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RESEARCH ARTICLE

MITIGATION OF EARLY BLIGHT OF TOMATO BY THE INTERVENTION OF FUNGAL AND BACTERIAL BIOAGENTS

[*1]Bellishree, K. [1]Girija Ganeshan, [2]Ramachandra, Y.L. [1]Archana, S Rao and [3]Chethana, B.S

[1]Division of Plant Pathology, Indian Institute of Horticulture Research, Bangalore, Karnataka, India
[2]Department of Biotechnology, Kuvempu University, Shankaraghatta, Karnataka, India
[3]ZARS, VC Farm, Mandya

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ABSTRACT

Tomato is one of the most important vegetable crop and it is affected by number of diseases, major one is early blight of tomato caused by *Alternaria solani* (Ell. and Mart) Jones and Grout, triggering substantial loss in yield up to 79%. To mitigate early blight of tomato four bioagents, *Trichoderma harzianum*, *Chaetomium* sp., *Bacillus subtilis* and *Pseudomonas fluorescence* were evaluated for their efficacy against *Alternaria solani.* These bioagents were screened for their antagonistic activity by dual culture assay, in this method *B.subtilis* recorded 92.85% inhibition of the pathogen growth and *Cheatomium* sp showed lowest inhibition of 62.79%. In volatile compound assay, *Cheatomium* sp inhibited the pathogen growth by 81.6% and least inhibition of 56.86% recorded by *T.harzianum B.subtilis* treated leaves recorded maximum inhibition of lesion by 94.90% followed by *T.harzianum* with 77.18% and *T.harzianum* recorded least inhabition of 54.7%. Under field studies *Cheatomium sp* recorded lowest PDI of 21.00, with 26.91% decrease over control, and in the second season disease incidence decreased upto 22.46% with PDI of 9.13. In both the seasons *Cheatomium sp* recorded highest marketable yield of 74.08 tonnes/ha and 75.62 tonnes/ha respectively. Subsequently *B.subtilis* and *T. harzianum* reduced the disease severity of early blight significantly when compared with control. These results suggest that the fungal and bacterial isolates studied have a good potential to be used as biocontrol agents against *A. solani* in tomato.

INTRODUCTION

Tomato belongs to the family Solanaceae. Tomato (*Lycopersicon esculentum* Mill.) crop is the second most important vegetable crop. The world total production of tomato is 161.8 million tonnes (FAOSTAT, 2012). Among the tomato producing countries in the world China is the largest producer, accounted for about one quarter of the global output, followed by India and the United States. Area under tomato cultivation in India in the year 2012-13 is 880000 HA with a production of 18227000 MT and productivity of 20.7 MT/HA. Major tomato growing states of the country are Andhra Pradesh (28.63%), Karnataka (10.52%), Madhya Pradesh (10.12%), Orissa (7.59%), Gujarat (6.35%), Bihar (6.18%), West Bengal (6.18%), Maharashtra (5.76%), Chhattisgarh (4.18%), Himachal Pradesh (2.27%) and others (12.23%). Karnataka is the second major tomato growing state with a production of 1916.60000 MT (Indian horticulture database, 2013).

***Corresponding author: Bellishree, K.** Division of Plant Pathology, Indian Institute of Horticulture Research, Bangalore, Karnataka, India*

Tomato is affected by number of diseases, major one is early blight of tomato caused by *Alternaria solani* (Ell. and Mart) Jones and Grout, triggering substantial loss in yield up to 79% due to early blight damage were reported from Canada, India, USA, and Nigeria (Basu 1974b; Datar and Mayee 1981; Sherf and Mac Nab 1986; Gwary and Nahunnaro 1998). This disease, which in severe cases can lead to complete defoliation, is most damaging on tomato (Peralta *et al.*, 2005) in regions with heavy rainfall, high humidity and fairly high temperatures (24-29^{o}C). Epidemics can also occur in semiarid climates where frequent and prolonged nightly dews occur (Rotem and Reichert 1964). *Alternaria solani* causes diseases on foliage (early blight), collar rot of seedlings, stem lesions on adult plants, and fruit rot of tomato. Early blight symptoms begin as small dark brown lesions on the older foliage. The tissue surrounding lesions may turn yellow, turning entire leaves yellow when the spots are abundant. Stem lesions on seedlings can girdle the plants. The disease appears on leaves, stems, petiole, twig and fruits under favorable conditions resulting in defoliation, drying off of twigs and premature fruit drop and thus causing loss from 50 to 86 percent in fruit yield (Mathur and Shekhawat, 1986).

