

UNVEILING ANTIFUNGAL PROTEINS FROM *TRAMETES VERSICOLOR* AS BIO-PESTICIDE TO INHIBIT *ALTERNARIA SOLANI*

ASHRAF I^{1,3}, ALI M², ASIF M³, ANWAR W³, AKHTER A³, KHAN HAA⁴, BUKHARI, NT⁵, ALI Q⁶, SHAFIQ M⁷

¹Agriculture Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

²Sustainable Development Study Centre, Government College University, Lahore, Pakistan

³Faculty of Agricultural Sciences, Department of Plant Pathology, University of the Punjab, Lahore, Pakistan

⁴Faculty of Agricultural Sciences, Department of Entomology, University of the Punjab, Lahore, Pakistan

⁵Department of Microbiology and Clinical Laboratory Sciences, Women University Swabi, Swabi, Pakistan

⁶Faculty of Agricultural Sciences, Department of Plant Breeding and Genetics, University of the Punjab, Lahore, Pakistan

⁷Rawalpindi Medical University, Tipu Road, Chamanzar Colony, Rawalpindi, Pakistan

*Correspondence author email address: adnanakhter.iags@pu.edu.pk

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Abstract: *Alternaria solani* was isolated from infected tomato leaves, and *Trametes versicolor* from northern areas of Pakistan established mycelial culture on PDA. Crude Protein extract was prepared in 50mM Sodium phosphate buffer by macerating *C. versicolor* (T/C) mycelium. Extracted protein solution (Crude extract) was filtered and tested for antifungal activity by using different concentrations (0.5mg/ml, 1mg/ml, 1.5mg/ml, and 2mg/ml) against *A. solani*. A protein with antifungal potential was purified using the DEAE- Cellulose column followed by the gel filtration column of Superdex75. MIC was also observed for purified antifungal protein by using micro spectrophotometry. Results showed that *T. versicolor* has the potential to inhibit the fungal growth of *A. solani*. Crude extract with a concentration of 2mg/mL inhibited 83% of the growth of *A. solani*. However, only 47% of inhibition was observed in the case of 0.5mg/ml of protein extract. The protein having antifungal potential purified by anion exchange chromatography has an approximate size of 15 kDa. MIC for the purified protein is 200µg, which inhibited the 100% growth of *A. solani*.

Keywords: Antifungal proteins, Bio-pesticides, Crude proteins, Thermal stability, biological formulations

Introduction

Agrochemicals in general and chemical pesticides have become a part of agricultural practices nowadays (Puglisi et al. 2012). Their application and environmental risks increase due to the persistence of chemical residues in soil, water, and even agricultural products (Cui et al., 2020). Chemical pesticides impact the environment, public health, and other populations. These formulations can play a vital role in reducing a specific population and disturbing normal environmental functioning (Feld et al., 2015). Although each pesticide company provides a procedure for safe usage, it can still risk that pesticides impose on public health and the environment (Hoffman et al. 2003). Irrigation water and wastewater effluents that fled into the field can be a better way for pesticide residues to get into the aquatic environment (Kock-Schulmeyer et al., 2013). One of the most well-known vegetables and a significant horticultural crop, the tomato

(*Solanum lycopersicum*), is grown all over the world. It holds a prominent role in the global economy of vegetables. In terms of processing crops, it comes in top place and is the second most popular vegetable after potatoes. According to agricultural statistics of Pakistan 2017-18, tomatoes are grown on about 52300 ha area, and the production was 529.6 million tons (GOP 2017). Thirty of the 200 tomato diseases that are known to exist are relevant commercially. Viruses and bacteria are significantly smaller organisms than fungi. They might create numerous plant problems that result in the loss of a sizeable amount of the harvest each year (Juanet al., 2015).

