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Pythium aphanidermatum Suppression by Antagonistic Action of *Trichoderma longibrachiatum**

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Abstract

Excessive use of pesticides represents a growing problem in agriculture sustainability and food safety. The use of antagonistic fungal interactions represents a promising approach to achieve reduced pesticide input. Beneficial saprotrophic fungi from the genus *Trichoredma* are able to express different mechanisms of antagonistic activity and effectively suppress plant pathogens. The aim of the present research was to investigate the potential use of the antagonistic effect of *T. longibrachiatum* 10/5 strain against the plant pathogen *Pythium aphanidermatum*. The antagonistic activity of *T. longibrachiatum* 10/5 strain against *P. aphanidermatum* was tested in vitro by dual culture test. A glasshouse trial was conducted with cucumber plants grown on commercial substrate. Ten plants per container were inoculated at the cotyledon growth stage with *P. aphanidermatum* mycelium. The experiment was conducted in five replications. The number of inoculated plants without symptoms per treatment was recorded. *P. aphanidermatum* expressed asymmetrical growth in the dual culture test, and the colony diameter was reduced by 77% in comparison to the control. Sterile liquid culture filtrate of *T. longibrachiatum* 10/5 showed 50% efficiency in the suppression of cucumber damping-off caused by *P. aphanidermatum*. Metabolites produced by *T. longibrachiatum* 10/5 showed a suppressive effect on *P. aphanidermatum* damping-off, which has a potential in application in sustainable agriculture.
Key words: *Trichoderma longibrachiatum*, *Pythium aphanidermatum*, antagonism, extracellular metabolites, disease suppression

Резюме

Екстензивната употреба на пестициди е нарастващ проблем за устойчивото земеделие и безопасността на храните. Прилагането на антагонистичните взаимоотношения между фунги е обещаващ подход за намаляване на приноса на пестицидите. Полезните сапрофитни фунги от род *Trichoredma* могат да експресират антагонистична активност с различни механизми и ефектовно подтискат растителните патогени. Цел на настоящото изследване е да се проучи потенциалната употреба на антагонистичния ефект на щам *T. longibrachiatum* 10/5 срещу растителния патоген *Pythium aphanidermatum*. Антагонистичната активност на щам *T. longibrachiatum* 10/5 срещу *P. aphanidermatum* беше проверена *in vitro* чрез двойно култивиране. Тестовете в оранжерия бяха проведени с краставици, култивирани върху търговски субстрат. По десет растения в контейнер бяха инокулирани на етапа на котиледон с мицел на *P. aphanidermatum*. Експеримента проведохме с пет повторения. Регистрирахме броя растения без симптоми след третиране. *P. aphanidermatum* показа асиметричен растеж при теста с двойно култивиране и диаметърът на колониите беше редуциран 77% в сравнение с контролата. Стерилен филтрат на *T. longibrachiatum* 10/5 показа 50% ефикасност в подтискането на изсъхването на краставиците, причинено от *P. aphanidermatum*. Метаболитните продукти от *T. longibrachiatum* 10/5 показаха подтискащ ефект върху изсъхването, причинено от *P. aphanidermatum*, което сочи потенциал за приложение в устойчивото земеделие.

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Introduction

Raising requirements for agricultural sustainability and reduction of environmental and human health risk has indicated the need for a decrease in pesticide input. In addition, excessive use of pesticides leads to pest resistance development and has a negative impact on the whole agro-biodiversity (Sharma, 2012). The use of antagonistic fungal interactions represents a promising approach to achieving reduced pesticide input. These interactions occur naturally in suppressive substrates (Kinkel *et al.*, 2011), but their effects can be enhanced with soil inoculation practices or use of some antagonists' bioactive metabolites. *Trichoderma* members are known as the most effective plant pathogen biocontrol agents, due to their fast growth and competitive abilities, production of different kinds of antifungal metabolites and mycoparasitism (Klein and Eveleigh, 1998). Selected *Trichoderma* strains are able to suppress numerous plant pathogens such as *Pythium*, *Fusarium*, *Sclerotinia*, *Botrytisan and Rhizoctonia* sp. (Benítez *et al*., 2004).

Pythium aphanidermatum is one of the most important soilborne pathogen, which threatens plants at the earliest stages of development, and causes severe damping-off in many crops worldwide (Dick, 1990). It occurs primarily in cold and wet soils where young seedlings of directly seeded crops may be killed before or soon after their emergence (Watson *et al.*, 1992). The pathogen causes seed and seedling diseases in bedding plants, as well as in greenhouse-grown transplants (Abbasi and Lazarovits, 2006). In addition, roots of mature plants may also be attacked (Zitter *et al.*, 1996; Al-Sa'di *et al.*, 2007).