The management of the fungal diseases can be done by several

methods. In them chemical control is effective when combined with rigorous cultural practices. Solicitation of fungicides to control Alternaria infection though successful, but it is associated with several problems such as increased production costs, development of resistance in the target organism, residue toxicity in the produce, environmental hazards and danger to human health. To overcome such problems it is vital to use safe and alternative approaches like biological control of the pathogen. The application of biocontrol agents is successful for controlling various plant diseases in many countries (Sivan, 1987). In recent years, several fungal and bacterial antagonists were applied to control tomato diseases (Hanafi 2003; Giotis *et al.,* 2009). Keeping this vision the present study has been undertaken to investigate efficacy of several fungal and bacterial bioagents against *Alternaria solani,* causative agent of early blight of tomato under laboratory and field conditions.

MATERIALS AND METHODS

The pathogen and bioagents used in the present study were procured from the culture repository of Division of plant pathology, Indian Institute of Horticulture Research, Bangalore, India. *Trichoderma harzianum, Chaetomium sp., Bacillus subtilis* and *Pseudomonas fluorescence* were taken to study their efficacy against *Alternaria solani*. Culture of pathogen and bioagents maintained on PDA and NA slants at 25 ± 2°C were used for the present study.

In vitro* evaluation of antagonists against *A. solani

Biological activity of these bioagents was determined by Dual Culture Technique (Dennis & Webster, 1971). In this method, 5mm culture disc of both the pathogen and fungal bioagents from periphery of 7 days old culture were inoculated on PDA in a single Petri plate at certain distance. In case of bacterial bioagents nutrient agar media and potato dextrose agar media in 1:1 ratio mixed and poured to petri plates and allowed to solidify. Later bacterial bioagents streaked at the centre of petri plate using a flame sterilized inoculation loop and 5mm of fungal pathogen disc kept at both the peripheral region of plate. Each treatment was replicated four times and incubated at 25 ± 2°C. Growth of bioagents, pathogen and zone of inhibition was recorded after 7 days of incubation. The percent inhibition of the isolates over pathogen was calculated using formula given by Vincent (1947).

$$\text{Percent inhibition of pathogen} = \frac{\text{Growth in control- Growth in Treatment}}{\text{Growth in control}} \times 100$$

The bioagents were further evaluated by detached leaf bioassay. In detached leaf bioassay the healthy mature tomato leaves were smeared with liquid culture of bioagents at a concentration of 0.4% and allowed to dry. Then the leaves were pricked in centre with sterile needle. The 7 day old pathogen disc of 5mm was inoculated and kept in moist chamber for incubation at room temperature. The development of lesion was observed and lesion size was recorded upto 5 days. The experiments were replicated thrice.

Volatile compounds Production assay

In this method, bottoms of two petri dishes were individually inoculated with 5mm disc of fungal bioagents on PDA/ bacterial bioagents are streaked on the NA media and 5mm pathogen disc was kept. The bottoms were adjusted (one base placed over the other one) and sealed by Para film. The control sets did not contain the antagonist. The cultures were incubated at room temperature (29 ±1°C), and diameter of radial growth of fungi was measured at 72 hours of incubation. The percent inhibition was obtained using the formula given by (Khare *et al,* 2010).

$$\text{Per cent inhibition} = \frac{D1 - D2}{D1} \times 100$$

(D1 – diameter of radial growth of *Alternaria solani* in control,

D2 - diameter of radial growth of *Alternaria solani* in treatment)

Preparation of talc formulation for bacterial bioagents

A loopful of bacterial bioagents were inoculated into the nutrient broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature. After 48 h of incubation, to the 400 ml of bacterial suspension, 1 kg of the purified talc (sterilized at 105^0C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxy methyl cellulose (CMC) 10 g (adhesive) were added and mixed under sterile conditions, following the method described by Vidhyasekaran and Muthuamilan (1995). The product was shade dried to reduce the moisture content and then packed in polypropylene bags and sealed. At the time of application, the population of bacteria in formulation was found to be $2.5\text{-}3x10^{-8}$cfu/g.

