

ANTAGONISTIC EFFECTS OF *TRICHODERMA* SPECIES IN BIOCONTROL OF *ARMILLARIA MELLEA* IN FRUIT TREES IN IRAN

Mohammad Reza Asef¹, Ebrahim Mohammadi Goltapeh^{1*}
Younes Razaee Danesh¹

¹Department of Plant Pathology, College of Agriculture, Tarbiat Modares University
P.O.Box 14115-336, Tehran, Iran

²Department of Botany, Plant Pests and Diseases Research Institute
P.O. Box 145419395, Tehran, Iran

Received: January 16, 2008

Accepted: May 20, 2008

Abstract: Root and butt rot caused by species of *Armillaria* is one of the most serious diseases of fruit and forest trees in Iran. In this study, antagonistic effects of *Trichoderma* in biocontrol of *Armillaria* were investigated. *Armillaria mellea* was isolated from infected roots and butts of cherry and almond trees and identified with pairing tests method. *Trichoderma* species were recovered from rhizomorphs and around soil of *Armillaria* infected roots. *Trichoderma* species identified were *T. virens* (nine isolates) and *T. harzianum* (three isolates). *Trichoderma* discs were placed onto cultures of *Armillaria* to study antagonistic effects. All isolates of *Trichoderma* colonized *Armillaria* colonies within 5–7 days. Volatile compounds of *Trichoderma* isolates inhibited *Armillaria* colony growth and rhizomorph formation. Mechanisms of biocontrol were investigated by light and scanning electron microscopy, these included penetration of *Trichoderma* hyphae in rhizomorphs, colonization of rhizomorphs by *Trichoderma* mycelia, colonization of apex meristemic center and apical buds of rhizomorphs, sporulation of *Trichoderma* in outer and inner surface of rhizomorphs, degeneration and lysis of rhizomorph tissue, and discharge of rhizomorph content.

Key words: *Armillaria* root rot, biocontrol mechanisms, pairing tests, *Trichoderma harzianum*, *Trichoderma virens*.

INTRODUCTION

Fungi belonging to the genus *Armillaria* (Fr.:Fr.) Staude, cause root disease of trees and shrubs in forests, plantations, orchards and gardens throughout the world (Hood

*Corresponding address:
emgoltapeh@yahoo.com

et al. 1991). In Iran, *Armillaria* root disease was first reported on apple trees in 1956 (Ershad 1995). Since then, *Armillaria* has been reported in associated with other cultivated and forest tree species throughout the country and is a well known cause of root rot and decline of Oak in the Northwest and is associated with mortality of many economically important hardwoods such as almond and cherry (Asef and Mohammadi Goltapeh 2003).

From studies on biocontrol of *Armillaria* it can be concluded that the most thoroughly studied antagonists of *Armillaria* are *Trichoderma* species (Hagle and Shaw 1991). *Trichoderma* is a worldwide fungus and biocontrol agent in soils, plant residues and other substrates. After Weindling (1932) showed the antagonistic potential of *Trichoderma*, it has been extensively studied for antagonistic activity against different plant pathogens (Cook and Baker 1983; Papavizas 1985). Bliss (1951) demonstrated the ability of *Trichoderma* to replace *Armillaria* in artificially infected root segments of citrus, fumigated with carbon disulphide. Aytoun (1953) studied *in vitro* interactions of *Trichoderma* and *Armillaria* and concluded that *Trichoderma* must be considered as a possible agent in the control of *Armillaria*. Sokolov (1964) found that several fungi including *Trichoderma* antagonized *Armillaria* and recommended using *Trichoderma* as a biocontrol agent for *Armillaria*. Reaves *et al.* (1990) found isolates of *Trichoderma* species that were antagonist to *Armillaria ostoyae*, reducing colony growth and rhizomorph formation in culture. Dumas and Boyonoski (1992) investigated the myco-parasite mechanisms of *Trichoderma* species against rhizomorphs of *Armillaria gallica* using scanning electron microscopy. They observed events such as direct penetration, coiling of the *Trichoderma* hyphae around the *Armillaria* hyphae and disintegration of rhizomorph content. Onsando and Waudó (1994) found that different isolates of *T. longibrachiatum*, *T. koningi* and *T. harzianum* reduced the mycelium and rhizomorph growth of *Armillaria* infecting tea in Kenya. Raziq (2000) investigated the antagonistic effect of isolates of *T. harzianum*, *T. virens* and *T. hamatum* against *A. mellea*, and reported differences between *Trichoderma* isolates in antagonistic effects.

