
Research Article

In vitro assessment of protease production and stress tolerance of mutant isolates of *Trichoderma* sp.

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Abstract

Soil-borne plant pathogenic fungi cause serious losses in agricultural products. The antagonistic fungi for the control of plant diseases have increased efficiency and use of space has emerged as an alternative to other methods for the protection of agricultural products. One of the fungi used for this purpose is *Trichoderma* species. In this study mutant isolates of *Trichoderma* spp. were used. The resistance and protease enzyme activities of mutant isolates against abiotic factors such as temperature, drought and salinity were investigated. Mutant isolates of *Trichoderma* sp. were showed differed in tolerance to different abiotic stress factors. Protease enzyme activity produced by isolates was influenced by the tested abiotic factors. In the medium containing 30% PEG, the highest protease activity was determined in Tm13 isolate. Indigenous *Trichoderma* strains produced proteases in high temperature, drought and saline conditions. This indicates that isolates may be promising candidates in agricultural production.

Introduction

The use of chemical pesticides is very common among methods used to protect agricultural products from diseases. Fungicides and fumigants are used more than herbicides and insecticides in agricultural production (Ghanbarzadeh et al., 2014; Reetha et al., 2014; Schmoll, 2010). In 1969, the morphological characteristics of *Trichoderma* were divided into 9 genotypes; *T. harzianum* Rifaii, *T. viride*, *T. hamatum* (Bonord.) Bainier, *T. koningii* (Oudem.) Duche R. Helim, *T. polysporum* (Link) Rifaii, *T. piluliferum* J. Webster Rifaii, *T. aureo-viride* Rifaii, *T. longibrachiatum* Rifaii ve *T. pseudokoningii* Rifaii (Btaszczyk ve ark, 2014). The

research has focused on the identification of isolates resistant to high temperature and high salt concentration (Poosapati et al., 2014). *Trichoderma* isolates tolerated to drought and high temperature were identified from hot soils (Poosapati et al., 2014). In one study, temperature tolerant *Trichoderma* isolates were identified. Such isolates have been shown to be antagonistic to pathogenic microorganisms, which are resistant to temperature fluctuations caused by global warming (Poosapati et al., 2014). Ferre and Santamarina (2010) reported that temperature and water activity were effective in *T. harzianum* antagonism.

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Karmel-Reetha et al. (2014) and Mohamed and Haggag (2006) found that *T. harzianum* mutant isolates produced higher levels of chitinase, cellulase, β -glucuronase enzymes under salt stress conditions than non-mutant isolates. The researchers reported that mutant isolates showed growth and formed spores in medium containing 69 mM NaCl.

The isolates mutated with UV rays were found to have higher sporulation abilities with the survival time to the soil than the natural isolates. It has been reported that *Trichoderma* mutant isolates have inhibited the diseases caused by *Pythium aphanidermatum* and *Sclerotium rolfsii* in tobacco, *M. phaseolina* in sunflower (Rao et al., 2015; Reetha et al., 2014). These isolates were found to be more successful than natural isolates in preventing the growth of *Sclerotium* sp., agent of white root rot disease (Mukherjee and Raghu, 1997; Qualhato et al., 2013; Rao et al., 2015). Szekeres et al. (2004) found that the ultraviolet-exposed *Trichoderma harzianum* T334 isolate increased the biocontrol activity, while the mutant isolate secreted a high amount of protease enzyme compared to the non-mutant. Rey et al. (2001) found that the mutant isolate of *Trichoderma harzianum* was effective in inhibiting the growth of *Botrytis cinerea*. *T. harzianum* mutant isolate produced four times more enzymes, and it was highly inhibited by the pathogen growth (Rey et al., 2001; Qualhato et al., 2013).

Trichoderma species have been reported to be the best protease producers (Alamri et al., 2015; Gajera and Vakharia, 2012; Kumari et al., 2012). Extracellular protease production potentials of *Trichoderma* isolates have been investigated. Researchers have found that the produced protease is altered by isolation (Szekeres et al., 2004).