Early blight is a common fungal disease of tomatoes caused by *Alternaria solani* (Saleem et al. 2016). It is a widespread and damaging disease causing the loss of millions of dollars annually worldwide, including in Pakistan. Warm temperatures and protracted

leaf wetness owing to dews, overhead irrigation, or heavy rains are conducive to the early blight disease. The fungus may exist on tomato plants or solanaceous weeds that are vulnerable (Foolad et al. 2008). For crop protection, a variety of chemical fungicides are offered in the market. Some of them have high effectiveness and cost-benefit ratios. However, due to the toxicity of their residues, their indiscriminate use has led to serious health risks, difficulties with air, soil, and water pollution, the emergence of resistance in target species, and problems with target organisms. By using less synthetic pesticides or augmenting with biological pesticides, it is possible to satisfy market demands while also protecting human health and the environment. Biopesticides are used in addition to synthetic pesticides as substitutes for controlling pests and diseases (Engindeniz et al. 2013). Traditional eastern remedies have a well-established history of using medicinal mushrooms. Japan, China, Korea, and other Asian nations still use preparations made from mushrooms in their modern healthcare practises. A medicinal fungus called *Trametes versicolor*, formerly known as *Coriolus versicolor*, is frequently used as a co-treatment to boost patients' immune systems. (Chu and Chow 2002). In addition to its medical applications, *T. versicolor* is widely used to degrade recalcitrant organic pollutants such as pentachlorophenol (PCP). Pesticides are among the most studied and investigated categories of pollutants by scientists. However, fewer investigations are carried out to find their replacement for a healthy environment, which was the focus of this study. Therefore, this research aimed to find viable replacements for chemical pesticides to recover environmental and public health.

Materials and methods

Collection of infected Tomato leaves

Areas with intensive pesticide usage and a high rate of disease severity were selected for the study. Tomato leaves infected with early blight were collected from ten different fields in the outer periphery of Lahore city. Leaf samples were randomly collected from each area in a zig-zag sequence. After field photography, the samples were sealed in labeled sampling bags. Samples were further processed for pathogen isolation in PARB 437 laboratory at the Department of Plant Pathology, Faculty of Agriculture, University of the Punjab, Lahore, Pakistan. Infected portions of the samples were cut into small pieces and surface sterilized with 1% sodium hypochlorite (NaOCl) for one minute. Sterilized pieces were then rinsed in

distilled water for 30 seconds and placed on blotter paper for drying. Dried samples were inoculated on potato dextrose agar (PDA) nutrient media and incubated in WITEG Labortechnik Wisd low-temperature incubator at 28°C for five days. As the mycelial growth appeared, culture purification was done by the hyphal tip method.

Collection and Mycelial Preparation of *T. versicolor*

Basidiocarps of *T. versicolor* were collected from Northern areas of Pakistan (Ayubia National Park, Khanspur, Neelum Valley, and Swat valley). Basidiocarps were cut into small pieces of 1 mm² and surface sterilized with 1% NaOCl and inoculated on PDA plates to develop mycelial culture.

Antagonistic Effect of *T. versicolor* against *A. solani*

Using the Dual Culture Method, the antagonistic action of *T. versicolor* against the early tomato blight was identified. A 6mm diameter agar disc was removed from the agar media plate's edge, and it was then replaced with an *A. solani* disc. The plug of *T. versicolor* was also inoculated on the opposing edge of the media plate. The inoculated plates were incubated at 28°C for seven days. As a control, *A. solani* was placed similarly on a fresh PDA plate. By comparing the radius of the *A. solani* colony in the control plate to the radius of the colony facing the *T. versicolor* colony (T) five days after incubation, the antagonistic activity was evaluated (C). Using the Skidmore and Dickinson methodology, the two measurements were converted into percentage inhibition of radial growth (PIRG) (Anupama et al. 2010).

$$\text{Percentage inhibition} = \frac{C - T}{C} \times 100$$

Where, C = control; T = Treatment

Crude Protein Extraction of T. versicolor:

A plug of *T. versicolor* from stock culture was inoculated into fresh PDB and incubated at 28°C on a shaking incubator at 150 rpm for five days. Mycelia filtered through a Whatman No 4 filter paper. Twenty grams of mycelia were extracted overnight with 50mM phosphate buffer (pH 6.0), filtered and centrifuged at 7000 rpm for 30 minutes to remove the insoluble residues. The supernatant was added with ammonium sulfate to get 85% saturation and incubated overnight at 4°C. The solution was centrifuged at 7000 rpm for 30min and the pellet was dissolved in 25mM Tris-Cl buffer with pH 7.5. The solution was dialyzed against the same buffer to remove salts. Activity assay was performed to observe the inhibition potential crude extract.