The objective in this study was to investigate the antagonistic effects of two *Trichoderma* species, isolated from soil and infected rhizomorphs, in biocontrol of *A. mellea* and their potential use as biocontrol in fruit trees in Iran.

MATERIALS AND METHODS

Cherry and almond trees with root and butt rot symptoms were identified in several orchards in Iran. *Armillaria* isolates were collected from these, and cultured onto malt extract agar (MEA) amended with benomyl, sulphate streptomycin or penicillin (Worral 1991). Species of *Armillaria* were identified by pairing tests method as has been described previously (Asef *et al.* 2003). *Trichoderma* species were isolated from rhizomorphs and rhizospheres of infected roots using the method of Davet (1979) and identified according to Rifai (1969), Domsch *et al.* (1980) and Bisset (1991). All isolates of *Trichoderma* and *Armillaria* were deposited in the culture collection of department of Plant Pathology, Tarbiat Modares University (Table 1).

For evaluation of colonization of *Armillaria* by *Trichoderma* in culture, 5-mm discs cut from *A. mellea* cultures, were placed at one side of plates of 2% MEA and incubated at $24 \pm 1^\circ\text{C}$. Twenty four to thirty days later, when *Armillaria* isolates had grown the entire surface of MEA medium, 5-mm discs of *Trichoderma* mycelium from the

margins of 4-day-old cultures were placed on the surface of the *Armillaria* colony near the center and plates were incubated at $24 \pm 1^\circ\text{C}$. The colonies were examined for type and rate of colonization 3–10 days later.

For study on interactions between *Trichoderma* hyphae and *A. mellea* rhizomorphs, a thin layer of 2% MEA was spread on sterile slides which were then placed in the middle of plates containing 2% MEA. The 5-mm disc of *A. mellea* were placed at the edge of plates and after 16–20 days, when *Armillaria* approached microscope slides, a 5-mm disc of *Trichoderma* was placed on the opposite side of the plate and then re-incubated at $24 \pm 1^\circ\text{C}$. Records of the interaction on the slide between the opposing colonies were made after a further 6–10 days using light and scanning electron microscopy (SEM). For SEM studies, samples were prepared using the methods of Dumas and Boyonoski (1992) and King and Brown (1983) with slight modification and examined with a Philips XL30 scanning electron microscope, operating at 20 KV.

Study of effects of volatile metabolites of *Trichoderma* spp., on *Armillaria* was carried out in accordance to Mohammadi Goltapeh and Danesh (2000) in two phases. Firstly, the 5-mm discs of *A. mellea* and *Trichoderma* spp. were placed on 2% MEA. The lids of the petri plates were removed and the bottoms of *A. mellea* were placed over *Trichoderma* bottoms and taped together by Parafilm. In the second phase, a disc of *A. mellea* was placed on MEA and after 14–16 days, when the colony had attained some growth and in first steps of rhizomorph formation, the lids of the petri plates were removed and the bottoms of *A. mellea* were placed over *Trichoderma* bottoms and taped together. In the control, *A. mellea* plates were placed over another MEA plates without *Trichoderma*. All of the plates were incubated at $25 \pm 1^\circ\text{C}$ and percentage inhibition was calculated by comparing growth using the following equation:

$$\text{Percentage inhibition (\%)} = \frac{\text{Colony growth rate in control} - \text{Colony growth rate in each treatment}}{\text{Colony growth rate in control}}$$

The data were analyzed in completely randomized design and results were grouped using Duncan's multiple range test (MSTAT-C computer program).

