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Antagonistic activity of *Trichoderma* spp. and *Bacillus* spp. against *Pythium aphanidermatum* isolated from tomato damping off

E. Christy Jeyaseelan*, S. Tharmila and K. Niranjan

Department of Botany, Faculty of Science, University of Jaffna, Jaffna, Sri Lanka

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ABSTRACT

Present study was aimed to determine in vitro antagonistic efficacy of two different species of Trichoderma and nine different species of Bacillus against tomato damping off causing fungi Pythium aphanidermatum. Different types of assays were carried out with Trichoderma spp., in all tests they revealed growth inhibition on P. aphanidermatum. In dual culture assay and assay for volatile metabolites, Trichoderma harzianum revealed significantly (P <0.05) higher inhibition on tested pathogen, but in assay for nonvolatile metabolites Trichoderma viride showed higher inhibition. Interestingly, assay for nonvolatile metabolites of P. aphanidermatum demonstrated growth induction on both of the tested Trichoderma spp. The extracted metabolites of Trichoderma spp. showed growth inhibition on P. aphanidermatum even at 10,000 times dilution. Out of tested nine Bacillus species, four revealed antagonistic effect on tested pathogen, B. polymyxa showed significantly (P < 0.05) higher effect at 48 hours incubation. In conclusion, both of the tested Trichoderma spp. and B. thracis, B. circulans and B. polymyxa and B. sphaericus have antagonistic effect on P. aphanidermatum. Therefore, these microbes can be used for further greenhouse and field studies to confirm the feasibility of using in tomato damping off disease management.

Keywords: *Pythium aphanidermatum*, Antagonistic effect, *Trichoderma*, *Bacillus*, Tomato damping off

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INTRODUCTION

Pythium aphanidermatum, a member of class oomycetes, is one of the important soil-borne plant pathogen and it causes great loss in agriculture production. This fungus like organism is an unspecialized parasite that has a wide host range. Young tissues and plants are infected and affected much more severely by this pathogen [1]. It causes damping off disease in several plants including tomato; it affects the plant both in pre and post emergence stage in nursery beds. Even though this pathogen can be controlled by some fungicides, nowadays researchers are more interested on biological control agents and their antifungal metabolites due to the notification of resistance development in the pathogen [2].

Biological control is an alternative approach to the chemical fungicides and it may be a safe, effective and ecofriendly method for plant disease management [3]. Some soil borne fungi, bacteria and actinomycetes have been identified and used as antagonistic microbes against some soil borne pathogens including members of genus *Pythium*. The most commonly using biological control agents include three fungi: *Gliocladium virens* for the control of seedling diseases of ornamental and bedding plants; *Trichoderma harzianum* for the control of several soil borne plant pathogenic fungi; and *Trichoderma harzianum/T. polysporum* for the control of wood decays. The other three commercially available microorganisms are bacteria: *Agrobacterium radiobacter* K-84 for use against crown gall; *Pseudomonas fluorescens* for use against *Rhizoctonia* and *Pythium* damping-off of cotton; and *Baccillus subtilis* used as a seed treatment [1]. With this background several works have been carried out by different researchers through out the world to find out new bio-control agents, and to evaluate the effectiveness of already existing agents against different pathogens [2, 3, 4, 5 and 6].

Tomato is one of the most popular vegetable crops throughout the world and it is also widely cultivated in Jaffna peninsula Sri Lanka. Damping-off is an important disease of tomato, causing significant losses in nurseries where young susceptible transplants are produced [4]. Therefore, in the present study an attempt was made to test the feasibility of bio-control ability of two different *Trichoderma* spp. and nine different *Bacillus* spp. against tomato damping off causing pathogen *Pythium aphanidermatum.*

MATERIALS AND METHODS

Fungal and bacterial isolates

Two species of *Trichoderma* (*Trichoderma viride* and *Trichoderma harzianum*) and nine different species of *Bacillus* (*Bacillus megaterium, Bacillus stearothermophilus, Bacillus polymyxa, Bacillus circulans, Bacillus firmus, Bacillus thracis, Bacillus brevis, Bacillus coagulans, Bacillus sphaericus*) selected for this study were obtained from culture collection, Department of Botany, University of Jaffna, Sri Lanka. The fungal pathogen *Pythium aphanidermatum* was isolated from infested tomato nursery beds at Farm School, Thirunelveli, Jaffna, Sri Lanka, and identified based on its morphological features. Bacterial and fungal isolates were maintained on nutrient agar and potato dextrose agar (PDA) slants respectively at 4 ^{o}C until used for the study.