Preparation of talc formulation for fungal bioagents:

The formulation of *Trichoderma* and *Cheatomium* species were prepared as per the procedure described by Jeyarajan & Ramaksirhnan (1991). In this regard, the cultures of bioagents were prepared on PDB in 250 ml conical flasks. After inoculation, the flasks were incubated at 25 ± 2°C. The mycelial mat of bioagents were harvested after 7 days of inoculation and blended in a known volume of distilled water in blender and finally purified talc with CMC (adhesive) were added and mixed under sterile conditions. The product was shade dried to reduce the moisture content and then packed in polypropylene bags and sealed (Jayarajan *et al.,* 1999). At the time of application, the population of fungi in formulation was found to be $3x10^{-8}$cfu/g.

Field evaluation studies of antagonists against early blight of tomato

The bioagents and chemicals were evaluated for the mitigation of early blight disease of tomato under field conditions at IIHR, Bangalore for two consecutive seasons. The tomato seeds were treated with fungal and bacterial formulation of bioagents for 1hr @10g/kg seed dried and sown in sterile coir pith filled protrays. The 25 day old plants were transplanted to experimental plots of 5x4 m^2 having 40 plants per plot. The treatments in Table 1 were adopted for the study.

All the treatments were replicated thrice following randomized block design. A total of four sprays were given at 15 days interval during the cropping season. Observations on percent

disease index were recorded at different intervals of crop growth. Scoring was done by following 0-5 scale. Percent disease index (PDI) was calculated by using the following formula (Wheeler, 1969).

$$PDI = \frac{\text{Sum of numerical ratings}}{\text{Total number of leaves examined}} X \frac{100}{\text{Maximum grade value}}$$

Area under disease progress curve (AUDPC) was estimated as per the equation suggested by Shaner and Finney, 1977.

$$AUDPC = \Sigma [(X^{i+1} + X^{i}) / 2] [t^{i+1} - t^{i}]$$

Where, X^{i} = PDI at i^{th} day
X^{i+1} = PDI at $i+1^{th}$ day
t^{i} = The time in days after appearance of the disease at i^{th} day
n = The total number of observations

The tomato fruits were harvested at colour break stage and yield was recorded in each plot and expressed as kg/ plot. On the basis of yield per plot, the total yield was computed and expressed in tonnes/hectare. The data was analyzed statistically.

Table 1 Treatments taken for evaluation of bioagents against Early blight of tomato in field conditions:

Sl No.	Treatments
T1	Talc formulation of *Bacillus subtilis* @ 20g/l
T2	Talc formulation of *Pseudomonas fluorescence* @ 20g/l
T3	Talc formulation of *Trichoderma harzianum* @ 20g/l
T4	Talc formulation of *Cheatomium sp.* @ 20g/l
T5	Copper oxy chloride (COC) @ 3g/l
T6	Mancozeb @ 2g/l
T7	Propineb @ 2g/l
T8	Chlorothelonil @ 2g/l
T9	Untreated control

RESULTS

In vitro evaluation of antagonists against *A. solani*

The data presented in Table 2 indicated that all the four bioagents inhibited the growth of *A. solani* after 7 days of inoculation. In dual culture assay, *B.subtilis* exhibited the maximum biocontrol activity with 0.53cm growth of pathogen, where as in control plate pathogen growth was recorded as 7.35cm. *B.subtilis* found to be the most potent bioagent by recording highest percent inhibition of the pathogen growth with 92.85% and 12mm inhibition zone was observed at the periphery region where pathogen and bioagents colony meets. Fallowed by *P.fluroscence* with 78.12% inhibition, 1.60cm growth of pathogen and *T. harzianum* showed 71.40% inhibition of the pathogen. The least inhibition of 62.79% among all the four bioagents was exhibited by *Cheatomium* sp. with 2.75cm growth of the pathogen. The percent inhibition was statistically significant in all the treatments. The inhibition zone around the bactorial colony was observed only in *B.subtilis* bioagent.