RESULTS

Identification of *Trichoderma* isolates showed that nine isolates were *T. virens* and three were *Trichoderma harzianum* (Table 1). Using pairing tests method, *Armillaria* isolates were compatible with test strains of Iranian *A. mellea* and were identified as *A. mellea*. In the colonization study, all isolates of *Trichoderma* colonized the surface of *Armillaria* colonies completely within 5–7 days.

Details of microscopic studies on the interaction of *Trichoderma* hyphae and *A. mellea* rhizomorphs revealed several different methods of colonization of *Armillaria* by *Trichoderma*.

Trichoderma hyphae developed in the surface of the medium and one or more hyphal branches penetrated the rhizomorphs directly, making penetration holes. The entrance hole of hyphae was observed in the surface of rhizomorphs using SEM. *Trichoderma* hyphae grew 10-mm towards the rhizomorphs in less than 40 h. After penetration and development of *Trichoderma* hyphae on rhizomorphs, the surface of rhi-

zomorphs was colonized and *Trichoderma* mycelium coiled around the rhizomorphs. Only one *Trichoderma* isolate (T1) sporulated in internal tissue of rhizomorphs, which was observed after the crushing of rhizomorphs. (Fig. 1 a–e).

Table 1. Characteristics of *Trichoderma* isolates used in this study

species	substrate	Isolate
T1	soil	<i>T. harzianum</i>
T2	soil	<i>T. virens</i>
T3	basidiocarp	<i>T. virens</i>
T4	basidiocarp	<i>T. virens</i>
T5	rhizomorph	<i>T. virens</i>
T6	rhizomorph	<i>T. virens</i>
T7	rhizomorph	<i>T. virens</i>
T8	rhizomorph	<i>T. harzianum</i>
T9	rhizomorph	<i>T. harzianum</i>
T10	basidiocarp	<i>T. virens</i>
T11	basidiocarp	<i>T. virens</i>
T12	soil	<i>T. virens</i>

The tip of rhizomorphs is an active meristemic center and all longitudinal growth of rhizomorphs is supported by the rhizomorph apex (Motta 1971). Therefore, colonization of apical center is very important. All isolates of *Trichoderma* penetrated to the rhizomorph tip and then colonized and sporulated. Also, *Trichoderma* mycelia colonized apical meristems of rhizomorphs that supported apical growth of rhizomorphs, similar to apex meristems (Fig. 1 f–j).

Two to three days after colonization of *A. mellea* rhizomorphs, the fungus sporulated and produced more conidiophores on the surface of rhizomorphs (Fig. 1 K, L) and (Fig. 2 a–f).

Ten to 15 days after inoculation of *Armillaria* colonies with *Trichoderma*, degeneration of the rhizomorphs was observed and rhizomorphs cracked longitudinally (Fig. 2 g, h); rhizomorphs and apical buds changed to only one hollow tube with transparent sheath (Fig. 2 i, j, k).

Results of effect of *Trichoderma* volatile metabolites showed that these compounds have high potential to control *A. mellea* growth. Comparing the results of inhibition of *Armillaria* growth with *Trichoderma* isolates showed that, there was significant difference between treatments and controls, but there was no difference among *Trichoderma* isolates (Fig. 3).