Colony growth inhibition assay with *Trichoderma* spp. in dual culture method

Firstly, the antagonistic activity of *T. viride* and *T. harzianum* against *P. aphanidermatum* was studied in dual culture method [6]. Culture discs of both *P. aphanidermatum* and *Trichoderma* spp. were placed on opposite end of PDA plate, and in control plates only *P. aphanidermatum* was placed. The plates were incubated at room temperature (29 ±1 °C) and the colony interactions were measured as percentage of inhibition of radial growth of *P. aphanidermatum* by following formula:

$$\text{Percentage of inhibition} = \frac{R1-R2}{R1} \times 100$$

(R1 – Radius of the radial growth of the pathogen towards opposite side in control plate, R2 - Radius of the radial growth of the pathogen towards the opponent antagonist in test plate)

Assay for volatile metabolites of *Trichoderma* spp.

Bottoms of two Petri dishes containing PDA were individually inoculated with a disc of *P. aphanidermatum* and *Trichoderma* spp. The bottoms were adjusted (one base placed over the other one) and attached 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 24 and 48 hours. The percent inhibition was obtained using the formula [6]:

$$\text{Per cent inhibition} = \frac{D1 - D2}{D1} X100$$

(D1 – diameter of radial growth of *P. aphanidermatum* in control, D2 - diameter of radial growth of *P. aphanidermatum* in treatment)

Assay for non-volatile metabolites of *Trichoderma* spp. and *P. aphanidermatum*

Sterilized cellophane discs (90 mm) were placed on the PDA medium, and then 8 mm diameter fungal discs obtained from the edge of a young culture of *Trichoderma* spp. were transferred to the center of above medium. The fungi were grown for two days at room temperature (29 ±1 °C). The cellophane containing the antagonist was removed, and on the same medium a disc of *P. aphanidermatum* was placed. The control had *P. aphanidermatum* growing similarly on PDA medium where previously there was a cellophane disc without antagonist. The plates were incubated at room temperature (29 ±1 °C) up to 48 hours [7]. The inhibition percentage was obtained using the formula as described above. The above procedure was repeated for the assay of non-volatile metabolites of *P. aphanidermatum* against *Trichoderma* spp., where the *P. aphanidermatum* was initially grown on the cellophane disc for three days, then after removing the cellophane disc, a disc of each of the *Trichoderma* spp. was placed on the medium separately. In control, *Trichoderma* spp. maintained on PDA medium separately where cellophane disc was kept without pathogen. Finally the percentage of inhibition was calculated as mentioned earlier.

Extraction of metabolites of *Trichoderma* spp. and *P. aphanidermatum*

Extraction of metabolic substances was done in liquid cultures of *Trichoderma* spp. growing in 100 ml of potato dextrose broth, and shaken on a rotary shaker at 150 rpm, for 72 hours at room temperature. Then the broth was

centrifuged at 9000 rpm for 20 minutes. The supernatant was filtered through Whatman no1 filter paper, and passed through 0.34 µm Millipore filter. Finally, the resulting sterilized liquid was used for further study. Likewise, metabolic substances of *P. aphanidermatum* were extracted as the method used for *Trichoderma* spp. [7].

Assay for extracted metabolites of *Trichoderma* spp. and *P. aphanidermatum*

The purified sterile metabolites of *Trichoderma* spp. were diluted four times (ten fold dilution) and their inhibitory effect was tested by poison food technique [8]. 1 ml of the original and dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) of the extracts were mixed with 20 ml of molten PDA separately and then poured into Petri dishes. PDA supplemented with sterile distilled water served as control. A mycelia disc of *P. aphanidermatum* was transferred on the centre of both test and control plates and incubated for 24 hours at room temperature. The linear growth of mycelia was measured and the percentage of inhibition was calculated by using above equation. Same procedures were applied for metabolites extracted from *P. aphanidermatum* against *Trichoderma* spp. All the experiments were repeated three times.

Evaluation of the antagonistic effects of *Bacillus* spp.

Each of the test bacteria was spotted on the edges of PDA plate and incubated for 24 hours. Then, a mycelia disc of *P. aphanidermatum* was cut from the colony margin by an 8 mm diameter sterile cork borer and placed onto the center of above PDA plates which already inoculated with bacteria. The Petri dishes were sealed with Para film and incubated at room temperature (29 ±1 °C) and clear zone caused by bacterial isolates between fungal and bacterial colony margins were recorded at 24 and 48 hours incubation [3].

