

Effectiveness of a Biological Agent (*Trichoderma harzianum* and its culture filtrate) and a Fungicide (methyl benzimidazole-2-ylcarbamate) on the Tomato Rotting Activity (growth, cellulolytic, and pectinolytic activities) of *Alternaria solani*

Tarek M. Abdel-Ghany,^{a,b,*} and Marwah M. Bakri^c

Fungicides can have a good result against phytopathogens, but alternatives, such as a biological control agents, have also been found to be effective and ecofriendly. In this experiment, *Alternaria solani* was isolated from rotten tomato fruit. The culture filtrates of *Trichoderma harzianum* gradually inhibited the radial growth of *A. solani* at higher concentrations, but growth was not completely inhibited until a high filtrate percentage of 75% was reached (75% by volume). The microscopic study revealed that the *T. harzianum* culture filtrate and its spores changed the size, number, and shape of the *A. solani* conidiospores. *A. solani* produced both cellulolytic and pectinolytic enzymes *in vitro*. The activity of these enzymes decreased with an increase in the *T. harzianum* filtrate percentage and chemical fungicide concentration. *A. solani* failed to produce hydrolytic enzymes, particularly pectinase, at high concentrations of fungicide (100 ppm and 150 ppm). When *T. harzianum* was used as a biocontrol agent, the detected hydrolytic enzyme activities increased compared with the other treatments.

Keywords: *Alternaria solani*; Fruit rot; Tomato; Cellulolytic activity; Pectinolytic activity

Contact information: a: Biology Department, Faculty of Science, Jazan University, Jazan, Saudi Arabia; b: Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Egypt; c: Academic Campus for Girls, Jazan University, Jazan, Saudi Arabia;

* Corresponding author: tabdelghany@yahoo.com

INTRODUCTION

Tomato (*Lycopersicon esculentum* L.) is an important fruit crop that can be infected by numerous serious diseases (Iftikhar *et al.* 2017). Diseases are a major impediment in the production of crops, as they can decrease productivity by up to 100%. Viruses, bacteria, fungi, and nematodes attack tomato plants at all stages of development and disseminate by means of air, soil, water, seeds, and vectors (Agrios 2005). Many phytopathogenic fungi are known to cause damage to the aerial organs of tomato plants, especially under storage conditions. Several studies have reported that the main pathogenic fungi that cause post-harvest disease in tomatoes are *Rhizopus stolonifer* (Schena *et al.* 1999), *Botrytis cinerea* (Lee *et al.* 2006), and *Alternaria alternata* (Feng and Zheng 2007; Kokaet *et al.* 2017). Chaerani and Voorrips (2006) reported that the diseases appeared on the foliage (early blight), seedling basal stems (collar rot), adult plant stems (stem lesions), and fruit (fruit rot) of tomatoes as a result of *A. solani* infection. Chaurasia *et al.* (2013) stated that fruit rot caused by *A. solani* is the most destructive disease that occurs during storage,

transportation, and selling of tomatoes. The *Curvularia* sp. was recently reported to be another causative agent leading to rotting of tomato fruit (Iftikhar *et al.* 2017).

Changes in agricultural technology have been a great operator in shaping the future of agriculture. The development of natural biocontrol agents and nanomaterials could open up novel applications in plant biotechnology and agriculture (Scrinis and Lyons 2007; Abdel-Ghany *et al.* 2016, 2017, 2018a,b; Abdel-Ghany and Alawlaqi 2018). Numerous applications of chemical pesticides may adversely affect the soil microflora, soil enzymes, and physico-chemical characteristics such as the pH, salinity, and alkalinity, which is deleterious to soil fertility. Microorganisms are considered to be the origin of enzymatic activity in soil (Mohiuddin and Mohammed 2014). Therefore, there is a close relationship between pesticides and microorganisms because these chemicals may have a damaging effect on non-target microorganisms, and most of these pesticides could be metabolized by various microorganisms, which results in modulation of their activity (Walia *et al.* 2014). Various bioagents and plant extracts are reported to effectively repress several plant pathogens. The biological control of plant pathogenic fungi has received considerable attention as an alternative strategy. The utilization of the antagonistic properties of *Trichoderma* spp. against several plant diseases has been the subject of various studies (Wolffhechel and Jensen 1992; Ushamalani *et al.* 1997; Devi *et al.* 2017).