Successful biological control is achieved with antagonistic microorganisms that reduce the activity of plant pathogens (Rao et al., 2015). Antagonistic property of *Trichoderma* spp., which involves direct micoparasitism involving the production of enzymes that break down the cell wall of the pathogenic fungus (Lorito et al., 2010; Qualhato et al., 2013).

The mutant isolates of *Trichoderma* related studies in Turkey is far from each other and

quite a few. Importance of organic agriculture and environmental health issues brought the preparations prepared from microorganisms to the agenda. For this purpose, in our study, mutant isolates were obtained by exposure to ultraviolet light 18 isolate of *Trichoderma* sp. previously isolated from in field soil Şanlıurfa, Turkey.

Materials and Methods

Isolates were taken from Harran University, Microbiology laboratory. All isolates were stored in Potato Dextrose Agar (PDA) at 4 °C.

Resistance to abiotic stress conditions of isolates

Resistance of the isolates to different stress conditions (temperature, salinity, drought) were investigated. To determine the tolerance to temperature; 5 mm discs from cultures (48 hours) were inoculated into the PDA medium and incubated at 25, 30, 35, 45 and 50°C for 5 days (Mukherjee and Raghu 1997). To determine salt tolerance; 70, 150, 240, 300 and 350 mM NaCl were added to the PDA medium, 5 mm diameter discs of the isolates were inoculated into the media and incubated for 5 days at 30°C (Abdel-Latif et al., 2005). To determine tolerance to drought; 10, 20, 30, 35 and 40% polyethylene glycol (PEG, 6000 Da) was added separately to the PDA medium. The prepared media were autoclaved. Petri is poured into boxes. The 5 mm diameter discs of the isolates were inoculated and incubated for 5 days at 30 °C (Amalraj et al., 2011). In all experiments, the mycelial growth of isolates was measured and the effect of stress factors was determined.

Protease production of isolates

Azocasein prepared with 50 mM Tris HCl (pH 8.1) was added onto 3 mm disks of the isolates on the PDA medium. The content was incubated for 1 hour at 27 °C. At the end of incubation, 300 μ l of trichloroacetic acid (10% w v⁻¹) was added and the reaction was terminated (Lopez-Mondejar et al., 2011). The contents were centrifuged at 15000 g for 10 minutes. Supernatant (350 μ l) and 1 M NaOH (300 μ l) were mixed and the centrifuged at 15000 g for 10 minutes. The content was measured at 440 nm against the standard (Gajera and Vakharia,

2012). Protease activity was calculated as U ml⁻¹. The experiment was done in 3 replicates.

Protein measurement

Protein measurement was performed according to the Coomassie brilliant blue G-250 method (Bradford, 1976). To determine the effect of temperature, salinity and drought on the enzyme; the best growth values of the isolates were selected.

Results and Discussion

In our study, the resistance of our isolates to NaCl at different concentrations was investi-

gated. Isolates were developed in medium containing 70 mM NaCl. In the medium containing 240 mM NaCl; the growth of Tm14 isolate was reduced by 28.5%, Tm15 was reduced by 45.1%, Tm16 isolate was reduced by 16.8% and Tm18 was reduced by 15.3%. Tm5, Tm8, Tm11, Tm13 and Tm17 isolates were not affected from 240 mM NaCl. In the medium containing 300 mM NaCl; was determined to be the most inhibited Tm7 (246.1%) isolate, this was followed by Tm17 (172.7%) and Tm15 (114.3%). Tm8 was observed as the most resistant isolate to 300 mM NaCl (Figure 1).