Statistical analysis

Statistical significance of antagonistic effect of *Trichoderma* spp. was tested by 't' test (P = 0.05), results produced by the *Bacillus* spp. were expressed as mean ± SD of three experiments, and were subjected for analysis of variance and Tukey test at P = 0.05 using statistical software SPSS Windows version 13.0.

RESULTS AND DISCUSSION

Table 1: Effect of *Trichoderma* spp. on the radial growth of *P. aphanidermatum* in dual culture method

Antagonists	Radial growth at 48 hours*		Per cent inhibition	Radial growth at 72 hours*		Per cent inhibition
	Control	Test		Control	Test	
T. viride	70.3 mm	59.3 mm	15.6 %	90 mm	55.7 mm	38.1 %
T. harzianum		50.7 mm	27.9 %		19.7 mm	78.1 %

** Values are mean of three replicates*

The results of dual culture of antagonists and pathogen demonstrated that both *T. viride* and *T. harzianum* had antagonistic effect on *P. aphanidermatum*. The results produced by antagonists were significantly (P < 0.05) different from control as well as within them. *T. harzianum* highly inhibited the growth of test pathogen compared to *T. viride*, and the percentage of inhibition increased about two times to *T. viride* and three times to *T. harzianum* from 48 hours to 72 hours incubation (Table 1).

Table 2: Effect of volatile metabolites of *Trichoderma* spp. on the radial growth of *P. aphanidermatum*

Antagonists	Radial growth at 24 hours*		Per cent inhibition	Radial growth at 48 hours*		Per cent inhibition
	Control	Test		Control	Test	
T. harzianum	64.2 mm	31.0 mm	51.7 %	90.0 mm	90.0 mm	0 %
T. viride		40.0 mm	37.7 %		90.0 mm	0 %

** Values are mean of three replicates*

Table 3: Effect of non volatile metabolites of *Trichoderma* spp. on the radial growth of *P. aphanidermatum* in cellophane disc method

Antagonists	Radial growth at 24 hours*		Per cent inhibition	Radial growth at 48 hours*		Per cent inhibition
	Control	Test		Control	Test	
T. viride	64.2 mm	16.7 mm	74.0 %	90.0 mm	22.3 mm	75.2 %
T. harzianum		-	100 %		-	100 %

** Values are mean of three replicates*

The volatile metabolites of *T. harzianum* and *T. viride* showed significant (P < 0.05) growth inhibition against *P. aphanidermatum* at 24 hours incubation. The inhibition produced by the *T. harzianum* was significantly (P < 0.05) higher than that produced by *T. viride*. However, there was no significant difference between the radial growths of *P. aphanidermatum* present in test and control plates at 48 hours incubation (Table 2).

Assay for non volatile metabolites of *Trichoderma* spp. showed that the products of *T. harzianum* completely inhibited the growth of *P. aphanidermatum* and the percentage of inhibition was 100% even at 48 hours incubation. On the other hand, *T. viride* had significantly ($P < 0.05$) lower inhibition on the test pathogen compared to *T. harzianum* (Table 3).

Table 4: Effect of non volatile metabolites of *P. aphanidermatum* on the radial growth of *Trichoderma* spp. in cellophane disc method

Antagonists	Radial growth at 24 hours*		Per cent induction	Radial growth at 48 hours*		Per cent induction
	Control	Test		Control	Test	
T. viride	24.2 mm	32.3 mm	33.5 %	65.4 mm	70.7 mm	8.1 %
T. harzianum	39.7 mm	43.3 mm	9.1 %	84.3 mm	88.0 mm	4.4 %

** Values are mean of three replicates*

The study for the effect of pathogenic metabolites on antagonists revealed interesting results; *P. aphanidermatum* induced the growth of both *T. harzianum* and *T. viride*. At 24 hours incubation, *T. viride* showed 33.5 % growth induction while *T. harzianum* expressed just 9.1 %. However, at 48 hours incubation it was greatly reduced to 8.1 % in *T. viride* and 4.4 % in *T. harzianum* (Table 4).

Table 5: Effect of extracted metabolites of *Trichoderma* spp. on the radial growth of *P. aphanidermatum*

Antagonists	Dilutions	Diameter of radial growth (mm)	Per cent inhibition
T. harzianum	10^{0}	36.3	43.5 %
	10^{-1}	40.0	37.7 %
	10^{-2}	41.7	35.0 %
	10^{-3}	44.7	30.4 %
	10^{-4}	47.3	26.3 %
T. viride	10^{0}	33.0	48.6 %
	10^{-1}	40.0	37.7 %
	10^{-2}	42.0	34.6 %
	10^{-3}	43.7	31.9 %
	10^{-4}	45.7	28.8 %

Radial growth of P. aphanidermatum in control – 64.2 mm

Assay with extracted metabolites of *Trichoderma* spp. revealed the inhibitive bioactivity on the growth of *P. aphanidermatum*. However, the inhibitory effect produced by the *T. viride* is slightly higher than that produced by *T. harzianum*. Furthermore, both extracts showed inhibition even at 10, 000 time dilution (Table 5). In contrast, the results of extracted metabolites of *P. aphanidermatum* did not show any inhibition or induction activity against the growth of both of the tested *Trichoderma* spp.

