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Pathogenicity of *Alternaria solani* on tomato plants and its management by Entomopathogenic fungi under laboratory conditions

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Abstract
Early blight of tomato caused by *Alternaria solani* is most destructive disease in Pakistan and worldwide. Use of microbial antagonists like Entomopathogenic fungi currently gaining interest to manage Phytopathogens as suitable alternative of synthetic chemicals. In present study, pathogenicity of *A. solani* was performed by using five different isolates and two Entomopathogenic fungi *B. bassiana* and *P. lilacinus* (known as *Purpureocillium lilacinum*) were evaluated against early blight pathogen. Results showed that isolate 1 showed highest disease severity of early blight followed by isolate 4, isolate 2, isolate 3 and isolate 5 respectively. Both Entomopathogenic fungi inhibited the mycelial growth of *A. solani* under dual culture, volatile and non-volatile metabolites assays. *P. lilacinus* showed highest pathogen growth inhibition as compared to *B. bassiana* in all assays. Non-volatile metabolites showed higher antifungal ability as compared to volatile metabolites. To our knowledge present study is first report which described the antifungal potential of *P. lilacinus* against plant pathogen.

Keywords: early blight, tomato, pathogenicity, antagonism, entomopathogenic fungi

1. Introduction

Tomato (*Lycopersicon esculentum* Miller) belongs to Solanaceae (nightshade) family, is an important “fruity vegetable” which is grown in Pakistan as well as all over the world due to its high rate of consumption after the potato (Mirza, 2007; Abdussamee *et al.*, 2014) [7, 1]. It is a rich source of Vitamin A, C and have remarkable amount of lycopene, which is known as best antioxidant (Chohan *et al.*, 2015) [7]. Total cultivated area of tomato during 2014-2015 in Pakistan was 60.6 thousand hectares with the annual production of 620.1 thousand tons (PBS, 2017-18). Average production of tomato is very low as compared to other vegetables due to biotic and abiotic factors. About 200 different diseases attacks on tomato crop globally (Abdussamee *et al.*, 2014) [1]. However, fungal diseases especially early blight of tomato is the most destructive diseases which cause 50-91 % yield losses (Saleem *et al.*, 2015) [28].

Alternaria solani (Ellis and Martin) is causal agent of tomato early blight which is air borne soil inhabiting pathogen responsible for blight and fruit rot. This disease can infect whole plant but infection starts firstly at lower older leaves and then moves to upper part of the plant. At the result of this disease drying of twigs, defoliation and premature fruit drop occur which directly leads to yield losses (Abdussamee *et al.*, 2014) [1].

Different fungicides are being used to control the early blight disease but continuous and regular use of these chemicals have negative impact on environment, human health hazards and pathogen attain resistance against chemicals (Latha *et al.*, 2009) [17]. So, there is a need of best alternatives of chemical fungicides which are safe to use and ecofriendly. Currently, many beneficial microorganisms are used by many researchers to manage different plant diseases and insect pests. Among the beneficial microorganisms which are used for biocontrol of plant diseases are fungi and Bacteria are very important (Khan *et al.*, 2012; Ownely *et al.*, 2010; Mckinnon *et al.*, 2017) [16, 20]. Entomopathogenic fungi also proved its antagonistic potential against phytopathogens and can be used as best alternative of chemical fungicides (Ownely *et al.*, 2008; Jaber, 2015) [15]. *B. bassiana* and *P. lilacinus* are well known Entomopathogenic fungi which are under development as biological control agent against different pathogens like *Rhizoctonia* spp, *Pythium* spp, *Fusarium* spp (Ownely *et al.*, 2008; Jaber, 2018) [16]. Present study investigated the antifungal potential of two Entomopathogenic fungi against early blight pathogen by using dual culture, volatile and nonvolatile metabolites and pathogenicity of *A. solani*.

2. Materials and Methods

The present research work was carried out in the laboratory of the Department of at Plant Pathology, College of Agriculture, University of Sargodha, Sargodha.

2.1. Collection, isolation and identification of Pathogen

Leaf samples showing typical symptoms of early blight disease were collected from tomato fields at district Sargodha (32° 5' 1" North, 72° 40' 16" East), Punjab, Pakistan. Samples were packed in zipper bags and brought to the laboratory of the Department of Plant Pathology and stored at 4 ºC until isolation.