Purification of Proteins

Dialyzed protein fraction with antifungal potential was applied on the DEAE-Cellulose column previously equilibrated with 25mM Tris HCl buffer (pH 7.5). Un-adsorbed fractions were removed with the same buffer. Adsorbed fractions were eluted with a 0-1M NaCl linear concentration gradient in 25mM Tris HCl buffer (pH 7.5). All fractions were collected and checked in their activity assay using micro-spectrophotometry described by Broekaert *et al.* (1990). Active peak (S2) was concentrated and further purified on Superdex 75 column (120ml) by using 200mM NaCl with 25mM Tris HCl buffer (pH 7.5). A small peak eluted between 83- 85ml fraction was found active against fungal pathogen confirmed by micro-spectrophotometry. This peak fraction was subjected to electrophoresis, which revealed a single band.

Electrophoresis

To measure the level of protein purity attained by the superdex 75, the purified protein was processed to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie brilliant blue R-250 was used to stain the gel, and a solution of 30% methanol and 10% acetic acid was used to remove the stain.

Estimation of Protein

Using bovine serum albumin as a reference, protein concentrations in crude extract, ammonium sulphate precipitation, DEAE-Cellulose column, and gel filtration column were calculated using the Lowry technique.

MIC of Purified Protein

The microdilution method was used to determine the minimum inhibitory concentration (MIC). Inoculum of fungal strains were prepared from overnight broth cultures, and suspensions were adjusted to 1.0×10^6 . Various concentrations of the protein $10\mu\text{g L}^{-1}$, $20\mu\text{g L}^{-1}$, $30\mu\text{g L}^{-1}$, $40\mu\text{g L}^{-1}$, $50\mu\text{g L}^{-1}$, $75\mu\text{g L}^{-1}$, $100\mu\text{g L}^{-1}$, $150\mu\text{g L}^{-1}$, $200\mu\text{g L}^{-1}$ were added to an equal volume of inoculated fungi in 96 wells plate. The plate was incubated at room temperature for 30 minutes, and absorbance at 595nm was measured. The plate was incubated for 72hrs. and absorbance was measured at 595nm again. The absorbance values of 30min subtracted with the absorbance values of 72hrs. Then the percentage of growth inhibition of *A. solani* was observed with a comparison of the growth of control (*A. solani*).

Thermal Stability of Purified Protein

The thermal stability of the purified protein was determined by incubating the sample at various temperatures varied from 80°C, 90°C, and

100°C for 15 minutes. After incubation, the sample tubes were rapidly cooled by ice, and antifungal assays were carried out using micro-spectrophotometry at 595nm. In addition, growth percentage inhibition was measured to observe the effect of temperature. The minimal activity was detected at pH 6 and 7. The activity was undetectable at pH 8 and 9 and at 95°C its activity was detected 55%.

Results

Morphological Characterization of *A. solani*

The fungal colony exhibited concentric zonation with uniform and smooth borders; the early stages of the colony had a matte texture and a grayish brown color, but the mature colony was black. Conidiophores range in length from 35 to 80 microns and are colored dark brown. 55-95 microns in length and 12-20 microns in breadth, having 1-5 longitudinal and 4-9 transverse septa. Hyphae in the mycelium were branched, septate, and a light brown hue until they darkened with age (Figure 1).

Characterization of *Trametes versicolor*

T. versicolor has a thin, solid, and oval pileus. This is a bracket-shaped mushroom with a wavy margin-colored concentric zone. *C. versicolor* had 3-5 cm across brackets that were semicircular, flattened, thin, and tough. Young brackets were flexible. The upper surface was velvety and attractively marked with concentric zones of varying colors, which were brown, yellow, grey, greenish, or black.

Antagonistic Activity of *Trametes versicolor*

A clear zone of inhibition was observed between *T. versicolor* and *A. solani*. *T. versicolor* inhibited more than 60% growth of *A. solani* compared to the growth of control of *A. solani* (Figure 2). The results clarified that *T. versicolor* had the antagonistic activity against the pathogenic fungus.