In the present work, we tested the potential of extracellular metabolites of *Trichoderma longibrachiatum* 10/5 strain to suppress cucumber damping-off caused by *P. aphanidermatum*. *T. longibrachiatum* 10/5 strain originating from compost has been selected as a potent plant pathogen antagonist in previous studies (Jovičić-Petrović *et al*., 2012; Jovičić-Petrović and Raičević, 2015). The results of previous research showed that *T. longibrachiatum* 10/5 exhibited some indirect mechanism of antagonistic action related to the production of some extracellular metabolites (Jovičić-Petrović and Raičević, 2015). In addition, it was shown that those bioactive extracellular metabolites are thermostable, and their effect was examined in this study.

The aim of the present research was to consider the potential application of the antagonistic effect of *T. longibrachiatum* 10/5 strain against the plant pathogen *P. aphanidermatum*.

Material and Methods

Fungal cultures

T. longibrachiatum 10/5 belongs to the collection of Department for Environmental Microbiology, Faculty of Agriculture, Belgrade. The strain was isolated from composted agro-industrial waste, and defined as a potential biocontrol agent (Jovicic-Petrovic, 2014; Jovicic-Petrovic and Raicevic, 2015). The culture of *P. aphanidermatum* used in this study is a part of the collection of The Institute of Pesticides and Environmental Protection.

Dual culture test – in vitro antagonistic activity

T. longibrachiatum 10/5 and *P. aphanidermatum* were precultured on Potato Dextrose Agar (PDA, Merck, Germany) for three days. Mycelia obtained from the edge of the formed colonies were used for dual culture test. Five-mm-diameter mycelial disks of *T. longibrachiatum* 10/5 and *P. aphanidermatum* were placed on PDA plate 3 cm apart. The experiment was performed in three replicates, and plates were incubated in dark at 25°C for 3 days. Evaluation of the fungal interaction was done by inhibition percentage calculation. The diameter of *P. aphanidermatum* colony was measured and compared with the colony diameter of the same fungi, grown in the pure culture under the same conditions.

Inhibition percentage was calculated as:

$$\text{inhibition percentage (\%)} = \frac{(Dc - Da)}{Dc} \times 100$$

Dc – *P. aphanidermatum* colony diameter in the control Petri dish

Da – *P. aphanidermatum* colony diameter in the dual culture plate

In vivo trial

Preparation of *P. aphanidermatum* inoculim. Inoculum of *P. aphanidermatum* was prepared on wheat grains using the procedure described by Chellemi *et al*. (2000). A mixture of 25 ml of deionized water and 20 g of wheat grains was allowed to soak for 24 h in each of two 250-ml flasks. The flasks were then autoclaved twice on two consecutive days. Each flask was inoculated with five 5-mm disks cut from a 2-day-old culture grown on PDA (potato dextrose agar) medium. The flasks were incubated for 2 to 4 weeks in the dark at 25°C and shaken periodically to ensure uniform growth of the inoculum. Then, the inoculum was mixed thoroughly with substrate at the rate of 2% and then put

in pots (Chellemi *et al.,* 2006).

Preparation of sterile liquid culture filtrate of T. longibrachiatum 10/5 (SLCF)

SLCF of *T. longibrachiatum* 10/5 was prepared by culturing the isolate in potato dextrose broth (Merck, Germany) for seven days at 25°C and 160 rpm in horizontal shaker-incubator (Biosan, Latvia). After incubation, the culture was filtrated through cheesecloth. The supernatant was collected and autoclaved at 121° for 15 min.

Glasshouse trial

One-week-old cucumber plants, grown in 60-cell polystyrene trays, were transplanted into 10 cm × 5 cm pots filled with 400 ml of sterile growth substrate (Floragard®, Germany). After inoculation, plants were treated with 100 ml of: water (positive control), SLCF (the effect of *T. logibrachiatum* extracellular metabolites) and propamocarb hydrochloride (Bayer Cropscience, Germany). Plants grown without addition of *P. aphanidermatum* inoculum were also treated with 100 ml of water per each container represented the negative control (NC).

The pots were kept in a greenhouse (20±2°C) and regularly watered to ensure good development of seedlings. The seedlings were scored as either healthy or infected 7 days after SLCF and fungicide application. Symptoms ranged from brown necrotic lesions at the collar to collapse and death (EPPO, 2004).

Statistical analysis

The experimental design was a complete randomized block with five replicates per treatment and one pot with 10 seedlings per replicate. The experiment was conducted twice (EPPO, 2004). The data were analyzed using ANOVA and the means were separated by Duncan's multiple range test. The efficacy was calculated using Abbott's formula.