Isolation of pathogen was performed by using tissue segment method (Rangaswamy, 1958) [27]. Infected leaves were cut into small pieces of about 2-3 cm and surface sterilized with 0.5% NaOCl for two minutes and rinsed thrice with distilled water in laminar flow chamber. After sterilization, samples were placed on paper towel to absorb the moisture. Potato dext agar (potato starch 4g L^{-1}, agar 20g $L^{-1}$and dextrose 20g L^{-1}) was used for pathogen isolation. Samples placed on PDA medium plates and incubated at 25 ± 1 ºC under dark conditions. After four

Days petri plates were checked for fungal growth. Purification was performed by using single spore technique (Choi *et al.*, 1999) [8] and preserved the culture plate at 4 °C for further experiments.
Identification of pathogens was confirmed on the basis of morphological characteristics and by using compound microscope. Spore size, shape, color and hyphal pattern were compared to the literature (Barnet and Hunter, 1972) [6].

2.2. Pathogenicity test
Pathogenicity of five different *A. solani* isolates was performed by using Koch's postulates at the screen house, College of Agriculture, University of Sargodha. Fungal spores were harvested from seven days old culture plates of *A. solani* by adding 10ml distilled water and conidial suspension (1×10^6 ml^{-1}) was prepared by using haemocytometer (Chohan *et al.*, 2015) [7]. Fifteen tomato plants of variety Rio Grande (moderately susceptible) per isolate were used for artificial inoculation of early blight pathogen. Foliar application of above mentioned conidial suspension was applied to five weeks old plants while control plants were sprayed with distilled water. All plants were covered with polythene bags for 48 hours to create the humidity. After 48 hours polythene bags were removed and plants were kept in screen house. Disease severity was calculated after ten days of inoculum spray and re-isolation of pathogen was performed to fulfill the Koch's postulates.

2.3. Dual culture test
Eight days old 4mm mycelial plugs of *A. solani* and Entomopathogenic fungi were placed opposite to each other on PDA media plates and sealed with para film. For control, petri plates contained a mycelial plug of pathogen in the center and incubated at 25ºC±1 (Anjum *et al.*, 2019) [4]. Experiment was repeated twice with nine replications per treatment. The percent inhibition data was calculated on 3rd, 5th and 8th days after inoculation by following formula.

$$\text{Inhibition percentage} = \frac{C - T}{C} \times 100$$

Where;
C = mycelial growth of pathogen in control
T = mycelial growth of pathogen in dual culture

2.4. Volatile metabolites
The effect of volatile metabolites of two Entomopathogenic fungi (*B. bassina and P. lilacinus*) on mycelial growth of *A. solani* was determined by using method with some modifications described by Dennis and Webster (1971b) [10]. Eight days old 4mm mycelial plugs of both tested Entomopathogenic fungi were placed in center of sterilized PDA plates. Petri plates containing PDA medium were inverted and kept over the plates having plug of *B. bassiana* and *P. lilacinus* in sterilized condition, sealed with Para film and incubated for the period of three days. After that inverted Petri plates were removed and eight days old 4 mm mycelial plug of *A. solani* placed on a PDA plate and incubated at 25±1ºC. Experiment was repeated twice with nine replications per treatment. The percent inhibition rate was calculated on 3rd, 5th and 8th days after inoculation by using above mention formula.

2.5. Non-volatile metabolites
Effect of non-volatile metabolites of both tested entomopathogenic fungi on mycelial growth of *A. solani* was determined by using method with some modifications described by Dennis and Webster (1971a) [9]. Cellophane membranes of 90mm were autoclaved and placed on 9 cm Petri plates containing sterilized PDA. Eight days old, 4 mm mycelial plug of each entomopathogenic fungi placed in the center of different petri plates above the cellophane membrane and incubated at 25±1ºC. After three days, cellophane membranes along with the plug of *B. bassiana* and *P. lilacinus* were replaced by eight days old 4 mm mycelial plug of *A. solani*. Control plates only contained 4mm mycelial plug of *A. solani* in center and incubated at 25±1ºC. Experiment was repeated twice with nine replications per treatment. The percent inhibition rate was calculated on 3rd, 5th and 8th days after inoculation by using above mention formula.