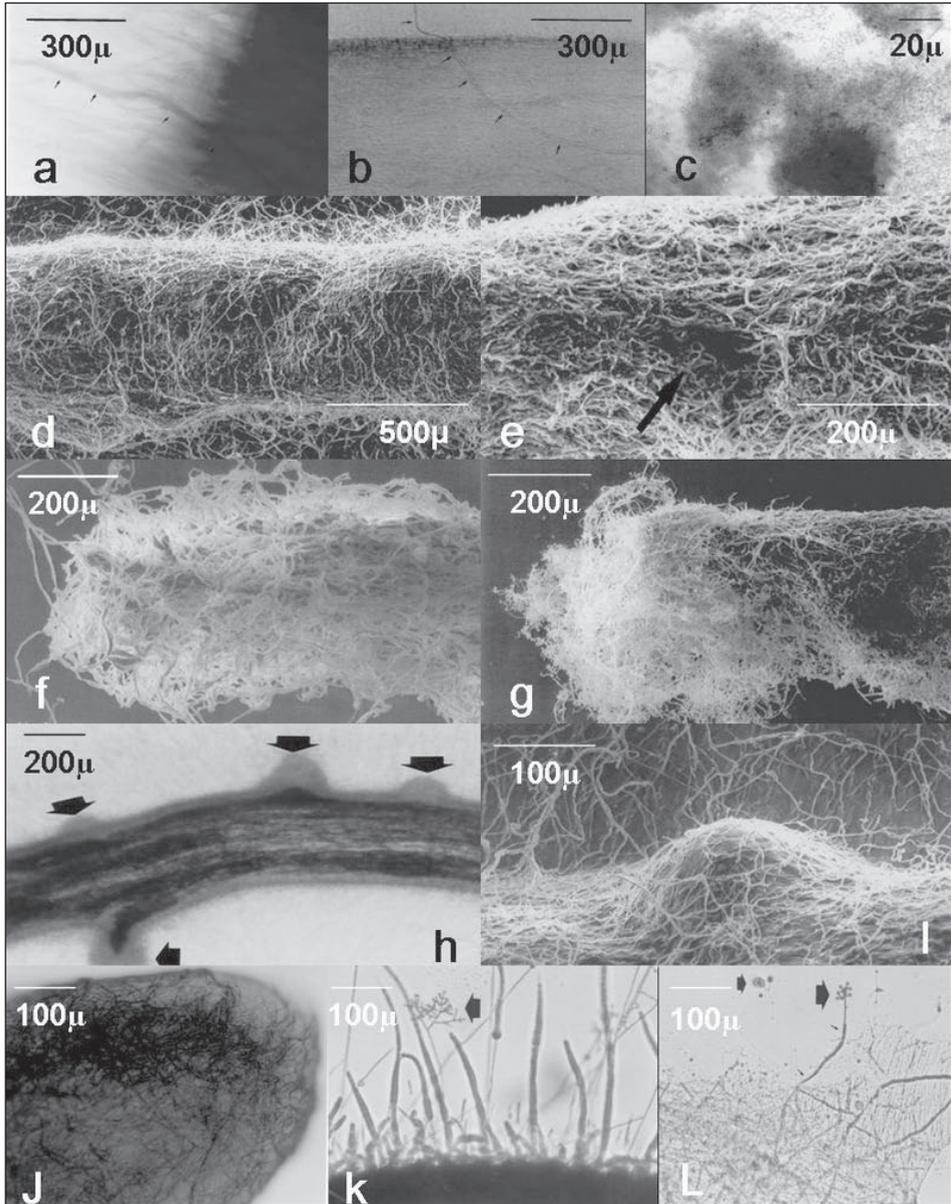


Fig. 1. (a), (b), Penetration of rhizomorph by hyphae (arrowhead) of *Trichoderma harzianum* (a) and *T. virens* (b). (c), Crushed rhizomorph, showing *Trichoderma* spore mass, produced internally in rhizomorph. (d), SEM of extensive growth of *T. virens* on the surface of a rhizomorph. (e), SEM of surface of the rhizomorph showing holes (arrowhead) where *Trichoderma* hyphae entered the rhizomorph. (f), (g), (J), Extensive growth and sporulation of *T. virens* and *T. harzianum* on the tip of rhizomorph. (h), (I), Colonization of apical buds of rhizomorph by *T. virens*. (K), (L), Sporulation of *T. harzianum* in surface of infected rhizomorph