Results and Discussion

Diseases caused by *Pythium* spp. are leading to significant losses in glasshouse production where *P. aphanidermatum* is one of the most abundant plant pathogen. *P. aphanidermatum* proved to be sensitive to competition and antagonism during its saprotrophic stage, which represents a key factor in biological control possibilities (Martin and Loper, 1999). However, disease control practices in glasshouses showed much better results than in the field, and biocontrol has already gained success in *P. aphanidermatum* suppression (Paulitz and Bélanger, 2001). Compost addition has also given significant results in the suppression of *P. aphanidermatum*. Hadar *et al*. (1992) reported suppression of *Pythum* dumping-off of up to 90% by compost addition to soil. Many studies show that antagonistic interactions between plant pathogens and compost saprotrophic microflora represent one of the mechanisms of disease suppression by compost amendment (Van Elsas *et al.*, 2002; Kavroulakis *et al.*, 2006; Chen and Nelson, 2008; Pane *et al.*, 2011; Suárez-Estrella *et al.*, 2012).

The examined strain of *T. longibrachiatum* 10/5 is also a member of compost microflora and results indicate its potential for *P. aphanidermatum* inhibition. *P. aphanidermatum* showed asymmetrical growth in the confrontation test with *T. longibrachiatum* 10/5, and its colony diameter was reduced by an average of 77% in comparison to the control (Fig. 1). Those results are comparable with some other established biocontrol agents belonging to genus *Trichoderma*, such as *T. harzianum* and *T. viride* strains that showed inhibition percentages between 56.5 and 86.4% (Arunachalam and Sharma, 2012).

Knowing that *T. longibrachiatum* represents an opportunistic pathogen, soil inoculation may bring a certain health risk and its application in agriculture should be conducted with special care (Hatvani *et al.*, 2013). That was why the present study aimed to evaluate the suppressive effect of *T. longibrachiatum* 10/5 secondary metabolites contained in the SCLF.

Table 1 summarizes the results of the Pythium damping-off severity and the efficacy of SLCF of *T. longibrachiatum* 10/5 applied prior to inoculation. The SLCF of *T. longibrachiatum* 10/5 showed satisfactory efficiency of 50% in suppression of cucumber damping-off caused by *P. aphanidermatum*.

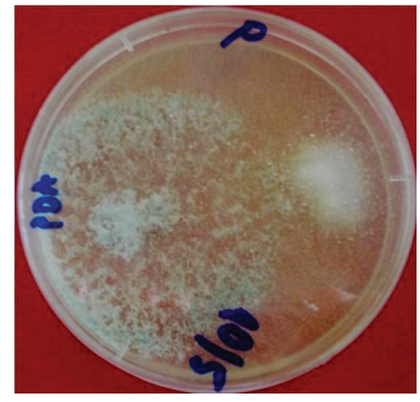

Fig. 1. Dual culture test: *T. longibrachiatum* 10/5 (left) and *P. aphanidermatum* (right)

Table 1. Effect of *T. longibrachiatum* sterile culture filtrate (SCLF) on *P. aphanidermatum in vivo*

Treatments	MS.	Sd.	Efficiency (% of plants without symptoms)
Negative control (NC)	0.0[a]	0.0	100
Positive control (PC)	9.4[c]	0.5	6
P. hydrochloride (P)	1.4[a]	1.1	86
Sterile liquid culture filtrate of *T. longibrachiatum* 10/5, SLCF	5.0[b]	2.0	50

The highest disease supression was recorded in cucumber plants treated with the synthetic fungicide propamocarb-hydrochloride (86%) compared to the inoculated untreated control (Figure 2).

Fig. 2. The effect of *T. longibrachiatum* sterile culture filtrate (SCLF) on *P. aphanidermatum*, *in vivo*: a. negative control; b. infected plants treated with SCLF; c. infected plants treated with propamocarb hydrochloride; d. positive control

The achieved efficiency of SCLF was significantly lower in comparison to the applied chemical pesticide, which showed a number of healthy plants comparable to the negative control. Further research is needed in order to examine the possibilities of application of SCLF in combination with a lower dose of pesticide aiming to increase efficiency.

Presented results indicate the role of thermostable extracellular metabolites of *T. longibrachiatum* in antagonistic interaction, although some other mechanisms are not excluded. Prapagdee *et al.* (2008) indicate that secondary metabolites of fungal antagonists of plant pathogens originating from the stationary growth phase affect the activity of the autoclaved culture, while filtrates obtained in the exponential phase of growth more often lose their activity after high-temperature treatment. *T. longibrachiatum* 10/5 extracellular metabolites retained their activity against *P. aphanidermatum* despite the treatment at high temperature that opens up the possibilities for application.

Conclusions

T. longibrachiatum 10/5 expresses a significant antagonistic activity towards *P. aphanidermatum*, *in vitro*. Also, *in vivo* experiments showed that thermostable extracellular metabolites produced by *T. longibrachiatum* 10/5 exerted a suppressive effect on *P. aphanidermatum* damping-off with the disease incidence reduced to half, which represents a potential for application in sustainable agriculture.

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