The volatile compounds assay revealed that maximum percent inhibition of 81.60% pathogen growth was observed in *Cheatomium* sp. by recording 1.38 cm growth of pathogen. Subsequently, volatile metabolites of *T. harzianum* and *B.subtilis* found to inhibit the growth of pathogen by 56.86%, 83.27% with 3.25cm and 1.27cm growth of the pathogen over 8.2 cm in control plate. The *P.fluroscence* showed 80.35% inhibition of the pathogen. The inhibition produced by the *Cheatomium* sp. was significantly higher than that produced by *T. harzianum*, *B.subtilis* and *P.fluroscence*.

In detached leaf assay, the tomato leaf smeared with *B.subtilis* showed a lesion size of 0.80 cm where as in

Table 2 *In vitro* evaluation of antagonists against *A. solani*:

Isolate	Dual culture assay		Volatile compounds assay		Detached leaf bioassay	
	*Average growth diameter of pathogen (cm)	Percent inhibition	*Average growth diameter of pathogen (cm)	Percent inhibition	*Average lession size in cm^2	Percent inhibition
Bacillus subtilis	0.53	92.85	1.27	83.25	0.47	94.90
Pseudomonas fluorescence	1.60	78.12	1.48	80.35	0.75	69.67
Trichoderma harzianum	2.15	71.40	3.25	56.86	0.50	77.18
Cheatomium sp.	2.75	62.79	1.38	81.60	0.57	73.46
Control	7.35		8.2		16.42	
CV	0.32		0.20		0.44	
CD at 5%	0.71		0.45		1.00	
CD at 1%	0.99		0.63		1.46	

*The values are mean of four replications.

Table 3 Efficacy of bioagents against early blight of tomato in field conditions:

Isolates	1st season				
	Average percent disease incidence	Percent decrease over control	AUDPC	Marketable yield in tonnes/ ha	Percent increase over control
Bacillus subtilis	21.36[ab] (27.49)	25.67	678.53	74.07[ab]	108.95
Pseudomonas fluorescence	24.38[ab] (29.53)	15.15	756.98	55.93[ab]	57.76
Trichoderma harzianum	21.42[ab] (27.56)	25.44	608.93	56.30[ab]	58.81
Cheatomium sp.	21.00[ab] (27.28)	26.91	677.48	74.08[ab]	108.96
Copper oxy chloride	19.53[ab] (26.21)	29.46	664.05	57.41[ab]	61.94
Mancozeb	16.16[ab] (23.66)	28.03	521.25	47.44[ab]	33.81
Propineb	20.49[ab] (26.85)	28.68	654.00	30.48	-14.02
Chlorothelonil	18.09[ab] (25.1)	29.38	624.30	30.37	-14.32
Control	28.74 (32.39)		893.03	35.45	
CV	0.42			1.95	
CD at 5%	0.88			4.12	
CD at 1%	1.22			5.69	

Table 4

Isolates	2nd season				
	Average percent disease incidence	Percent decrease over control	AUDPC	Marketable yield in tonnes/ha	Percent increase over control
Bacillus subtilis	8.51[ab] (16.95)	27.73	470.62	78.86[ab]	23.66
Pseudomonas fluorescence	10.37 (18.72)	11.93	564.97	57.25	-8.28
Trichoderma harzianum	11.30 (19.64)	3.99	629.25	58.49	-10.22
Cheatomium sp.	9.13[ab] (17.56)	22.46	529.57	75.62[ab]	18.58
Copper oxy chloride	10.13[ab] (18.53)	13.91	499.12	71.45[ab]	12.04
Mancozeb	8.75[ab] (17.15)	25.69	453.45	77.47[ab]	21.48
Propineb	8.95[ab] (17.36)	-23.99	462.22	65.43	2.61
Chlorothelonil	11.49 (19.73)	2.35	604.5	49.67	-22.1
Control	11.77 (20.00)		619.27	63.77	
CV	0.72			2.14	
CD at 5%	1.52			4.51	
CD at 1%	2.11			6.25	

Values in the parenthesis are angular transformed;
CD- critical difference
[ab] – significant at both 5% and 1% CD

untreated control the infection was observed to be 16.42cm, hence the *B.subtilis* treatment reduced the infection on leaf by 94.96% with that of control. Consequently *T.harzianum*, *Cheatomium* sp and *P.fluroscence* treated leaves recorded 77.18%, 73.46% and 69.67% inhibition of the infection on leaf over untreated control.