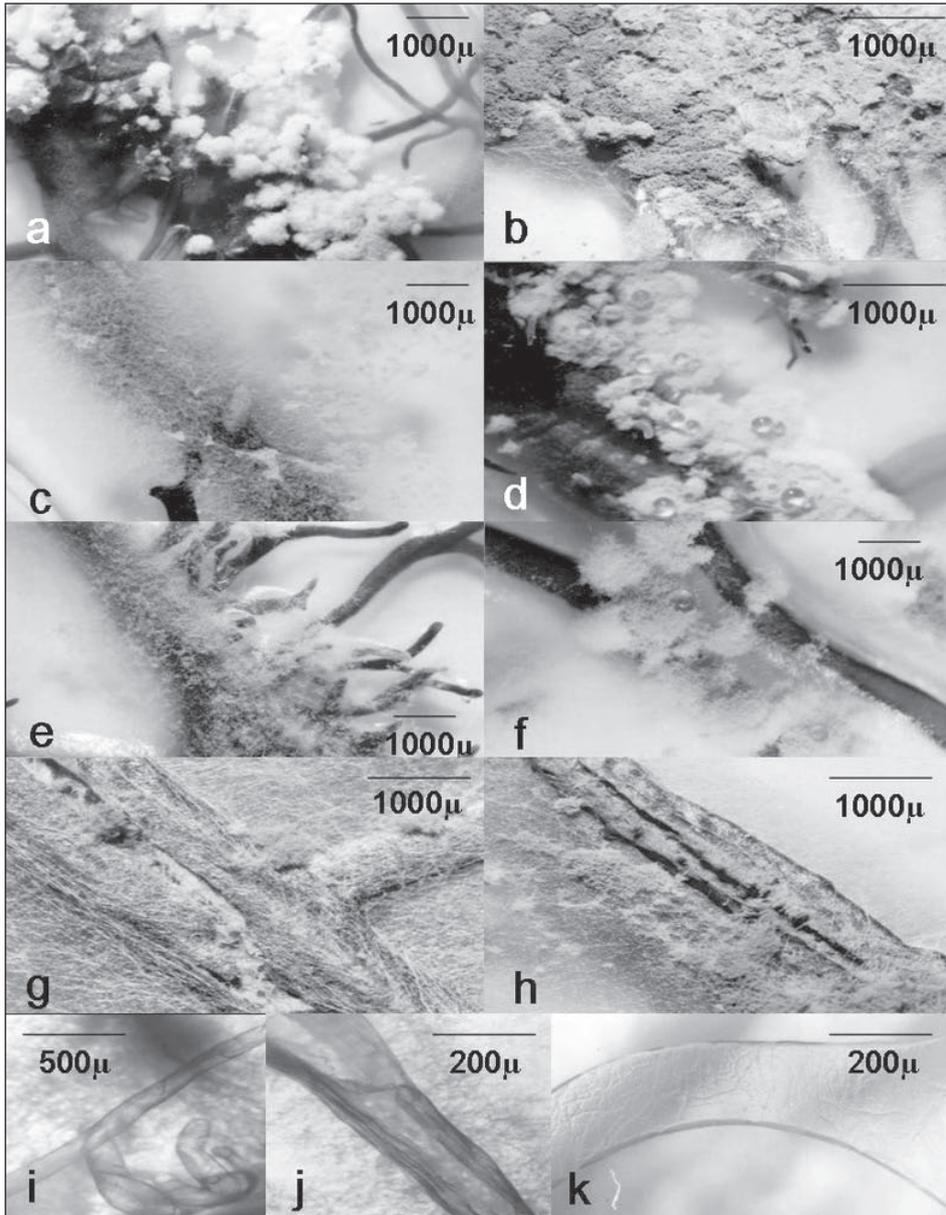


Fig. 2. (a-f), Extensive growth, colonization and sporulation of *T. harzianum* in the surface of rhizomorphs. (g), (h), Surface of infected rhizomorph showing longitudinally cracks. (i), (j), (k), Change of rhizomorphs and apical buds to hollow transparent tubes