3. Statistical Analysis
Statistix 8.1 software was used for statistical analysis. Data of disease severity for pathogenicity was analyzed by using Tukey HSD test and data of antibiosis assay (dual culture, volatile and non-volatile metabolites) were analyzed by using LSD to separate the means.

4. Results
4.1. Identification of Pathogen
Identification of five isolates of *A. solani* was confirmed on morphological bases (Ellis, 1976). Microscopic observations showed that color of conidiophore was olivaceous brown to brown and conidia were smooth, straight or flexuous and oblong tapering to beak with 160 to 300µm in length, 2 to 4 longitudinal and 8 to 10 transverse septa.

4.2. Pathogenicity test
Result showed that all tested isolates of *A. solani* caused infection on inoculated tomato plants while control plants showed no symptom of disease. Isolate 1 showed maximum disease severity as compared to other tested isolates of *A. solani* (Figure 1). Diseased samples were re-isolated and identified as *A. solani* to complete the Koch's postulates.

4.3. Dual Culture
In present study, both tested Entomopathogenic fungal biocontrol agents (*B.bassiana* and *P. lilacinus*) inhibited the mycelial growth of *A. solani* under dual culture technique as compared to control treatment. Maximum growth inhibition was showed by *P. lilacinus* about 66.2% while *B. bassiana* showed 62.7% inhibition of early blight pathogen (Figure 2) at final day of observations.

4.4. Volatile Metabolites
Volatile metabolites of both tested Entomopathogenic fungi (*B.bassiana* and *P. lilacinus*) inhibited the mycelial growth of *A. solani* (Figure 3). Maximum growth inhibition was showed by volatiles metabolites of *P. lilacinus* about 49.3% while 41.8% inhibition was showed by *B. bassiana* at eighth days of observations.

4.5. Nonvolatile metabolites
Nonvolatile metabolites showed best mycelial growth inhibition as compared to volatile metabolites. Nonvolatile

Metabolites of *P. lilacinus* gave maximum growth inhibition (59.6%) of early blight pathogen while metabolites of *B. bassiana* showed 53.3% inhibition (Figure 4) at final day of observations.

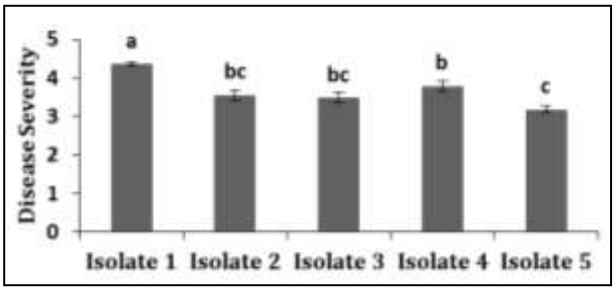

Fig 1: Pathogenicity test of five isolates of *A. solani* on Tomato (Rio grandi) ($P \leq 0.05$)

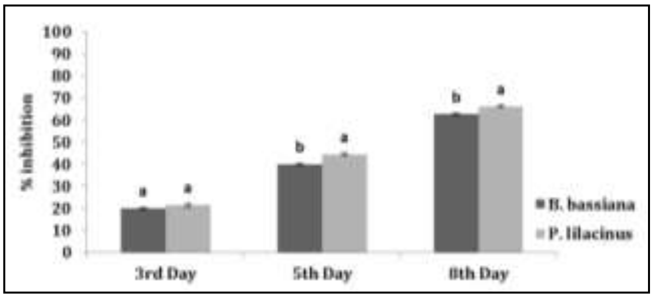

Fig 2: Antifungal potential of *B. bassiana* and *P. lilacinus* on percent inhibition growth of *Alternaria solani* by using dual culture technique ($P \leq 0.05$)