Hydrolytic enzymes play a great role in infecting fruit and vegetables with fungi, thus causing deterioration and spoilage. During infection, fungi and its hyphae spread in intercellular spaces and inside of the parenchyma cells of fruit and secrete enzymes that cause tissue maceration, leading to fruit corruption (Srivastava and Misra 2017). The degree to which fruits rot during infection depends on the ability of a pathogen to produce cellulolytic and pectinolytic enzymes. Various cell wall degrading enzymes have been shown to be secreted by phytopathogens (Yao *et al.* 1996). Pectin is a crucial ingredient for the organization of plant tissue. When it is combined with hemicellulose and cellulose microfibrils, it results in enhanced cell wall integrity and provides texture to vegetables and fruits (Pilnik and Voragen 1991). Pectinases are groups of enzymes that attack pectin and depolymerize it through hydrolysis and trans elimination, as well as by the de-esterification process, which is hydrolysis of the ester bond between the methyl and carboxyl of pectin (Gummadi and Panda 2003). Fungal pectinases are used in the food industry for clarification and to increase the production of fruit juice (Henriksson *et al.* 1999). In a recent study, approximately 50 fungus strains were isolated from infected fruits and tested for their pectinase production represented by polygalacturonase (PG) (Suryam and Charya 2018).

To the best of the authors' knowledge, no fungicides have been approved for postharvest treatment of tomatoes to prevent or inhibit the development of *A. solani* rot; therefore, this study compared the effectiveness of a safe natural control with a commercial fungicide at controlling tomato fruit rot. This study was also done to determine the pectinolytic and cellulolytic activities of the causative agents of tomato fruit rot.

EXPERIMENTAL

Fungi Diseased Sample and Isolation of the Causal Pathogen

Tomato fruits that showed symptoms of rot were collected from agricultural fields in the Jazan region, Saudi Arabia, and brought to the laboratory. Using a sterilized blade, the diseased parts of the fruits were cut into small pieces (3 mm to 4 mm) and their surfaces

were sterilized with 0.1% HgCl₂. The sterilized pieces were then inoculated on a potato dextrose agar medium and incubated at 25 °C. The developed fungal culture was purified by the hyphal tip cut method, and the pure culture was identified as *A. solani* based on the characteristics of the hyphae, conidiophores, and conidia (Ellis 1971; Rotem 1994).

Confirmation of the Pathogenicity of the Causal Pathogen

A pathogenicity test of the isolated fungus was conducted using healthy and uninjured tomato fruits of the same type as the infected fruits. The fruits were washed thoroughly with sterilized distilled water. A conidial suspension of isolated fungus (10⁶ spores/mL) in 50 mL of sterile water was prepared. The fruits were pricked using sterilized pins and inoculated by the spore spray inoculation method. The inoculated fruits were incubated in a moistened chamber at 25 °C for 5 d to develop disease symptoms, and fungus from the artificially induced diseased symptoms was re-isolated to confirm the pathogenicity of the fungus.

Effect of *T. harzianum* Culture Filtrates and Chemical Fungicide on Growth and Sporulation of *A. solani* in vitro

Trichoderma harzianum, used as a biological control agent, was provided by the Regional Center for Mycology and Biotechnology at Al-Azhar University (Cairo, Egypt). It was grown in 100 mL of Czapek-Dox medium (GEM Chem, Maharashtra, India) for 7 d at 25 °C, and then the metabolized medium was filtered through Whatman No. 1 paper. The filtrate was used at three concentrations (25%, 50%, and 75% filtrate volume). The Czapek-Dox agar medium components were prepared in 100 mL of filtrate metabolized medium with water. *A. solani* (6-d old 5-mm mycelial disc taken from an actively growing culture) was cultivated at the center of a plate containing the previously mentioned medium and incubated at 25 °C for 6 d. The Czapek-Dox agar medium was supplemented with Carbomar (methyl benzimidazole-2-ylcarbamate manufactured by Gyangsoetnal, China) as a chemical fungicide at concentrations of 50 ppm, 100 ppm, and 150 ppm. *A. solani* (6-d old 5-mm mycelial disc taken from an actively growing culture) was cultivated at the center of a plate containing Czapek-Dox agar medium with the different concentrations of fungicide and incubated at 25 °C for 6 d. The medium without any treatments served as the control. The inhibition percentage of the fungus was calculated after the incubation period using Eq. 1,

$$\% \text{ inhibition} = \left(\frac{C - T}{C} \times 100\% \right) \quad (1)$$

where *C* is the radial growth of the fungus in the control (cm), and *T* is the radial growth of the fungus in the treated sample (cm).