Table 6: Antagonistic effects of *Bacillus* spp. on *P. aphanidermatum*

Test Bacteria	Width of the clear zone (mm)*	
	24 hours incubation	48 hours incubation
B. megaterium	-	-
B. stearothermophilus	-	-
B. polymyxa	2.8 ± 0.3^{b}	5.2 ± 0.3^{a}
B. circulans	3.0 ± 0.5^{b}	4.0 ± 0.0^{b}
B. firmus	-	-
B. thracis	5.3 ± 0.6^{a}	3.3 ± 0.6^{bc}
B. brevis	-	-
B. coagulans	-	-
B. sphaericus	-	2.5 ± 0.5^{c}

**Values = mean ±SD; Values with different superscr ipt in the same column differ significantly (P<0.05); (-) – no clear zone.*

Among the tested nine *Bacillus* spp., *B. thracis*, *B. circulans* and *B. polymyxa* showed growth inhibition against *P. aphanidermatum* with the production of clear zones at 24 hours incubation (Table 6). The result produced by the *B. thracis* was significantly ($P < 0.05$) higher than that produced by other two bacteria. However, there was no significant ($P > 0.05$) difference between *B. circulans* and *B. polymyxa*. The antagonistic effect of the above bacteria varied significantly at 48 hours incubation, *B. circulans* and *B. polymyxa* revealed increases in the width of clear zone, but there was reduction in the width of clear zone produced by *B. thracis*. In addition to that the bacteria *B. sphaericus* which failed to show inhibition at 24 hours, however, it revealed inhibition at 48 hours. Even though *B. polymyxa* expressed lower effect at 24 hours incubation it demonstrated significantly ($P < 0.05$) highest inhibition at 48 hours compared to other tested bacteria.

A microbial biological control agent may express different mechanisms against pathogens during their antagonistic activity; it weakening or destroying the pathogen by parasitize the pathogen directly, by producing antimicrobial compounds, by compete for space and nutrients, by producing enzymes that attack the cell components of the pathogens [1]. Antibiosis, production of antibiotic compounds and inhibition of other microbes, is the most important mechanism expressed by the antagonistic bacteria [2]. In this study, the antagonistic effect expressed by the *Trichoderma* spp. in dual culture method might be due to the one or combination of all the above mechanisms. However, the results of all other three assays for *Trichoderma* spp. and assay for *Bacillus* spp. mainly depended on the ability of producing antimicrobial compounds and degradative enzymes by the tested antagonistic organisms. It has been already reported that *Bacillus* spp. has ability to produce a large number of antifungal metabolites such as bacitracin, gramicidin S, polymyxin, tyrotricidin, bacilysin, chlotetaine, iturin A, mycobacillin, bacilomycin, mycosubtilin, fungistatin and subsporin [9, 10, 11].

B. thracis, *B. circulans* and *B. polymyxa* and *B. sphaericus* had inhibitory effect on the mycelial growth of tested fungi. This shows that above bacteria are potent antagonists of *P. aphanidermatum*. Similar results were reported by some other researchers [2], where different strains of soil isolates of *Bacillus* spp. revealed antagonistic effect against *P. aphanidermatum.* In the present study among the tested nine *Bacillus* spp. five failed to show any inhibition zone. This may be due to the lack of ability to produce antimicrobial compounds which are inhibitive to *P. aphanidermatum* or may be inadequate production of antimicrobial compounds.

Further study can be conducted with tested fungi and bacteria which showed antagonistic effect against *P. aphanidermatum* as direct biological controlling agent or isolated and purified antimicrobial chemicals from those antagonistic organisms as bio-pesticides.

CONCLUSION

In conclusion, according to results obtained from this preliminary *in vitro* antagonistic study, the tested both of the *Trichoderma* spp. and bacteria, *B. thracis*, *B. circulans* and *B. polymyxa* and *B. sphaericus* are potent sources for further greenhouse and field studies against *P. aphanidermatum* causing damping off disease.

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