Analysis of Antagonistic Potential of Secondary Metabolites and Organic Fractions of Trichoderma Species against Alternaria Alternata

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Role of Trichoderma species is well documented as antagonists as well as plant growth enhancers. Presently, the fungicidal potential of three Trichoderma species namely, T. koningii (FCBP769), T. viride (FCBP904), and T. harzianum (FCBP1277) was assessed against Alternaria alternata that causes leaf necrotic spots of Syzygium cumini and broad range of other plants using 0, 15, 30, 45, 60 and 75% dilutions of filtrates. There was a significant reduction of around 40-95%, 22-86% and 52-91% in fungal biomass by T. koningii, T. viride and T. harzianum, respectively. In fractionation bioassays, Trichoderma metabolites were partitioned using organic solvents viz., n-butanol, n-hexane, chloroform and ethyl acetate. Antifungal activity at different concentrations (10, 20, 30, 40 and 50 ppm) was assessed against the pathogen. Ethyl acetate fraction of T. koningii extract displayed the most promising activity resulting in 10-90% suppression in biomass. In case of T. viride butanol fraction proved the most effective in retarding the growth of pathogen from 20 to 80%. While T. harzianum extract revealed 55-85% arrest in fungal biomass due to n-hexane fraction. Present study concludes that test Trichoderma species demonstrated a strong fungicidal activity against A. alternata. Current research offers the possibility of developing strategies for controlling pathogens with bioactive metabolites of Trichoderma.

Key words: Antagonists / Antifungal activity / Alternaria alternata / Fractionation / Trichoderma species.

INTRODUCTION

The antagonistic property of Trichoderma species was first demonstrated by Weindling (1932) who suggested their potential use as biocontrol agents for plant diseases. This is largely result of change in attitude of public toward the use of chemical pesticides and fumigants (Hjeljord and Tronsmo, 1998). Trichoderma strains are the most promising biocontrol agents being able to control a wide range of plant pathogens (Hermosa et al., 2000; Guo et al., 2002). They inhibit the growth by several mechanisms such as competition for nutrients, antibiotics and production of fungal cell wall degrading enzymes (Limon et al., 2004). According to Rosado et al. (2007), the main factor for ecological success of this genus is a combination of very active mycoparasitism mechanisms and an effective defensive strategy, induced in the plants. These fungi are used in biological control against various plant pathogens. Similar to soil pathogens, they successfully suppress leaf pathogens as well (Gal-Hemed et al., 2011; Kamal et al., 2013). The antifungal compounds produced can vary according to the microbial diversity of the mycoparasite. Earlier, the isolates of the biocontrol agents Trichoderma species were formulated by using different organic and inorganic carriers either through solid or liquid fermentation. They were applied as a seed treatment, bio-priming, seedling dip, soil application, foliar spray, fruit spray, sucker treatment and set treatment. They concluded that the management of plant diseases by employing microbial agents are the most suitable strategy to replace chemicals which are
concerned for health hazards and environmental pollution (Bhattaacharjee and Dey, 2014).

Keeping in view the problems of pathogens, the present study was designed to control Alternaria alternata using metabolites of three different Trichoderma species. Alternaria alternata isolated from jamun leaf spot was treated with Trichoderma metabolites and controlled successfully. Further, different solvents fractions from extracts of Trichoderma species were evaluated by the fractionation method to control the pathogenic fungi.

**MATERIALS AND METHODS**

**Selection of Phytopathogenic Fungus & Biocontrol Agents**

Pure cultures of Trichoderma species, namely T. viride, T. koningii, and T. harzianum, were obtained from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan which were selected to evaluate their natural antagonistic potential. The current research work signifies the use of these indigenous strains of Trichoderma that were available in Culture Bank at that time and the work on these strains is not conducted in this aspect before.

Alternaria alternata isolated from leaf necrotic spots of Syzygium cumini (L.) Skeels (Jamun) was selected as the test pathogen.