Effect of Chemical Fungicide on Growth *T. harzianum* in vitro

The Czapek-Dox agar medium was supplemented with Carbomar at concentrations of 50 ppm, 100 ppm, and 150 ppm. *T. harzianum* (6-d old 5-mm mycelial disc taken from an actively growing culture) was cultivated at the center of a plate containing Czapek-Dox agar medium with the different concentrations of fungicide and incubated at 25 °C for 6 d. The medium without fungicide served as the control. The inhibition percentage of the fungus was calculated according to Eq. 1

Test of Antagonism by the Dual Culture Technique

Trichoderma harzianum was used to evaluate the biocontrol potential against *A. solani* via the dual culture technique on Czapek-Dox agar medium. Mycelial plugs (6-d old 5-mm mycelial disc taken from an actively growing culture) of *A. solani* and *T. harzianum* antagonists were placed 3 cm apart from each other on the same Czapek-Dox agar medium plate. Each plate received two discs, one with *T. harzianum* mycelium and the other with *A. solani* mycelium. The plates were incubated at 25 °C for 6 d. After the incubation period, the antagonistic activity and morphological changes were recorded.

Test Conditions for Enzyme Production by Fruit Rot from *A. solani*

The medium used for enzyme production was treated with *Trichoderma harzianum* filtered at three concentrations (25%, 50%, and 75%), a chemical fungicide at different concentrations (50 ppm, 100 ppm, and 150 ppm), and a dual culture of *T. harzianum*/*A. solani* discs (1/2; 2/2; and 1/2 discs, each disc containing 10⁶ spores). Media without any treatment were used as the control.

Carboxymethyl Cellulase Enzyme Production and Assay

Two 6-d old 5-mm mycelial discs taken from an actively growing culture of *A. solani* were cultivated in a medium consisting of 5.0 g/L NaNO₃, 2.0 g/L yeast extract, 1.0 g/L KH₂PO₄, 0.5 g/L MgSO₄·7H₂O, and 0.001 g/L FeCl₃, with the addition of 1% carboxymethyl cellulose (CMCase) (Deacon 1985) and were incubated for 7 d at 25°C. At the end of the incubation period, the medium in each flask was filtered through Whatman No. 1 filter paper and the culture filtrate served as the enzyme solution. The CMCase (β-1,4-endoglucanase) activity was determined using 1.5 mL of 3,5-dinitrosalicylic acid (DNSA) (Eveleigh *et al.* 2009). The enzyme activity was expressed in international units (IU), which is defined as the micromoles (μmol) of glucose released per min per mL of culture filtrate.

Pectinase Enzyme Production and its Assay

Two 6-d old 5-mm mycelial discs taken from an actively growing culture of *A. solani* were cultivated in a medium consisting of 20 g/L NaNO₃, 1 g/L K₂HPO₄, 50 g/L MgSO₄, 50 g/L KCl, and 0.01 g/L FeSO₄, with the addition of 15% pectin and were incubated for 10 d at 25 °C (Mandels 1985). A 0.1M citrate buffer containing 1% pectin (pH =5.8, and 0.5 mL) was placed in a test tube and 0.5 mL of culture filtrate (obtained by filtration through Whatman No. 1 filter paper) was added for pectinase detection. The reaction mixture was incubated at 50 °C for 30 min and terminated by adding 1.5 mL of DNSA reagent. The mixture was then cooled at room temperature. The absorbance was read at 575 nm. The enzyme activity was expressed as the mmol glucose released per min·mL of culture filtrate. One unit (U) of pectinolytic activity is defined as the amount of enzyme that catalyzed the formation of 1μmol galacturonic acid (Minjares-Carranco *et al.* 1997).