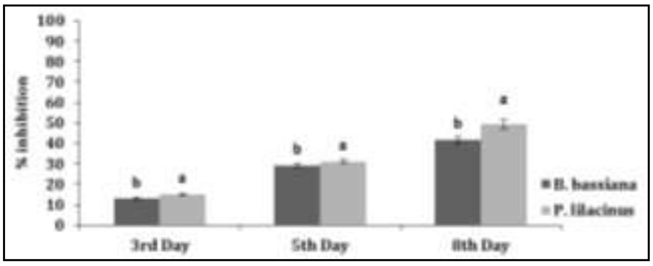

Fig 3: Antifungal potential of *B. bassiana* and *P. lilacinus* on percent inhibition growth of *Alternaria solani* by using volatile metabolites ($P \leq 0.05$)

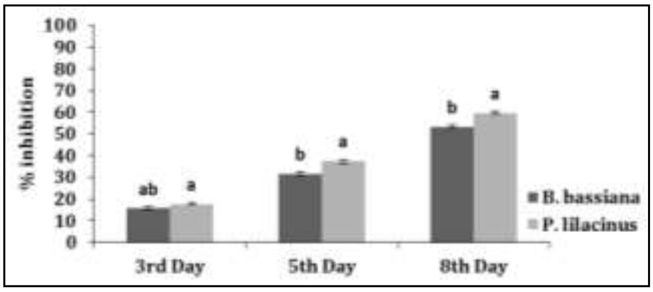

Fig 4: Antifungal potential of *B. bassiana* and *P. lilacinus* on percent inhibition growth of *Alternaria solani* by using non-volatile metabolites ($P \leq 0.05$)

6. Discussion

Entomopathogenic fungi are well known biological control agents against insect pests (Powell et al 2007; Mantzoukas *et al.*, 2015) [26, 19] and plant pathogens (Griffin *et al.*, 2006; Jaber, 2015; Zhou *et al.*, 2016) [13, 15, 30]. Present study confirmed that *P. lilacinus* and *B. bassiana* showed antagonistic ability against early blight pathogen of tomato under laboratory conditions. Metabolites of *P. lilacinus* and *B. bassiana* reduced the mycelial growth of *A. solani*. Ownely *et al.* (2004) confirmed that *B. bassiana* produced antifungal metabolites that inhibited the mycelial growth of phytopathogens under laboratory conditions. Toshinori *et al.* (2004) [29] also confirmed that *Verticillium psalliotae* (an Entomopathogenic fungus) produced metabolites that inhibited the growth of *A. solani, P. infestans* and *F. oxysporum*. Our results also matched with Gothandapani et al, (2015) [11] who reported that three Entomopathogenic fungi viz; *V. lecanii, B. bassiana* and *M. anisopliae* inhibited the mycelial growth of *A. porri* when evaluated by using dual culture and food poison technique. According to present results, non-volatile metabolites showed higher mycelial growth inhibition as compared to volatile metabolites. Results showed by Lee *et al.* (1999) [18] also confirmed that culture filtrate of *B. bassiana* reduced the growth of *R. solani* under laboratory conditions. *B. bassiana* also reduced the growth of *R. solani* and *Pythium myriotylum* when tested by dual culture (Griffin, 2007) [12]. In another study, culture filtrate of *B. bassiana* reduced the growth of *F. oxysporum* (Bark *et al.*, 1996) [5]. *B. bassiana* produced many toxins but are very difficult to isolate or production is very low. Some studies documented the production of beauvericin during colonization in tomato (Powell, 2005) [25] but it can assume that these type of metabolites reduced the growth of *A. solani*. In present study, maximum inhibition of *A. solani* in all assays was showed by *P. lilacinus* as compared to *B. bassiana*. *P. lilacinus* inhibited the growth of phytopathogenic nematodes and decreased the rate of egg hatching (Anastasiadis *et al.*, 2008) [3]. To our knowledge present study is first report of antifungal potential of *P. lilacinus* against fungal plant pathogen *A. solani*.

7. Conclusion

Entomopathogenic fungi are basically insect pathogenic but they also showed their dual biocontrol activity against insect pests and plant pathogens. On the basis of results, it is concluded that Entomopathogenic fungi (*B. bassiana* and *P. lilacinus*) have potential to inhibit the growth of *A. solani* causal agent of tomato early blight disease. Use of these Entomopathogenic fungi against phytopathogens can reduce the risk of chemical fungicides on environment and human health and also helps to produce organic crops.

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