**Extraction and Bioassays of Fungal Metabolites**

*Trichoderma* species were cultured in 2% Malt extract broth medium at 27°C. After 15 days of incubation, cultural filtrates were collected and used to check the antagonistic effect of metabolites against phytopathogen, A. alternata. For bioassays, 0, 15, 30, 45, 60 and 75% dilutions of stock were made and simmered at 70°C without pressure for 15-20 min. Then a disc of 5 mm from actively growing colony of A. alternata was inoculated in fungal growth medium amended with *Trichoderma* metabolites and control set and incubated at 25-27°C for seven days. After seven days, broth was filtered through pre-weighed filter paper. Biomass of A. alternata was collected and oven dried overnight at 40°C. Percentage reduction in fungal biomass due to various employed concentrations of the metabolites over control was calculated by applying the following formula:

$$\text{Growth inhibition (\%) } = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

**Evaluation of Organic Fractions of Fungal Metabolites**

Fractions of metabolites of *Trichoderma* species partitioned in different organic solvents, viz., n-butanol, n-hexane, chloroform and ethyl acetate, were evaluated according to the protocol of Lazarovits et al. (1979). A disc of 5 mm diameter of selected *Trichoderma* species from actively growing colony was inoculated in each medium flask and left for 15 days at 25-27°C for fungal growth. After incubation period, filtration was carried out through filter paper (Whatman No.1). This filtrate was preserved at 4°C in refrigerator. According to Lazarovits et al. (1979) a volume of 400 mL of crude cultural filtrate of each *Trichoderma* species was taken in 1000 mL separating funnel. Four organic solvents were used for extraction. First, a volume of 200 mL of n-butanol was added to 400 mL filtrate in separating funnel, shaken well and kept until two phases get separated. The clear layer of n-butanol was separated in pre-weighed beaker and dried in oven at 40°C. The remaining filtrates were extracted similarly in succession with n-hexane, chloroform and ethyl acetate. All the organic fractions were evaporated in a hot-air oven at 40°C to obtain the final residues. The quantities of residues obtained in different fractions are presented in Table 1.

**Antifungal Bioassays with Fractions**

Aqueous extracts of each organic fraction was prepared in sterilized distilled water to make the final volume of 10 mL. To the fraction obtained from organic solvents, firstly 500 µL DMSO (dimethyl sulfoxide) was added to dissolve the fractions. Then finally distilled water was added to make final volume of 10 mL. Different dilutions of stock extracts were prepared. To check the bioactivity of fractions, 0, 10, 20, 30, 40 and 50 ppm dilutions of the organic solvent residues solutions were formed by adding 1ml of residue solution and distilled water to 2 mL Malt Extract broth in order to make the final volume of each dilution to 3 mL in the test tubes. Each treatment of all fractions was replicated thrice. Control set contained only malt extract broth. Alternaria alternata was inoculated in each test tube by agar discs of 2 mm diameter. The test tubes were incubated at 25-27°C until the control set gets maximum growth. After incubation period, data was recorded by taking the dry

| Solvent          | Amount of Organic Fraction collected (mg) |
|------------------|-------------------------------------------|
| Butanol          | T. viride 1560  T. harzianum 1441  T. koningii 1380 |
| n-hexane         | T. viride 1014  T. harzianum 1200  T. koningii 1593 |
| Chloroform       | T. viride 963  T. harzianum 997  T. koningii 997 |
| Ethyl acetate    | T. viride 1216  T. harzianum 1307  T. koningii 1036 |
biomass of A. alternata. Biomass analysis predicted the growth inhibition of A. alternata caused by the application of different metabolite fractions of Trichoderma species.

**Statistical analysis**

Standard errors (SE) of means of three replicates of each treatment were computed using computer software Microsoft Excel. All the data were analyzed by analysis of Variance (ANOVA) followed by Duncan’s New Multiple Range (DMR) test to separate the mean differences (Steel and Torrie, 1980) and to detect the significant difference among the treatments. Inhibition constants (Kᵢ) values were determined by plotting the range of fungal biomass produced against the log of concentrations of Trichoderma metabolites. Non-linear regression was used as it provided the highest values for R². The inhibition constant (Kᵢ) was determined to calculate the inhibition concentration that reduced the biomass production to 50%.

**RESULTS**

**Identification of Alternaria alternata**

The fungal colony on Malt extract agar was sporulated having dark brownish black, reverse off-white to black reaching 4.5-5 cm in diameter, with regular margins and superficial mycelia. Sporulation pattern, conidiophores and conidial morphology was examined under compound microscope (Labomed CX2; Labo America, Inc. USA). Conidiophores were branched, septate, olive brown 70-80 x 3-4 µm in size. Conidia were long elliptical, pale brown to pale greenish as matured, produced in chains of 4-8. Mature conidia ranged in size from 25-30 x 5-9 µm, with 4-7 transverse septa and 1-2 longisepta. The spore wall was smooth but some had geniculations. Based on morphological characteristics, the pathogen was identified as A. alternata (Fig. 1).