RESULTS AND DISCUSSION

One fungus was isolated from the rotten tomato fruits obtained from the agricultural fields (Fig. 1A), and it was identified as *A. solani* (Fig. 1B). This assignment agreed with numerous studies (Oladiran and Iwu 1993; Chaerani and Voorrips 2006; Chaurasia *et al.*

2013). When the isolated fungus from the diseased samples was artificially inoculated on healthy tomato fruits, the fruits were found to be susceptible to the development of fruit rot, which was characterized by dark brown to black lesions with concentric rings. The present findings agreed with those of Devi (2017), who confirmed *A. solani* as a pathogen for fruit rot disease in tomatoes. During the regular survey of a local tomato growing field, it was observed that most of the tomato fruits suffered fruit rot disease caused by several fungi, and the highest frequency of occurrences was caused by *A. alternata*, followed by *A. solani* (Sajad *et al.* 2017).

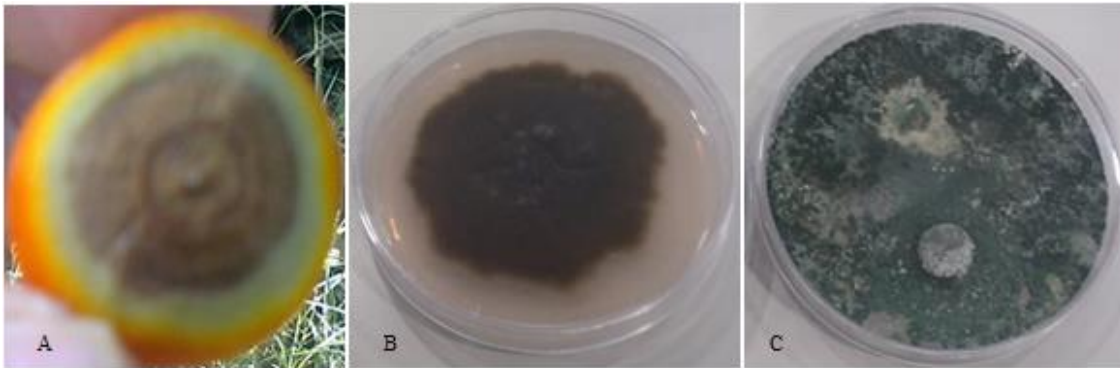


Fig. 1. (A) Tomato fruit rot by fungal pathogen; (B) *A. solani* pathogen isolate; and (C) dual culture technique of the biocontrol agent *T. harzianum* with *A. solani*

The obtained results indicated that the culture filtrates of antagonistic *T. harzianum* (Fig. 1C) gradually inhibited the radial growth of *A. solani* with an increasing filtrate concentration, but did not completely inhibit growth until a high filtrate percentage was reached (75% by volume), where there was 51.5% growth inhibition (Fig. 2). Generally, *Trichoderma* spp. are known to have the ability to produce some extracellular lytic enzymes that are involved in the process of antagonism against a variety of pathogenic organisms. Chohan *et al.* (2015) reported that *T. harzianum* and *T. viride* inhibited 67.8% and 59.6% of *A. solani* growth, respectively. Additionally, 79.5% of *A. porri* growth was inhibited by *T. harzianum* (Chethana *et al.* 2012). In contrast, the chemical fungicide inhibited 76.9% of *A. solani* growth (Fig. 2). To evaluate the using of chemical fungicide with the biocontrol agent *T. harzianum* against *A. solani*, these results in Fig. 3 confirmed that chemical fungicide affect the growth of the *T. harzianum*. Therefore, the combined application of filtrate metabolized medium of *T. harzianum* with the chemical fungicide is preferable than using *T. harzianum* spores.

In addition to the suppressive effect of the *T. harzianum* filtrate on *A. solani* growth, the microscopic study revealed that the *T. harzianum* culture filtrate changed the size, number, and shape of its conidiospores (Fig. 4), which are the conidial germination and sporulation of the *A. solani*. The chemical fungicide had a more deleterious effect on the morphological characteristics of *A. solani* than the *T. harzianum* filtrate, as the conidiospores were not developed and their longitudinal sections did not appear. Also, many chlamydospores were recorded as a result of fungicide treatment (Fig. 4). Kamala and Indira (2011) reported that *Trichoderma* isolates and their culture filtrates presented a high efficacy against phytopathogens. The present investigation indicated that *T. harzianum* was effective, and these results were in accordance with those of Ushamalini *et al.* (1997).