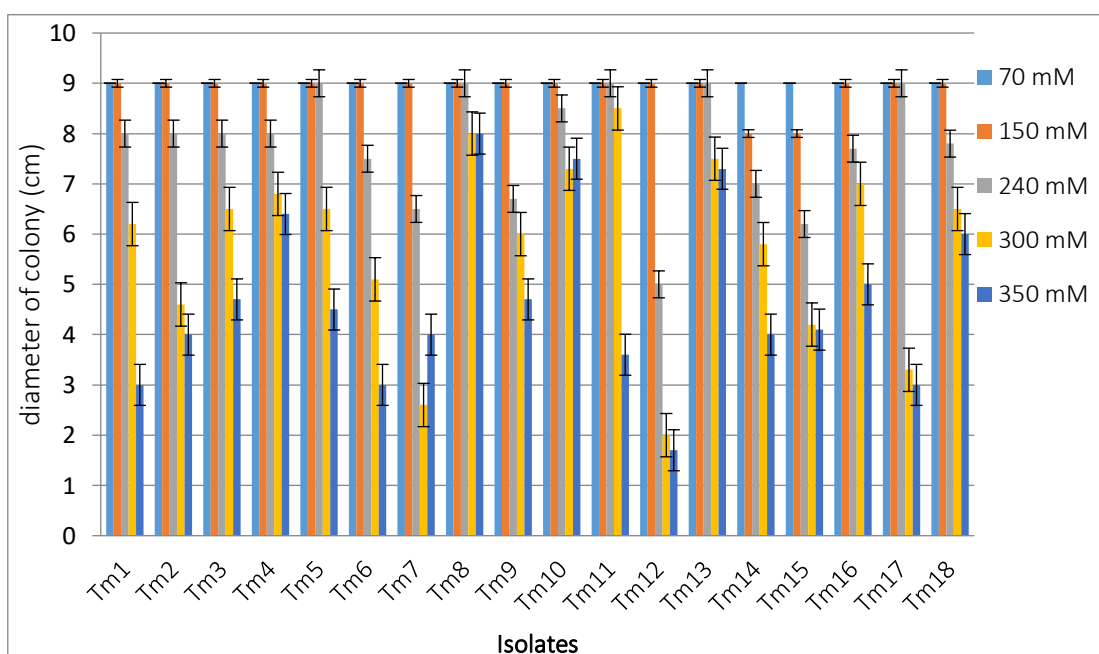


Figure 1. Tolerances of isolates to different concentrations of NaCl

In the medium containing 350 mM NaCl, the most resistant isolate was Tm8 (Figure 1) and the most sensitive isolate was identified as Tm12 (Figure 1). Mohammed and Haggag (2006) found that *Trichoderma harzianum* mutant isolates produced spores rapidly in medium containing 69 mM NaCl and *T. harzianum* mutant isolates produced gliotoxin, gliovirin and trichodermin antibiotics under salt stress. Researchers have also found that mutant isolates are also resistant to salt stress; chitinase, cellulase and β -galactosidase enzyme activities are producers. The rapid micelle formation of

our isolates in media containing 70 mM and 150 mM NaCl is similar to the findings of researchers. Regraui and Lahlou (2005) reported that salinity was the most important environmental factor limiting the antagonistic activity of *Trichoderma* species. In our study, it was determined that mycelial growth of isolates were not affected at 25 °C, 30 °C, and 35 °C (Figure 2). All isolates grew at 45 °C. The colony growths of the isolates were decreased at 50 °C. The isolates most affected by temperature at 50 °C are Tm1, Tm10, Tm13, Tm4, Tm11, Tm8, Tm6 and Tm17, respectively (Figure 2).