**Effect of Cultural Filtrates of Trichoderma species on Growth of Alternaria alternata**

The results obtained from the biomass assays of A. alternata in different concentrations of aqueous extracts of T. koningii grown on malt extract broth are presented in Fig. 2A. It was depicted from the recorded data that an increase in extract concentration of Trichoderma, resulted in a significant decrease in the biomass production of A. alternata. The lowest concentration (15%) exhibited around 42% inhibition in the growth of A. alternata and, the highest concentration (75%) caused approximately 94% suppression. Similarly a significant reduction in biomass production of A. alternata, ranging from 24-84%, was observed at all the concentrations of T. viride extracts and the higher concentrations (60 and 75%) were the most effective as they revealed 80-84% reduction in fungal biomass production (Fig. 2B). In case of T. harzianum metabolites, lower concentrations (15-30%) were proved more effective in comparison to T. koningii and T. viride as it reduced the growth of target fungus up to 45%. However, the fungal biomass greatly decreased as concentration of extract increased from 45-60%. Maximum reduction in the biomass of test fungus (90%) was achieved at 75% concentration (Fig. 2C).

**Determination of Growth Inhibition Kinetic Constants**

From the regression equations, the concentration of fungal metabolites was determined that reduced fungal biomass by 50% of the control (Fig. 2 A-C). The values of Ki are the mean ± SE of three replicates (Table 2). The least Ki value was calculated by T. harzianum that was 16.62% than other species against the growth of pathogen. The comparative analysis for Ki values of all the three Trichoderma species and growth inhibition of plant pathogen depicted that T. viride metabolites possessed the minimum efficiency to suppress the growth of the pathogen.

**Antifungal Activity of Fractions of Trichoderma koningii**

The antifungal effect of butanol fraction on the growth of A. alternata revealed a statistically significant suppression by all the concentrations over control treatment. The effect of lower concentrations of 10-20 ppm was less pronounced with 10-20% reduction in fungal biomass in comparison to control treatments. However, the maximum antifungal activity was recorded at the highest concentration 50 ppm with 75-80% reduction in fungal biomass over control (Fig. 3). In case of n-hexane fraction of filtrate of T. koningii, an inverse pattern of antymycotic activity was observed as there was an increase in biomass production of pathogen with the increase of concentration, however, this increment was significantly lower than control (Fig. 3). The lower concentrations (10-30%) of n-hexane fraction were found to be more effective in suppressing the growth of A. alternata as maximum reduction of 70% was depicted by 10 ppm
The butanol fraction depicted the most promising antifungal potential as compared to other fractions of *T. viride*. In general, the subduing effect of all the concentrations was statistically significant over the control (Fig. 4). Maximum antifungal activity was recorded at the highest concentration of 50 ppm with 80% reduction in fungal biomass. Growth response of n-hexane fraction of *T. viride* was parallel to the butanol fraction as it also significantly reduced the growth of *A. alternata* at all concentrations employed. A significant reduction ranging from 14 to 80% in the biomass production of pathogen was recorded in all concentrations of chloroform fraction as compared to the control. The maximum reduction of 70% was depicted by the highest concentration (50 ppm). Ethyl acetate fraction proved to be the most effective as it caused maximum suppression (around 90%) in biomass production of pathogen.

**Antifungal Activity of Fractions of *Trichoderma viride***

The butanol fraction depicted the most promising antifungal potential as compared to other fractions of *T. viride*. In general, the subduing effect of all the concentrations was statistically significant over the control treatment (Fig. 4). Maximum antifungal activity was recorded at the highest concentration of 50 ppm with 80% reduction in fungal biomass. Growth response of n-hexane fraction of *T. viride* was parallel to the butanol fraction as it also significantly reduced the growth of *A. alternata* at all concentrations employed. A significant reduction ranging from 14 to 80% in the biomass production of pathogen was recorded in all concentrations of chloroform fraction as compared to the control. The maximum reduction of 70% was depicted by the highest concentration (50 ppm). Ethyl acetate fraction proved to be the least effective in comparison to the other fractions of *T. viride* extract. Maximum inhibition of about 54% in

### TABLE 2. Kinetic Constants for fungal biomass inhibition by *Trichoderma* species

| Species           | $K_i$ (ppm) | SE    |
|-------------------|-------------|-------|
| *T. koningii*     | 24.23       | ±0.830|
| *T. viride*       | 32.24       | ±1.605|
| *T. harzianum*    | 16.61       | ±0.731|
fungal biomass was observed by 50 ppm extract fraction of ethyl acetate (Fig. 4).