The antagonistic effects of *Trichoderma* spp. against pathogens might have been because of the production of non-volatile toxic/antibiotic substances that were diffused in the medium. These explanation agreement with other authors (Zhang *et al.* 2014; Zhang *et al.* 2016), reported that the influence on the microorganism development and inhibition it is associated with antibiotic production, as well as, volatile compounds.

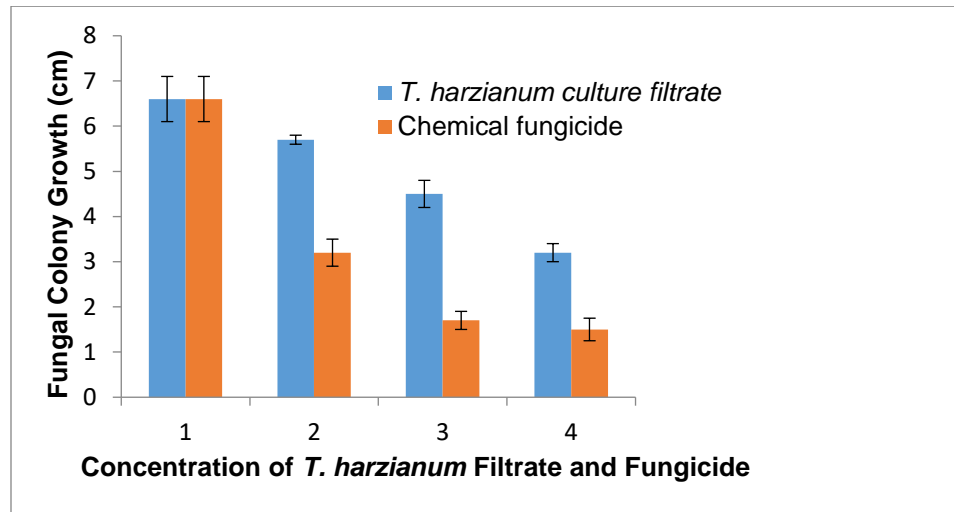


Fig. 2. Growth of *A. solani* treated by the *T. harzianum* culture filtrate (1: control; 2: 25%; 3: 50%; and 4: 75% v/v) and chemical fungicide (1: control; 2: 50 ppm; 3: 100 ppm; and 4: 150 ppm); the data is expressed as the average \pm standard deviation

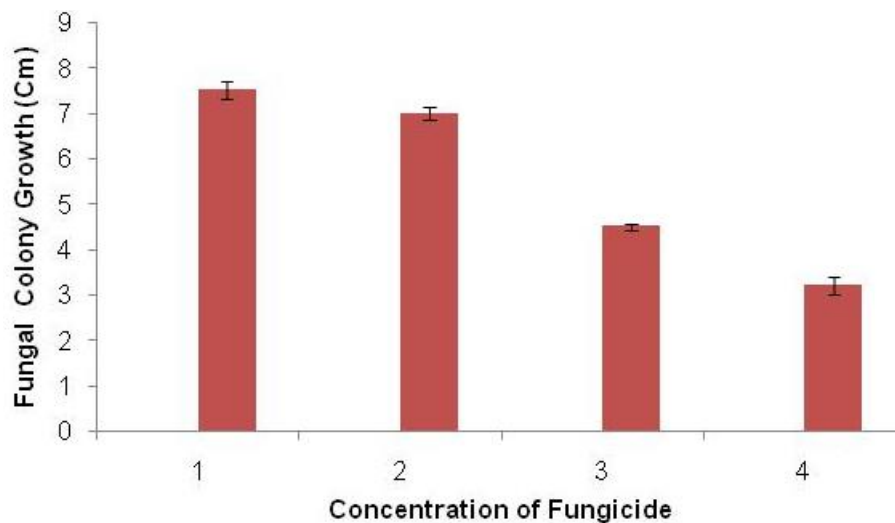


Fig. 3. Effect of chemical fungicide (1: control; 2: 50 ppm; 3: 100 ppm; and 4: 150 ppm) on growth of *T. harzianum*; the data are expressed as the average \pm standard deviation/

The results from the dual culture assay showed that *T. harzianum* inhibited the mycelial growth of *A. solani*. It actively grew as a single culture (Fig.1B), but the dual culture assays showed that *T. harzianum* was efficient at reducing the mycelial growth of the pathogen and it also sporulated on the pathogen colony (Fig. 1C).