Antifungal Assay of Crude Protein Extract

Crude protein extract was also applied to inhibit the growth of *A. solani*. Three concentrations (0.5mg/ml, 1mg/ml, and 2mg/ml) of crude extracts were prepared from *T. versicolor* to confirm the activity assay against *A. solani*. Among all the applied concentrations of crude protein extract, 2mg/ml concentration inhibited about 83% growth of *A. solani* followed by 1mg/ml concentration that inhibited 68% mycelial growth. At the same time, the minimum inhibition was observed with the 0.5mg/ml concentration, which was measured as 47%. These results confirmed the efficacy of crude protein extract of *T. versicolor* confirmed against *A. solani* (Figure 3, 4).

Purification of Antifungal Protein

After confirming the inhibitory potential of crude protein extract against *A. solani*, the extract was used to purify on DEAE-Cellulose Column equilibrated with 10mM Tris-Cl (pH 7.2). Un-adsorbed fractions D1 were collected with the same buffer. Adsorbed

fractions were desorbed with a linear gradient of NaCl from 0-1M that resulted in three peaks D2 and D3 and D4. Adsorbed and unadsorbed peaks were used to check the antifungal activity assay by using microspectrophotometry. Adsorbed peak D2 was found active against *A. solani*. Active peak D2 applied on gel filtration column superdex 75. Four different peaks were observed on superdex 75. Larger peak SU3 was found active against fungal pathogen estimated at 15 kDa with its molecular mass on SDS-PAGE (Figure 5, 6, 7).

Estimation of Protein

Protein concentration was estimated in all fractions from crude extract to gel filtration column. Approximately 1.3mg of purified protein was

obtained after purification by gel filtration column (Table 1).

MIC of Purified Protein

As a result, the value for the MIC of purified protein was calculated as 200µg, which was recorded as 100% growth inhibition of *A. solani*. (Figure 8).

Thermal Stability of Purified Protein

T. versicolor's pure protein was heated for 15 minutes at 80 °C, 90 °C, and 100 °C, and it was discovered that the purified protein has incredible thermal stability even at extremely high temperatures like 100 °C, exhibiting 100% suppression of *A. solani's* mycelial development at all applied temperatures (Table 2).

Table 1. Estimation of Protein

Protein Type	Total volume (ml)	Total conc.(mg)
Crude extract	100	62.9
DEAE-Cellulose	28	5.8
Superdex-75	5	1.3

Table 2. Thermal Stability of Purified Protein

Treatment	%age inhibition
80°C., 15min	100
90°C., 15min	100
100°C.,15min	100

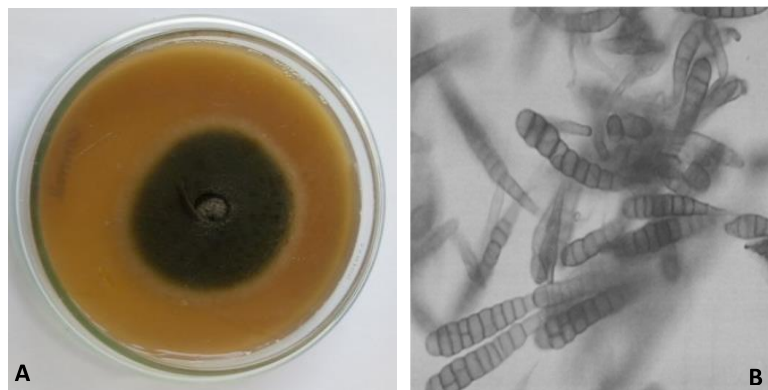


Figure 1. Pure culture of *Alternaria solani* growth at different stage on PDA medium