Field evaluation studies of antagonists against early blight of tomato

Under field conditions, in the first season, the percent disease incidence of early blight was less in the plants treated with *Cheatomium sp.* with PDI of 21.00 and 26.91% decrease over untreated control. The highest disease incidence among all the bioagents was observed in *T.harzianum* treated plants with PDI of 24.38 and 15.15% decrease over control. All the bioagents treated plants recorded least disease incidence when compared to that of untreated control. The fungicides COC, Mancozeb, Propineb and Chlorothelonil recorded PDI of 19.53, 16.16, 20.49 and 18.09 respectively. However, the percent incidence was high in bioagents treated plants over chemical check but the marketable yield was high in *Cheatomium sp* and *P.fluroscence* treatment with 74.08 tonnes/ha and 74.07 tonnes/ha. The least yield was recorded in chlorothelonil treatment with 30.37 tonnes/ha among all the treatments. In *B.subtilis* treatment the PDI was 21.36, with 25.67% decrease over untreated control and marketable yield of 55.93 tonnes/ha was observed. The AUDPC was high in *T.harzianum* by recording 756.98, followed by *B.subtilis*, *Cheatomium sp.* and *P.fluroscence.* The results are depicted in table-3.

In the second season, *B.subtilis* recorded lowest PDI of 8.51with 27.73% decrease over untreated control. The second potent biocontrol agent under field condition is *Cheatomium sp.* by recording PDI of 9.13 and 22.46% decrease over control. In *T.harzianum* and *P.fluroscence,* PDI of 11.30 and 10.37 with 3.99% and 11.93% decrease over control was observed. The marketable yield was maximum in *Cheatomium sp* with 75.62 tonnes/ha with 18.58% increase over control. Subsequently highest yield was recorded in *B.subtilis* and *P.fluroscence* with 65.43 tonnes/ha and 58.49 tonnes/ha. Other than bioagents in fungicide treatment highest yield was recorded in propineb and mancozeb with 78.86% and 77.47%.

DISCUSSION

Our present study reveals that, all the four fungal and bacterial bioagents inhibited the growth of pathogen in dual culture assay and inhibition zone was also observed in *B.subtilis* which indicates pathogen inhibition. Similarly, Yazici *et al,* 2011 recorded that, *B.subtilis* (IK-92) and (IK-83) also inhibited *A. solani* with inhibition zone of 26.6 and 17.8 mm, respectively. Production of zones of inhibition at the boundary with the pathogen agrees with the report of Basım (1990) that *in vitro A. solani* interactions of *B. subtilis* AB-27 and AB-2 strains resulted in production of the highest zone of inhibition. The zones of inhibition produced might be due to the production of antifungal metabolites by the *B.subtilis* isolates. It was reported that *B. subtilis* can secrete several antifungal metabolites such as subtiline, bacitracin, bacillin and bacillomycin (Alippi and Mónaco, 1994). *T. viride* inhibited the growth of pathogen significantly by recording 15.33 mm pathogen growth and appeared to be the most superior over all the antagonists tested (Deshmukh *et al,* 2010).

In volatile compound assay, the fungal bioagents effectively produced the volatile compounds rather than bacterial bioagents which results in inhibition of pathogen growth. The fungistatic or fungicidal nature of the inhibition caused by the antagonist can determine the success of the biocontrol agent. The production of antifungal volatiles by antagonists *in vitro* has also been reported in the work carried out by Swadling and Jeffries, 1998. Our results are in agreement with the work of Jeyaseelan *et al,* 2012, who observed the volatile metabolites of *T. harzianum* and *T. viride* showed significant growth inhibition against *P. aphanidermatum* at 24 hours incubation. Antibiosis, production of antibiotic compounds and inhibition of other microbes, is the most important mechanism expressed by the antagonists (Intana *et al,* 2008).