The microscopic study of the dual culture assays revealed that the *A. solani* conidiospores were deformed compared with when it grew alone (Fig. 5).

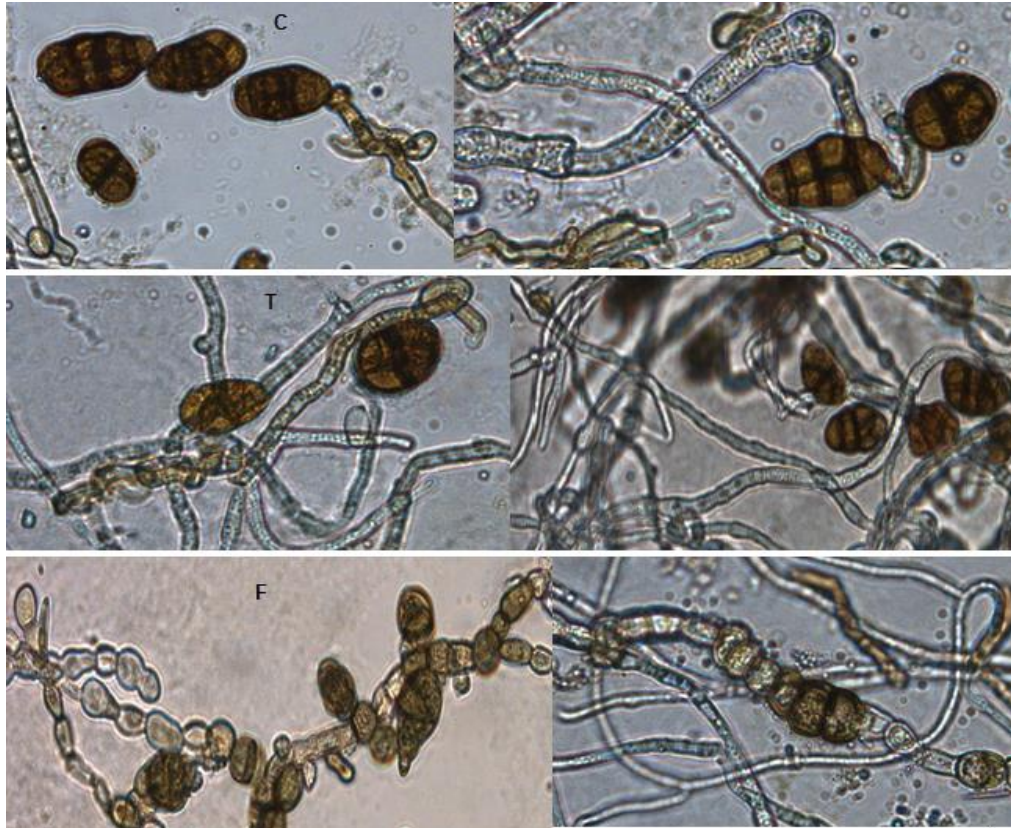


Fig. 4. Morphology of *A. solani* treated by the *T. harzianum* culture filtrate and chemical fungicide: C: control; T: 75% (by vol.) *T. harzianum* culture filtrate; and F: 150 ppm chemical fungicide