A=Colony culture of *A.solani*, B= Conidia of *A.solani*

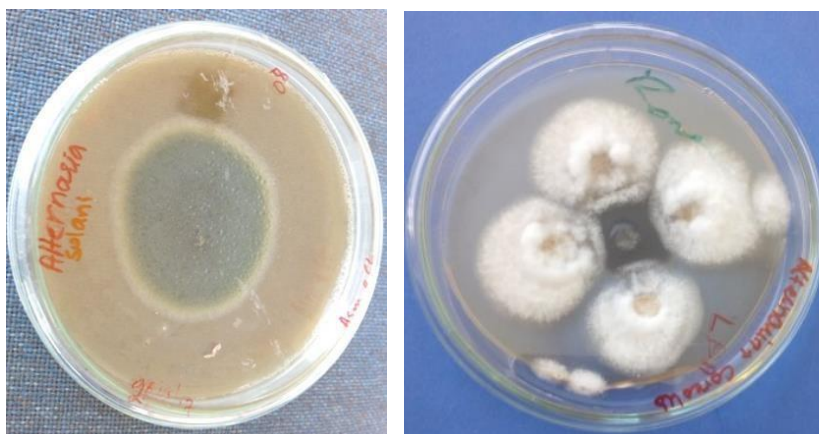
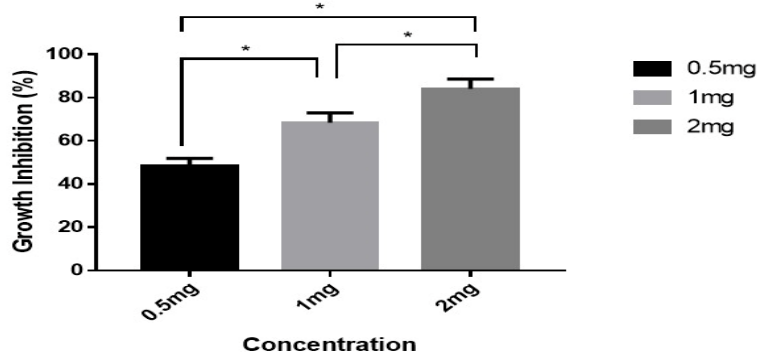


Figure 2. Antagonistic effect of *Trametes versicolor* A= *A. solani* as Control.

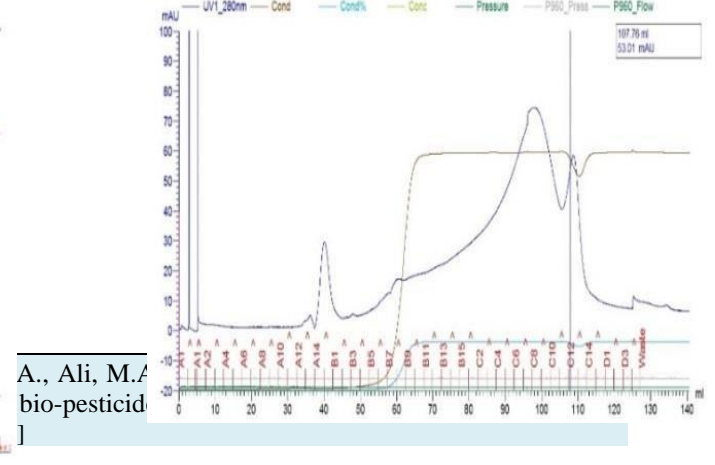
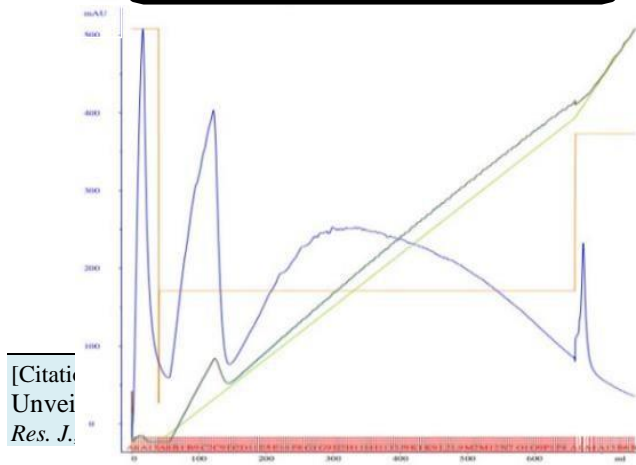
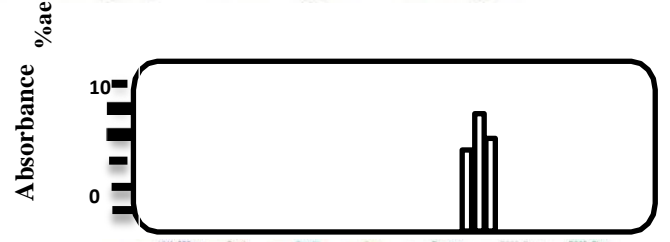
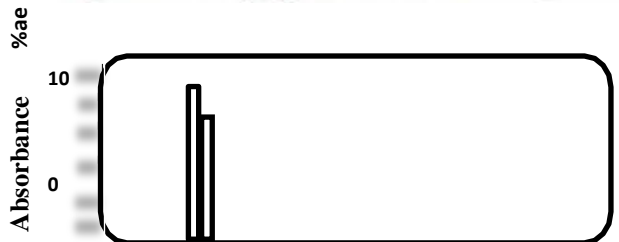