**Antifungal Activity of Fractions of *Trichoderma harzianum***

Data in relation to the effect of different concentrations of butanol fraction on the growth of *A. alternata* revealed a gradual decrease in fungal growth with the increase in concentrations of butanol fraction. The highest concentration (50 ppm) was proved highly lethal for the growth of *A. alternata* (Fig. 5). The results displayed that n-hexane fraction of filtrate obtained from *T. harzianum* was the most effective as compared to other organic fractions, even the lowest concentration of 10 ppm showed the pronounced suppression of 55% in the biomass production of pathogen. The percentage reduction in fungal biomass was observed in the range of 52-88%. The effect of lower concentrations i.e., 10 and 20 ppm of chloroform fraction on the growth of *A. alternata* was statistically insignificant among themselves but it was significant with respect to control treatment. There was about 16-78% reduction in biomass production of *A. alternata* due to 10-50 ppm concentrations of chloroform fraction. Ethyl acetate fraction was proved the least effective in suppressing the growth of target fungus as compared to other fractions. The overall growth of the target fungus was decreased in the range...
of 5-75%. Higher concentrations (30, 40 and 50 ppm) had more pronounced and statistically significant antifungal effect against the pathogen (Fig. 5).

### Kinetic Constants for fungal biomass inhibition and Different Fractions of *Trichoderma* Species

Calculated values for $K_i$ for all treatments are presented in Table 3. The kinetics results based on all experiments provided a range of 10.34 to 50.05 ppm of $K_i$. In case of *T. koningii*, n-hexane and chloroform fractions of metabolites provided the least $K_i$ values that were 16.20 and 16.71 ppm, respectively. However butanol fraction of *T. viride* metabolites showed minimum value of 26.92 ppm than its other fractions against the growth of phytopathogen. Finally $K_i$ for n-hexane fraction of *T. harzianum* metabolites was only 10.34 ppm which demonstrated that fungal growth was statistically most significantly inhibited by this fraction (Figs 3 to 5). Comparative analysis of $K_i$ values for all three *Trichoderma* species metabolites fractions and growth inhibition of plant pathogen revealed that ethyl acetate fraction of metabolites is least effective in controlling the growth of pathogen with highest $K_i$ value for *T. viride* metabolites (Table 3).

### DISCUSSION

A safest and economical way to control the phytopathogens without affecting the beneficial soil microorganisms is the use of biological control methods such as the implementation of antiphytopathogenic microorganisms (Karatyn, 1993) or the use of extracts from various plants and their sections (Bajwa and Iftikhar, 2005; Shafique et al., 2011). The active antiphytopathogens are a number of fungi, among which the genus *Trichoderma* is well recognized. A number of *Trichoderma* species have been used so far as potent biocontrol agents for a variety of phytopathogenic fungi (Mukhtar, 2010; Murtaza et al., 2012).

In the light of recent studies and discoveries, a research was carried out to study the *in vitro* effectiveness of secondary metabolites of three species of *Trichoderma* viz., *T. koningii*, *T. viride* and *T. harzianum* against a pathogenic test species, *A. alternata*. Different concentrations (0, 15, 30, 45, 60 and 75%) of fungal metabolites were employed. The outcomes from this study imitate that all the *Trichoderma* species have the innate capability to induce antagonistic effect on growth rate of pathogenic fungus. The comparative potency of this effect however varied within *Trichoderma* species and also among the concentrations of crude extracts of *Trichoderma* species used. Thus the efficiency of the extracts was found to be correlated with the resistance or susceptibility offered by different species of *Trichoderma* (Hanada et al., 2009; Murtaza et al., 2012). Earlier Faheem and coworkers (2010) reported six isolates of *Trichoderma* spp. in their study that were verified for their potential to prevent the growth of different soil borne pathogens viz., *Rhizoctonia solani*, *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* under *in vitro* conditions. The results showed that *T. viride* greatly retarded the growth of pathogen up to 71% in case of *Rhizoctonia solani* followed by *T. viride* and *T. harzianum* showing 66 and 60% inhibition over control, respectively. Similarly in case of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, *T. viride* proved to be the best for all isolates in suppressing mycelial growth of test pathogens. Recently, Kumari et al. (2016) worked on 26 isolates of *Trichoderma* species and screened out for their antagonistic and antibiosis efficacy against *Rhizoctonia solani*. All the isolates inhibited the growth of *R. solani* ranging from 33-54%.