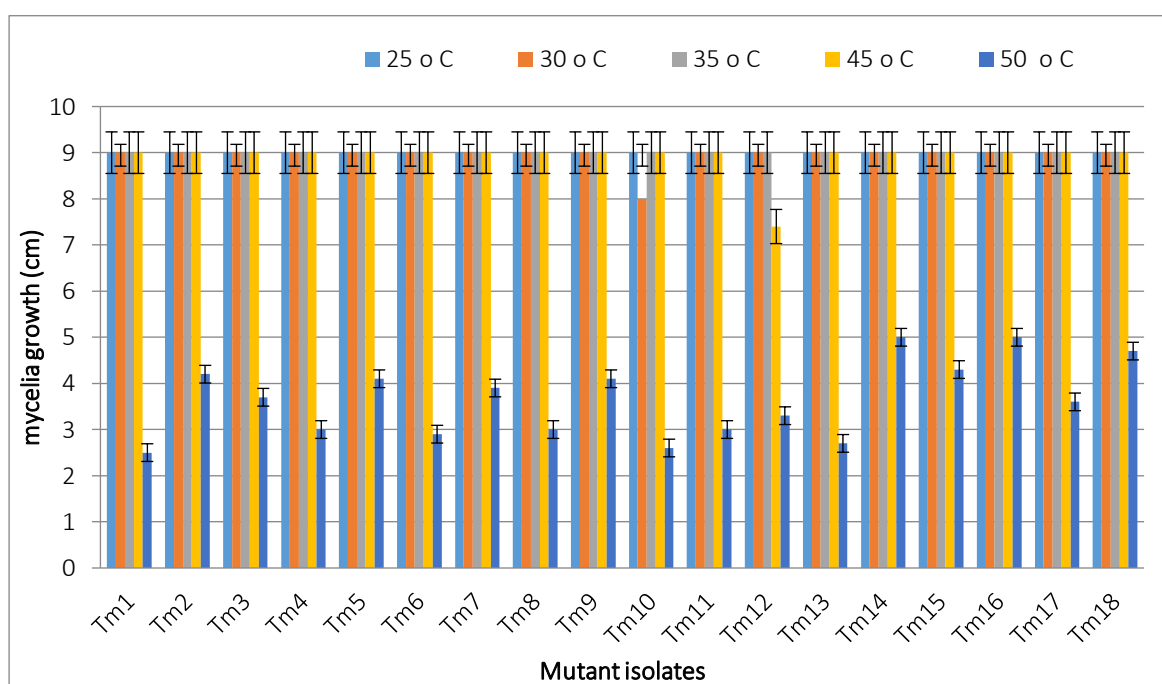


Figure 2. Tolerance of isolates to different temperatures

Nikolajeva et al. (2012) found that the effect of temperature on the growth of different *Trichoderma* isolates is important. It has been reported that *T. polysporum* is a psychrophile *Trichoderma* species (Begoude et al., 2007) and *T. polysporum* has been shown to grow rapidly at 4 °C (Nikolajeva et al., 2012). In our study, our isolates grew rapidly at 30, 35 °C, 45 °C (Figure 2). They grew at 50 °C. Since our region has hot climates, isolates are isolated from agricultural fields in Şanlıurfa, Turkey and therefore our isolates are adhered to high temperature and tested high temperature values are considered to be tolerant.

Amalraj et al. (2010) reported that the optimal temperature for the growth of isolates of *Trichoderma asperellum* is 30 °C. Similar results have been reported in other *Trichoderma* species (Begoude et al., 2007). Mukherjee and Raghu (1997) investigated that the biocontrol effect of *Trichoderma* spp. on *Sclerotium rolfsii* and *Trichoderma* spp. was showed the optimum activity at 25-30 °C. Begoude et al. (2007) reported that the optimal temperature for the growth of *Trichoderma asperellum* is 30 °C. In our work, the tested temperature values temperatures (25 °C, 30 °C, 35 °C, and 45 °C) did not limit the growth of our isolates. The rapid

growth of our isolates was at 25 °C and 30 °C and this data is also supported by researchers' work. At 50 °C, our isolates produced micelles, but the formation of spores was reduced (Figure 2). Poosapati et al. (2014) observed that the growth of *Trichoderma* isolates did not have a negative effect in 35 °C. In our study, all of our isolates showed growth at 35 °C. Poosapati et al. (2014)'s work supports our work.

One of the most important limiting factors in the growth of *Trichoderma* species used as biofungicides has been described as drought (Amalraj et al., 2010; Mishra et al., 2016). It has been reported that the amount of water present in the environment and substrate in medium is very important for fungal growth (Begoude et al., 2007). Begoude et al. (2007) found that *Trichoderma asperellum* was resistant to drought. Tm2 and Tm13 from our isolates were found to be resistant to all tested drought conditions. Mycelial growths of Tm2 and Tm13 isolates decreased at increasing PEG concentration. *Trichoderma* species have been used as potential biocontrol agents against agricultural pathogens since the last 20-30 years.