B=Antagonistic effect of *T. versicolor* with *A. solani*

Figure 3. Antifungal potential of Crude Protein Extract A= control, B=2mg/ml, C= 1.5mg/ml D=1mg/ml



Figure 4. Antifungal potential of Crude extract; A= control B=2mg/ml, C= 1.5mg/ml D=1mg/ml



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Figure 5. Crude extract of *T. vericolor* on DEAE-Cellulose. D1 is unadsorbed peak while D2, D3 and D4 are adsorbed peak desorbed with linear gradient of NaCl (0- 1M). D2 was found active against *A. solani*

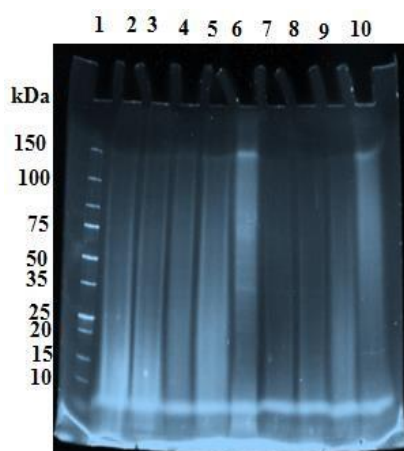


Figure 7. SDS-PAGE for Purified Protein. 1st Well from Left side represent Protein Unstained Marker (Biorad) and well 10 is showing single protein band with approximately 15kDa size.

Discussion

Chemical pesticides are usually manufactured with different chemical compounds targeting a wide range of applications as fungicides, insecticides, nematocides, and other plant growth and vigour promoters. Due to the widespread uses and toxic nature of these chemicals, these are considered pollutants of serious concern all over the world (Bashir et al. 2018). Pesticide application is rapidly increasing all over the world which has made it an important research concern especially for environmental scientists (Cui et al. 2020) because every chemical pesticide has its own specific nature of influence on environmental and public health. Toxicity, bioaccumulation, and alteration physiological functions of humans and wildlife are among the most significant effects of chemical pesticides (Hoffman et al. 2003). In order to identify some ecologically benign alternatives to chemical pesticides, it is necessary to investigate numerous avenues. The symptoms on tomato plants in the field first appeared as tiny brown to black necrotic patches approximately 1-2 mm in diameter on the elder leaves. A circular ring is frequently used to magnify these areas, creating the distinctive target board look.

Figure 6. Gel Filtration of peak D2 derived from the adsorbed fraction of DEAE-Cellulose) on a Superdex75 column. Peak SU3 were found active against *A. solani*.

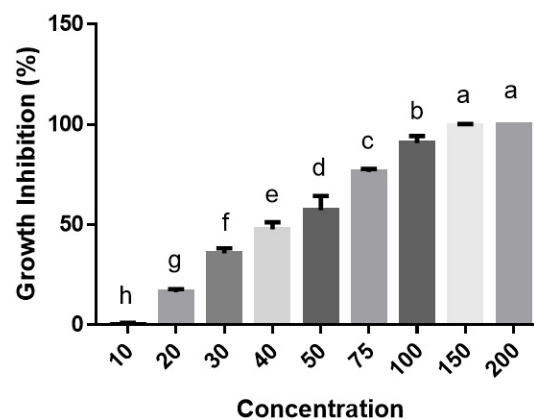


Figure 8. Minimum Inhibitory Concentration of Purified Protein against *A. solani*