In current study, aqueous extract of all the *Trichoderma* species significantly suppressed the pathogen growth. This is because the *Trichoderma* species are reported to secrete a wide range of secondary metabolites including gliovirin, gliotoxin, viridian, pyrenes and viridiol (Jones and Hancock, 1987; Jones et al., 1988). Presently a decreasing gradient was observed with an increase of metabolite concentrations which revealed that higher concentrations were more effective in suppressing the fungal biomass. Working on the same line, Shafique et al. (2012) documented that in case of methanolic extract, all the regimes (1-4%) of methanolic extract of *Cymbopogon citratus* caused significant inhibition in mycelial biomass production of the *Alternaria* species but the highest concentration of 4% was proved the most effective in suppressing the biomass of target fungal species.

### TABLE 3. Inhibition constants for fungal biomass by *Trichoderma* species in different organic solvents

| Species      | Fractions | K$_i$ (ppm) | SE  |
|--------------|-----------|-------------|-----|
| *T. koningii*| Butanol   | 25.44       | ±1.699 |
|              | n-hexane  | 16.20       | ±1.627 |
|              | Chloroform| 16.71       | ±1.510 |
|              | Ethyl acetate | 31.33       | ±1.473 |
| *T. viride*  | Butanol   | 26.92       | ±0.392 |
|              | n-hexane  | 27.68       | ±0.327 |
|              | Chloroform| 35.08       | ±3.898 |
|              | Ethyl acetate | 50.05       | ±0.970 |
| *T. harzianum*| Butanol | 29.86       | ±1.909 |
|              | n-hexane  | 10.34       | ±1.200 |
|              | Chloroform| 35.56       | ±2.471 |
|              | Ethyl acetate | 36.24       | ±0.283 |
In subsequent experiment, the fractions of four organic solvents (n-butanol, n-hexane, chloroform, ethyl acetate) obtained from the culture filtrates of three Trichoderma species were checked against the selected pathogen. When these organic solvents were successively employed, organic compounds from fungal metabolites having different polarities were dissolved in different solvents thus became separated. The concentrations of these fractions were applied in the range of 10–50 ppm. The disparity in fungistatic activity of extracts in different solvents may be ascribed to the different chemical nature of the solvents. It is due to the dissolution of different types of chemicals in different solvents that ensured the variable activity of metabolites of same source in different solvents. There are many examples in literature which support these findings (Mukhtar, 2010; Mulatu et al., 2013). Previously, Baiwa et al. (2007) worked on the pathogenic species of same genus Alternaria, namely A. alternata, A. citri and A. tenuissima to check out the antifungal potential of Aloe vera aqueous and n-hexane shoot extracts. Their study concluded that the inhibition in fungal biomass was found to be varied with the employed concentrations and gave significant reduction in biomass production. In a similar study, the antifungal potential of five species of Trichoderma viz., T. viride, T. aureoviride, T. reesii, T. koningii and T. harzianum was examined against Alternaria citri by Murtaza et al. (2012). Culture filtrate of T. harzianum was found highly effective in suppressing growth (upto 93%) of the test fungal species. In fractionation guided bioassay of T. harzianum metabolites, there was 68% reduction in growth of A. citri due to 1% concentration of ethyl acetate fraction was observed.

In present study, in case of T. koningii, significant results were presented by all the concentrations of ethyl acetate fraction which showed maximum fungistatic activity particularly at higher concentrations. Although significant reduction in biomass production of pathogen was recorded by all concentrations of all fractions, however, n-hexane and chloroform proved very toxic for fungal growth even at lower concentrations which induced more than 50% inhibition in fungal biomass production with least Ki values. The results regarding the n-hexane and chloroform fractions of T. viride are also comparable with T. koningii as their lower concentrations again exhibited more than 50% inhibition in fungal biomass production. The fractionation analysis of T. harzianum revealed that n-hexane fraction of extract was the most effective as its all concentrations induced the maximum inhibition in fungal biomass in the range of 52-88%. These findings in are good agreement with previously published results that showed the ethanol and butane crude extracts of Trichoderma isolates had good activity against plant pathogenic fungus (Shahverdi et al., 2007). Working on the same line, Mulatu et al. (2013) designed an experiment to evaluate and characterize effective antifungal extracts from Trichoderma isolates against coffee wilt pathogen (Gibberella xylarioides) using different organic solvents viz., chloroform, ethanol, methanol, ethyl acetate, n-hexane and butane. Inhibitory assays revealed that all Trichoderma isolates significantly reduced mycelial growth of the pathogen compared to the control under in vitro conditions.

This study concludes that secondary metabolites of all Trichoderma species contain potential antifungal compounds, and can be subjugated as an ideal treatment for future plant disease management programs eliminating fungal spread.

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