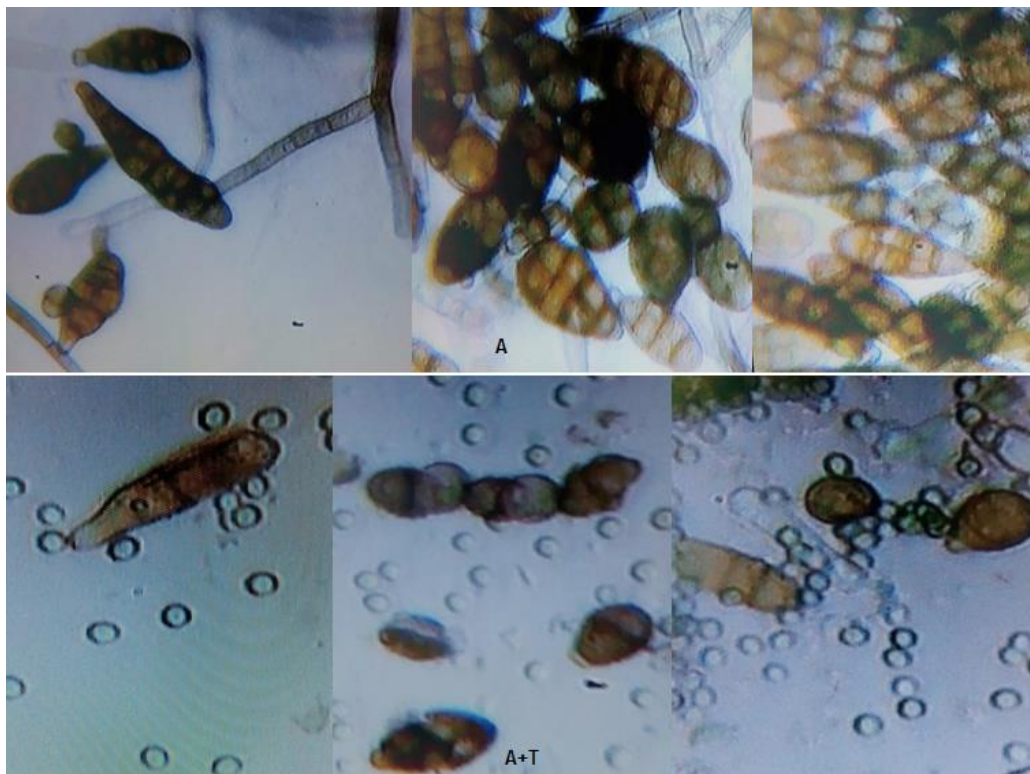


Fig. 5. Morphology of the *A. solani* (A) and of the dual culture of *T. harzianum* with *A. solani* (A+T)

The inhibition of β -glucan synthase activity in the pathogenic fungi by *Trichoderma* spp. has been reported in the literature (Fogliano *et al.* 2002), which prevents the rebuilding of the host cell wall. Recently, 12 isolates of *Trichoderma* spp. exhibited remarkable biocontrol potential against pathogenic fungi, including *T. harzianum* (Iftikhar *et al.* 2017). In the present investigation, *A. solani* produced both cellulolytic and pectinolytic enzymes *in vitro* (Figs. 6 and 7).

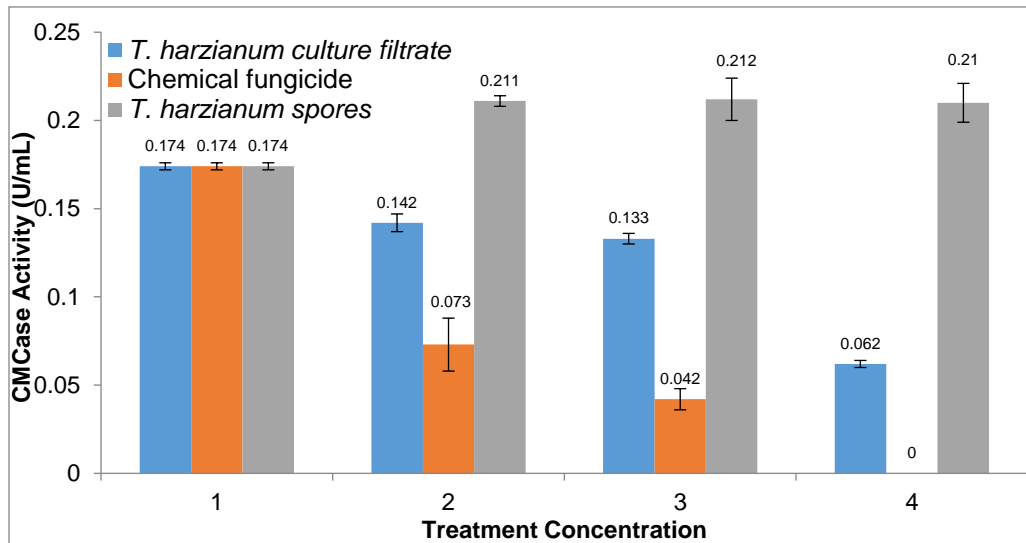


Fig. 6. CMCase activity of *A. solani* treated by the *T. harzianum* culture filtrate (1: control; 2: 25%; 3: 50%; and 4: 75%, by vol.), chemical fungicide (1: control; 2: 50 ppm; 3: 100 ppm; and 4: 150 ppm), and *T. harzianum* spores/*A. solani* spores (1: control; 2: 1/2; 3: 2/2; and 4: 1/2 discs); the data is expressed as the average \pm standard deviation