Qualhato et al. (2013) reported that extra-cellular enzyme systems important for competition and mycoparasitism and produced by

Trichoderma isolates are active even in conditions not suitable for mycelial growth. There are many mechanisms involved in *Trichoderma* antagonism. In mycoparasitism; *Trichoderma* sp. isolates directly inhibited the growth of plant pathogen by secreting lytic enzymes such as chitinase, β -1,3-gluconase and protease (Mendoza et al., 2015). Since cell walls of pathogenic fungi contain chitin, glucan and pro-

teins, enzymes secreted in the presence of successful antagonist play an important role in the degradation of the cell wall of the pathogen (Lorito et al., 2010). Filamentous fungal cell walls were contain lipids and proteins. Thus, the protease synthesized by the antagonist fungus has been shown to destroy the host cell fungal pathogen (Lorito et al. 2010; Srivastova et al., 2015).

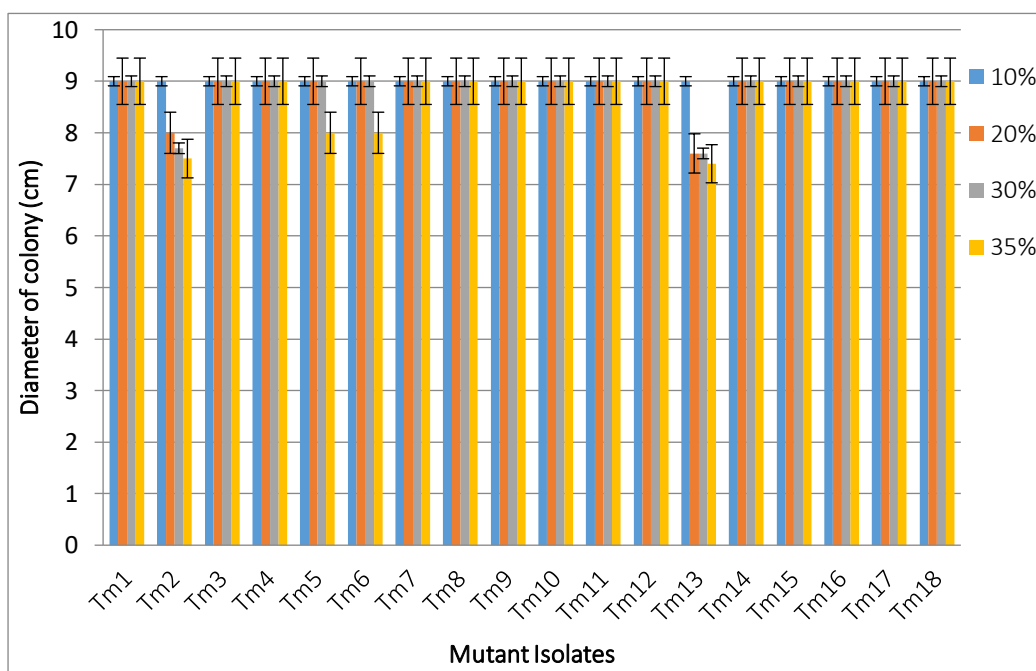


Figure 3. Tolerances of isolates to different PEG concentrations (drought)

Protease activities have been investigated taking into account stress factors that our isolates have best growth. Since all isolates showed growth at 240 mM NaCl, 30% PEG and 45 °C, protease activity was also examined at these values. Protease activities were investigated growth in media containing 240 mM NaCl, 30% PEG and 45 °C, respectively. The highest protease activity was obtained from the Tm15 isolate (74 U ml⁻¹). This was followed by isolates Tm16 (35.8 U ml⁻¹) and Tm17 (28 U

ml⁻¹). The protease activity produced by the isolates varied between 12.8-74 U ml⁻¹. The lowest activity was examined in the Tm9 isolate (Figure 3). In the salt-containing medium, the protease activity produced by the isolates varies according to isolation. The % reduction in protease activity when compared to control was observed in Tm5 isolate. Tm5 isolates were followed by Tm16, Tm3, and Tm2 isolates, respectively.