Later, upward disease progression was seen, and leaves dried out and drooped. Singh and Kuwar (2008) provided a comparable description of the symptoms on tomatoes and potatoes.. On fruits, spots at the stem end first appeared as black or brown sunken lesions that grew considerably and eventually covered the entire fruit, causing it to rot. Similar symptoms on stems, petioles, and fruits were also noted by Ramakrishnan et al. in 1978. The current study's findings were in agreement with those of *A. Solani's* earlier research (Nafisa et al. 2017). The *Alternaria* species produced septate mycelium with conidiophores growing alone or in small clusters on a culture media. The conidiophores were straight or flexuous, occasionally geniculate brown to olivaceous brown. The conidia were single, straight, muriform, or ellipsoidal, and light or olivaceous brown in colour. The same results were obtained by Nafisa et al. (2017) when they identified the pathogens isolated from tomatoes with early blight. Therefore, *A. solani* has been identified as the pathogen responsible for tomato early blight.

The *C. versicolor* mushroom, which was found in northern Pakistan, has a fan-shaped cap, a wavy

edge, and colourful concentric zones. The brackets of *C. versicolor* were semicircular, flattened, thin, and tough, measuring 3-5 cm wide. Those in their youth were adaptable. These morphological characteristics were similar to the findings of Soothill and Fairhurst, (1977). Concentric zones of varied hues, which were brown, yellow, grey, greenish, or black, were nicely delineated on the upper surface's velvety surface. *C. versicolor* had a wavy border. The antagonistic fungus *C. versicolor* interacts with the pathogenic fungus *A. solani*, showing that *C. versicolor* may be able to control the pathogens. Because of the production of inhibitory metabolites at the time of inoculation at the substrate, *C. versicolor* has the ability to limit the mycelial development of other fungi (Ofodile et al. 2012). On the antifungal experiment, crude protein extract extracted from *C. versicolor* was seen. *A. solani*'s growth was being inhibited by the extract by raising the crude extract concentration. This work's description and the description provided by Baig et al. (2015) were in line. According to Alessia et al. (2019) *T. versicolor* cultures' complete crude extract may effectively limit the growth of *F. langsethiae*. However, Fagade and Oyelade (2009) reported that the extract of *T. versicolor* showed no inhibition against of test bacteria, such as *Escherichia coli*, *Flavobacterium sp.*, and *Bacillus cereus*. This phenomenon might be because some antifungal proteins have a specificity of action against only fungal species (Pushpa and Purushothama 2010). Zjawiony (2004) and Parthasarathy et al. (2009) also used extracts of *T. versicolor* who gave good antifungal activity results.

Numerous studies showed that various basidiocarp and mycelium extracts of the *Trametes* species possess antifungal properties (Sivaprakasam et al. 2019). Yamaç and Bilgili (2006) reported that extract of *T. versicolor* fruiting bodies was highly active against *Saccharomyces cerevisiae* but without any effect on *C. albicans*. Although previous studies showed that terpenoids, polysaccharides PSK and PSP, cinnamic acid and phenolic compounds presented in fruiting bodies and mycelia of the *Trametes* species are the main carriers of antifungal activity, terpenoids, polysaccharides PSK and PSP and cinnamic acid also have this role (Cheng and Leung 2008, Alves et al. 2013). The protein responsible for inhibition of *A. solani* was isolated from the crude extract of *T. versicolor* by using the anion exchange chromatography technique. Approximately 15kDa size of purified protein from *C. versicolor* was responsible for inhibition of growth of early blight of tomato. An immunomodulatory protein from *T. versicolor*, named TVC has also been purified at the size of 15KDa by Li et al., (2011). MIC was defined as the lowest concentration of fungal mycelia extract to which no

growth of was observed after the incubation period (Teoh and Mat 2010). The antifungal activity was evaluated using minimum inhibitory concentration (MIC) assay as shown in Fig. Results showed MIC value of purified protein as 200 µg against *A. solani*.

Conclusion

In conclusion, in our knowledge, this result constitutes the first report about the antifungal activity showed by purified protein from *T. versicolor* against *A. solani*.

Conflict of interest

The authors declared absence of conflict of interest.

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