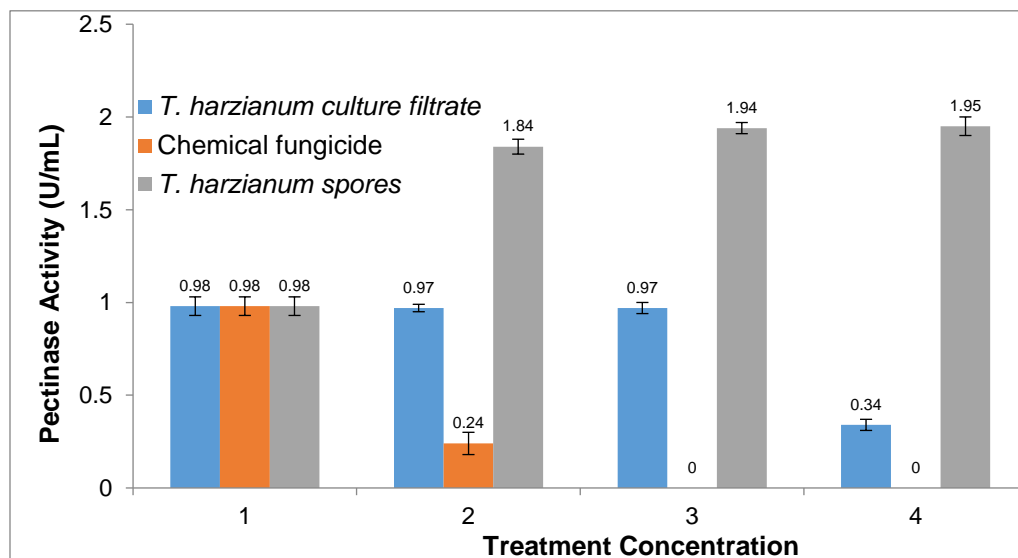


Fig. 7. Pectinase activity of *A. solani* treated by the *T. harzianum* culture filtrate (1: control; 2: 25%; 3: 50%; and 4: 75%, by vol.), chemical fungicide (1: control; 2: 50 ppm; 3: 100 ppm; and 4: 150 ppm), and *T. harzianum* spores/*A. solani* spores (1: control; 2: 1/2; 3: 2/2; and 4: 1/2 discs); the data is expressed as the average \pm standard deviation

It is widely accepted that the production of these enzymes is a major means by which these fungi invade the host tissue, which agrees with the experimental findings of Ramachandran and Kurup (2013), who showed that pectinase-producing fungi cause spoilage of fruits and vegetables. The activity of these enzymes decreased with an increase in the *T. harzianum* filtrate percentage and chemical fungicide concentration. *A. solani* failed to produce hydrolytic enzymes, particularly pectinase, at the high fungicide concentrations (100 ppm and 150 ppm). When *T. harzianum* was used as a biocontrol agent, the detected hydrolytic enzyme activities increased compared with the other treatments. The enzyme activities at different spore doses did not differ remarkably. The increase in the enzyme activities in these cases (*T. harzianum* co-cultured with *A. solani*) may have been because of *T. harzianum* activity, rather than the *A. solani*. This differed from the explanation of Smitha *et al.* (2017), who reported that hydrolytic enzyme assays of *T. viride* co-cultured with pathogens showed an increased activity of cellulase, chitinase, and pectinase over a monoculture, which confirmed the positive induction of enzyme secretion by *Pythium* spp., *Alternaria* spp., and *Fusarium* spp. The production and activity of pectinolytic and cellulolytic enzymes detected *in vitro* suggested their active role in the development of disease by *A. solani* in tomato fruits. An earlier study by Anand *et al.* (2008) confirmed the role of these enzymes in fruit rot pathogenicity.

CONCLUSIONS

1. Combined application of fungicide (methyl benzimidazole-2-ylcarbamate) with the *T. harzianum* metabolites were found to be more effective than its spores in controlling phytopathogens and its hydrolytic enzymes.
2. The *T. harzianum* metabolites were more effective than its spores at repressing hydrolytic enzymes.

Conflict of Interest

The authors declare that they have no conflict of interest.

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