the centre of the petri plate. Sterile filter paper discs (6 mm) were placed one cm away from the edge at four sides centring the fungal disc. Twenty five micro litres of bacterial broth culture (9×10^8 cfu ml^{-1}) of each strain were dropped over the filter paper discs. Observation was taken after seven days for the presence of inhibition zone. Control was maintained with sterile distilled water instead of bacterial inoculums.

Growth inhibition was calculated as:

% Inhibition= (1-(Fungal growth/Control) × 100

***In Vitro* Effect of Bacteria on *A. solani* using Tomato Seeds:** Seeds (One gram) of tomato cv. PKM1 surface-sterilized with 2% sodium hypochlorite for 20 seconds; rinsed in sterile distilled water and dried overnight were treated with ten ml of biocontrol inoculums containing not less than 3×10^8 cfu ml^{-1}. One hundred mg of Carboxy Methyl Cellulose (CMC) was added as an adhesive material. Positive controls where sprayed with nutrient broth plus *A. solani* spore suspension. The treated seeds were kept for two hours and air-dried overnight in a sterile Petri dish and used for sowing.

Greenhouse Experiment: The greenhouse experiment was conducted at the Department of Plant Pathology, Sam Higginbottom Institute of Agriculture, Allahabad based on the *in vitro* inhibition of the pathogen and percent disease control by the bacterial isolates. The five promising bacterial isolates *B. subtilis* (Bs12, Bs16) and *P.fluorescens* (Pf2, Pf9 and Pf17) were used to control early blight disease of tomato cv. PKM1. The experiment was laid out in a completely randomized design (CRD) with 8 treatments and 3 replications.

Pot Culture Experiment : The experiment was carried out in 20 × 15 cm diameter plastic pots. Mixed soil of cattle manure, sandy and clay loam (1: 1: 2) respectively was autoclaved at 121^OC for 2h. 3 kg of the sterilized soil at pH 6.8 was filled into the plastic pots. Two tomato plants were transplanted to each pot and were regularly watered with de-ionized water. Plants were harvested at 60 days after transplanting. The plant shoot and root lengths were determined and recorded. Disease infection percentage and percent disease control were also calculated.

Statistical Analysis: The experiment was laid in a completely randomized design (CRD) with three replications. Data were analyzed statistically using analysis of variance according to SAS procedure for a completely randomized design. The differences of means were identified by Duncan's Multiple Range Test (DMRT).

Results

Compatibility among Bacterial Strains: A total of 21 Bacterial isolates were tested for their efficacy in inhibiting growth of *A. solani*. Ten of them were considered as effective (showed clear inhibition zone). Five isolates showed higher inhibitory effect on the pathogen for their ability to suppress *A. solani* in an *in vitro* dual-culture assay. Strains of *B. subtilis* (Bs12 and Bs16) and that of *P. fluorescens* (Pf2, Pf9 and Pf17) were tested for their compatibility *in vitro*.

None of the antagonistic bacteria inhibited each other, suggesting that these bacterial biocontrol agents were compatible with each other.

Effect of Bacteria on Radial Growth of *A. solani* under *in vitro* Tests: The bacterial strains were tested individually and in combination to assess the radial growth of *A. solani*. All the treatments were effective in reducing the mycelia growth of the pathogen. However, the combined application of Bs12+Pf2 resulted in the least mycelial growth with zone of inhibition of 48.11mm. The control plates recorded the highest mycelial growth with no inhibition zone (Table i).

Effect of Bacteria on Early Blight Incidence under Greenhouse Conditions.: All the treatments with biocontrol strains significantly reduced the PDI (by 39- 63%) compared to untreated control (Table ii). Conspicuously, the treatment with a combination of Bs12+Pf2 resulted in a significantly lower early blight disease severity than any of the strains treated individually.

cillus and *Pseudomonas* displayed antagonism against *A. solani in-vitro*, due to the production of antimicrobial compounds. The efficient antibiotic-producing bacteria could be used for biological control of plant diseases carried by many workers. Isolates of Bacteria on agar plates produced a zone of inhibition of the pathogens, or grew rapidly over the pathogen and inhibited their growth [18]. Bacterial bio-control agents belonging to the genera Agrobacterium, *Bacillus, Pseudomonas, and Streptomyces*, have been found by observing zones of

Effect of Bacteria on Plant Growth Promotion of Tomato: The treatment with biocontrol combination of Bs12+Pf2 produced significantly tomato seedlings with higher plant vigour (40.65mm shoot length and 16.50mm root length) than did the individual biocontrol strains Bs12, Bs16, Pf2, Pf9 and Pf17, whose shoot and root lengths recorded were 38.55 and 14.53; 30.48 and 12.43; 32.38 and 12.25; 31.65 and 12.55; and 31.48 and 12.50 mm respectively. The untreated control seedlings had the lowest shoot and root lengths (26.43and 9.53mm) respectively (Table iii).