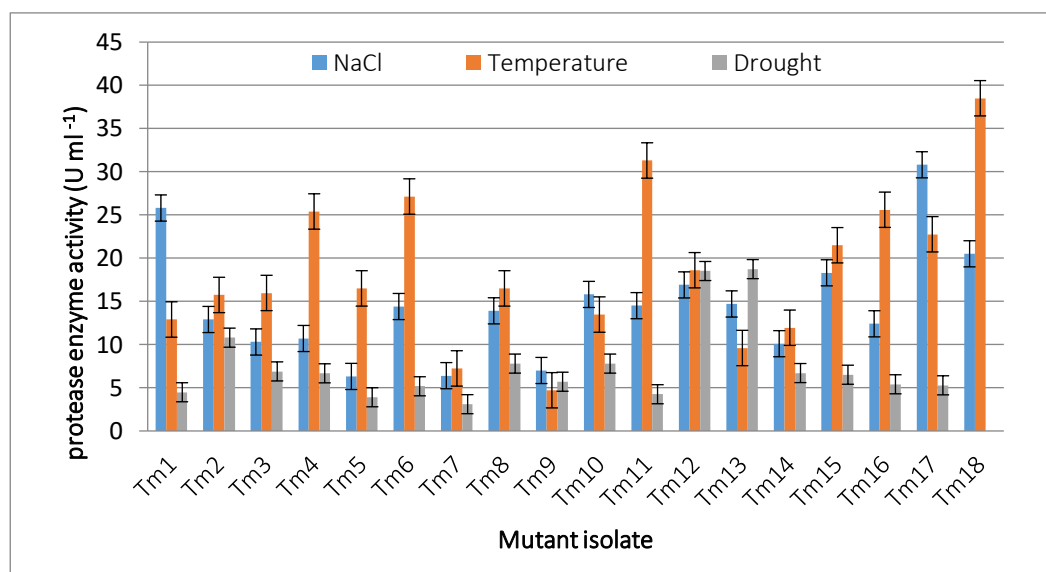


Figure 4. Protease activity of mutant isolates in different stress conditions

When 30% PEG was added to the medium, the protease activity produced by the isolates varied between 3.1-18.73 U ml⁻¹. The highest activity was taken from the Tm13 isolate under conditions where the drought was applied (Figure 4). Compared to control, the protease activity was decreased in Tm15, Tm16, Tm10, Tm1 and Tm5 isolates, respectively. When compared with control, Tm12 isolates were least affected by stress factors. In our study where heat is applied as a stress factor; the protease activity produced by the isolates ranged from 4.71 to 38.5 U ml⁻¹ (Figure 4). Nikolajeva et al. (2012) investigated that the effects of different temperature values on protease activity produced by *Trichoderma harzianum* performed. Investigators have demonstrated that the optimal temperature for the highest protease activity secreted by *T. harzianum* is 30-40 °C and the highest protease activity secreted by *T. flavus* is examined at 20-40 °C. The same investigators have found that the growth of *Botrytis cinerea* in the pod leaves is inhibited by the protease enzyme secreted by *T. flavus* and *T. harzianum*.

In the study of the temperature effect, the highest protease activity was taken from the Tm18 isolate, while the lowest activity was determined in the Tm9 isolate (Figure 4). When compared to control, the maximum % decrease in protease activity was determined at Tm15

and Tm10, respectively. Compared to control in protease production, Tm4 is least affected by temperature. Different responses of the isolates to the applied stress factors can be thought of as different isolates.

Conclusion

As a result mutant isolates tested in our study were identified as potential isolates of abiotic stress factors. For Sanliurfa, Turkey and similar climatic regions, farmers can be an economically viable alternative to disease control. Additional work needs to be done to assess our isolates for the protection of agricultural crops in high temperature, drought and salty soils. The rapid growth of our isolates, their ability to produce protease enzyme in high temperature, drought and saline conditions suggests that they may be promising candidates for use as biocontrol agents.

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Conflict of Interest

All authors declare that: there are no other relationships or activities that could appear to have influenced the submitted work.

Authors Contribution

TG (Post Graduate Student) conducted experiments and recorded observations, data analysis and references, conducted manuscript proofreading before submission. ÇK (Professor of Microbiology) conceived the idea, about the laboratory technique and supervised the experiment and wrote the concept and discussion. All authors read and approved the final version of the manuscript.

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