Detached leaf tests were convenient to conduct and usually had smaller coefficient of variation (Paul *et al,* 1995). In the present work, *B.subtilis* showed maximum inhibition of the infection on tomato leaf, followed by *T.harzianum*, *Cheatomium sp* and *P. Fluorescence.* Similar results were observed in the work carried out by Kumar *et al,* in their work among the strains, maximum inhibition of lesion development was noticed with one strain of *B. subtilis* (2.92% disease severity) (severity scale of 1) and one strain of *B. atrophaeus*

(32.08% severity and a mean severity scale of 1.67). Overall, lesion development was 70.41% with *Bacillus* sp; 89.84% with *Brevibacillus* sp; 71.61% with *Paenibacillus* sp and 50% with *Arthrobacter* sp. Chamber and Scott, 1995, observed that *Trichoderma hamatum* and *Gliocladium virens* prevented *P. cinnamomi* and *P. citricola* from causing infection symptoms on micropropagated shoots of chestnut in an *in vitro* excised shoot bioassay for bio-control.

Biocontrol with individual antagonists has been successfully demonstrated against onion leaf blight (Lokesh & Hiremath 1988; Ray *et al.,* 1990; Basin & Katircioglu 1994; Mohan 1996). In the present work, evaluation of efficacy of bioagents under field conditions against early blight of tomato revealed that, *Cheatomium sp, B.subtilis* and *T.harzianum* found to decrease the disease severity and increase the yield. *Cheatomium sp* was effective in controlling disease incidence in strawberry with increased yield (Soytong *et al.,* 2001). Poornima, (2011) reported that *Trichoderma* spp. and *Pseudomonas* spp. showed 62.6% and 36.1% disease control, respectively when applied individually. Numerous modes of action have been postulated and demonstrated for antagonistic effects of *Pseudomonas fluorescence* in controlling diseases which include synergistic effects observed on fungal pathogens with a combination of antifungal compounds (Dowling & O'Gara 1994; Dunne *et al.*, 1998), competition for nutrients (O'Sullivan & O'Gara 1992), production of cell wall lytic enzymes (Singh *et al.,* 1999) and induced systemic resistance (Dalisay & Kuc 1995; Nandakumar *et al.*, 2001a) and *B. subtilis* also having all these modes of action (Leifert *et al.,* 1995; Seddon & Schmitt 1999). The diversity of mechanism available to *Trichoderma* sp. for pathogen suppression (e.g. production of wide range of broad spectrum antifungal metabolites, mycoparasitism, competition with pathogen for nutrient and for occupation of the infection court, induced resistance, protease and fungal cell wall degrading enzyme) makes this fungi an attractive biocontrol agent (Denis & Webster 1971; Elad 2000; Perello *et al.,* 2003). The effect of biocontrol activity of these bioagents against tomato early blight pathogen in the present study is in accordance to the findings of Chet & Baker (1981) and Chand *et al.,* (1991). Coley-Smith *et al.,* (1991) also reported that bottom-rot disease of lettuce was suppressed by *T. harzianum* and *T. viride in vivo* condition. The biocontrol activity of these bioagents observed in the present study is similar to the finding of Hadar *et al.,* (1979), Mathew & Gupta (1998) and Rajappan & Ramaraj (1999), who reported effective inhibition of *Rhizoctonia solani* and *Fusarium moniliforme*. On the other hand, Kim & Roh (1987) also observed the same biocontrol activity of *T. harzianum, T. viride* and *G.virens* against *R. solani*. Benhamou & Chet (1993) were also reported the same efficacy of *T. harzianum* against *R. solani*. Vidhyasekaran *et al.,* (1997) obtained effective control of pigeon pea wilt caused by *F. udum* using talc- based formulation of *P. fluorescence*. Raguchander *et al.,* (1998) reported that seed pelting with *B. subtilis* effectively controlled soybean root- rot caused by *Macrophomina phaseolina* and increased the grain yield.

In conclusion, our results indicate that *Cheatomium sp, B. subtilis* and *T. harzianum* enhanced protection against early blight of tomato to a level of equal or better than fungicide treatment and increased tomato yield. Although the marketable yield and disease incidence was on par in bioagents and fungicide treatments in both the seasons, use of bio fertilizers increase the quality of the soil and also microflora which is a beneficial factor in controlling environmental hazards. Thus usage of bioagents is a farmer friendly and eco friendly approach in agriculture.

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