Discussion: A total of 21 Bacterial isolates were tested for their efficacy in inhibiting growth of *A. solani*. Ten of them were considered as effective (showed clear inhibition zone). Five isolates showed higher inhibitory effect on the pathogen for their ability to suppress *A. solani* in an *in vitro* dual-culture assay. Bs12+Pf2 exhibited the highest inhibition (48.11mm), followed by the isolates Bs16, Pf2, Bs12, Pf17 and Pf9 which recorded inhibition zones of 44.07, 42.20, 33.45, 31.70 and 31.10mm respectively (Table i). This suggests that the ten isolates of *Ba* inhibition in Petri plates [19].These results are in agreement with our results. *Bacillus* spp. isolates have shown the capacity to control early leaf spot of peanut [20], yam leaf spot [21], grey mould of strawberries [22] and post-bloom fruit drop of citrus [23] which support our results. The group fluorescent pseudomonads entails different species, which posses many beneficial activities such as production of antibiotics,chitinolytic enzymes, siderophore, HCN, plant growth hormones, mineral solubilisation, etc. (Davison 1988;)

Table I. Effect of Biocontrol Agents on the Mycelial Growth of *A. solani*

Trmt.	Bacterial Isolate	Inhibition Zone (mm)	D. Reduction (%)
T1	B. subtilis (Bs12)	33.45d	34.51d
T2	B. subtilis (Bs16)	44.07c	43.09c
T3	P.fluorescens(Pf2)	42.20c	41.96c
T4	P.fluorescens(Pf9)	31.10d	32.57d
T5	P.fluorescens(Pf17)	31.70d	32.89d
T6	Bs12+Pf2	48.11b	49.46b
T7	Mancozeb(0.2%)	52.02a	53.11a
T8	Pathogen alone	-	-

Values are mean of three replications. Means in the same column followed by different letters indicate significant differences at 5% level by DMRT.

Table II. Influence of Antagonistic Bacteria on Percent of Disease Incidence and Disease Reduction of *A. solani* (*in vivo*).

Trmt.	Bacterial Isolate	D. Incidence (%)	D. Reduction (%)
T1	B. subtilis(Bs12)	34.32 ± 1.64d	43.04 ± 0.79e
T2	B. subtilis(Bs16)	32.4 ± 0.82e	45.51 ± 0.86c
T3	P.fluorescens(Pf2)	32.8 ± 0.78e	44.62 ± 0.79d
T4	P.fluorescens(Pf9)	38.12 ± 0.02c	44.62 ± 0.79d
T5	P.fluorescens(pf17)	41.10 ± 1.64b	39.60 ± 086f
T6	Bs12+Pf2	21.90 ± 1.01f	63.00 ± 0.82b
T7	Mancozeb(0.2%)	18.99 ± 0.76g	63.00 ± 0.82b
T8	Pathogen alone	55.2 ± 0.82a	-

Values are mean of three replications. Means in the same column followed by different letters indicate significant differences at 5% level by DMRT.

Table III. Efficacy of PGPR Strains on Plant Growth Promotion under *in vivo* Condition.

Trmt.	Bacterial Isolate	Shoot Length (cm)	Root Length (cm)
T1	B. subtilis (Bs12)	38.55 ± 1.88b	14.53 ± 1.39b
T2	*B. subtilis* (Bs16)	30.48 ± 0.60^{d}	12.43 ± 1.53^{d}
T3	*P.fluorescens*(Pf2)	32.38 ± 0.95^{d}	12.25 ± 0.75^{d}
T4	*P.fluorescens(* Pf9)	31.65 ± 0.90^{d}	12.55 ± 0.79^{d}
T5	*P.fluorescens*(Pf17)	31.48 ± 0.60^{d}	12.50 ± 073^{d}
T6	Bs12+Pf2	40.65 ± 0.70^{a}	16.50 ± 0.73^{a}
T7	Mancozeb(0.2%)	34.43 ± 0.62^{c}	13.43 ± 062^{c}
T8	Pathogen alone	26.43 ± 1.11^{e}	9.53 ± 0.74^{e}

Values are mean of three replications. Means in the same column followed by different letters indicate significant differences at 5% level by DMRT.

Accumulating evidence from literature has shown that compatible multiple strains appear to be an important pre-requisite for the desired effectiveness of strains and more consistent disease suppression [24]. The results of the present study provide evidence that the *P. fluorescens* strains (Pf2, Pf17 and Pf9) and *B. subtilis* strains (Bs16 and Bs12) were compatible and effectively inhibited the growth of *A. solani*. Earlier it has been reported that the biocontrol agents such as *Trichoderma viride* and *P. fluorescens* significantly reduced the mycelial growth, spore germination, spore production and germ tube formation of *A. solani* and *A. alternata* [25]. Several strains of *Pseudomonas* and *Bacillus* spp. have been reported to produce wide array of antibiotics viz., 2,4, diacetylphloroglucinol, oligomycin, phenazine, pyoluteorin, pyrolnitrin, pyocyanin, iturin, bacillomycin, zwittermycin A and surfactin which are responsible for their antifungal action [26]. Results from the present study clearly indicated maximum reduction in mycelia growth due to the combination of biocontrol strains than individual strains suggesting the synergism among biocontrol agents in reducing the mycelia growth of the pathogen. Similarly, the treatment with combination of Bs12+Pf2 increased the plant growth of tomato more than did individual biocontrol strains. Similar results on increased root and shoot length due to combined application of Pf1+Py15+Bs16+Zimmu in tomato [4] and chilli [5] were also reported. In the pot culture studies also the treatment with combination of Bs12+Pf2 resulted in a significantly lower early blight disease severity than any of the strains treated individually. Earlier mixtures of PGPR strains were reported to suppress sheath blight in rice more than the individual PGPR strains [27]. Although the chemical treatment with Mancozeb (0.2%) recorded the least disease severity it is noteworthy to observe that the treatment with combination Bs12+Pf2 also produced almost comparable results in reducing the disease severity. Thus the eco-friendly nature of antagonistic bacterial formulations is advantageous over the use of chemical fungicides.

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