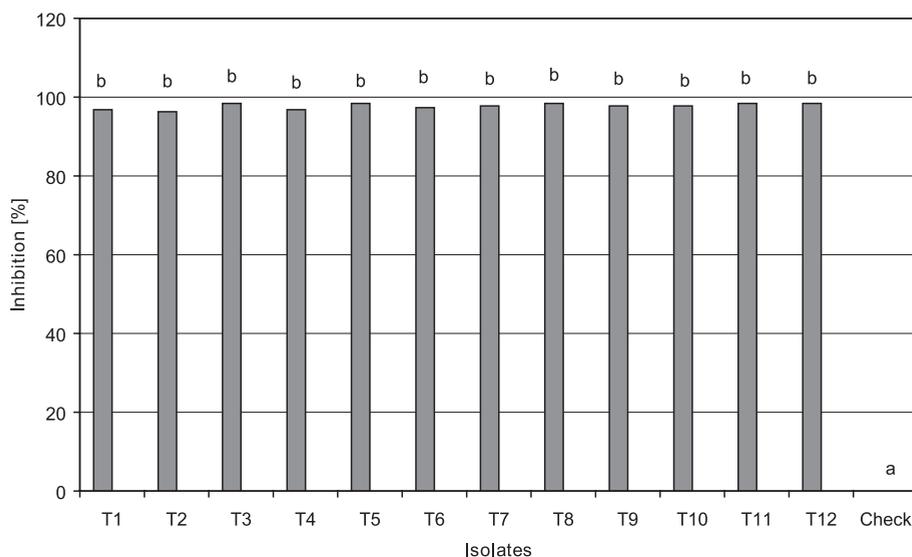


Fig. 3. Inhibitory effect of volatile metabolites of *Trichoderma* isolates in growth of *Armillaria mellea* colony when two fungi were cultured at the same time. Means sharing a common letter are not significantly different at $p \leq 0.05$

DISCUSSION

Trichoderma species have been extensively studied as a biocontrol agent against many plant pathogenic fungi throughout the world. The antagonistic effects of *Trichoderma* arises from various attributes, such as tolerance to changes in environmental conditions (Munnecke *et al.* 1981), ability to degrade various organic substances in soil, resistance to inhibitors and metabolic versatility, production of various toxic compounds, antibiotics and enzymes (Ishikawa 1976; Papavizas 1985; Vandriesche and Bellows 1996; Howell 2003). These abilities permit *Trichoderma* to compete, mycoparasite and to antagonize many fungi such as species of *Sclerotinia*, *Rhizoctonia*, *Rosellinia* and *Botrytis* (Tu 1980; Cook and Baker 1983, Elad *et al.* 1983, Elad and Kapat 1999).

Armillaria species have some unique abilities including production of antibiotic compounds with considerable inhibition against fungi and bacteria, and production of rhizomorphs, as highly differentiated organ with a special structure that enables the fungus to resist antagonistic effects of other organisms. Considering the slow growth rate of *Armillaria* compared to a high growth rate of *Trichoderma*, and also the antagonistic effects of this fungus, all isolates of *Trichoderma* were able to colonize the surface of *Armillaria* colonies, similarly to other pathogenic fungi such as: *Sclerotinia sclerotiorum* (Tu 1980), *Rhizoctonia solani* (Elad *et al.* 1983), *Rosellinia necatrix* and *Agaricus bisporus* (Cook and Baker 1983). Study on volatile metabolites effect, showed a significant control ability and inhibitory effect of *Trichoderma* isolates on mycelial growth of *Armillaria*, in comparison to the findings of Dennis and Webster (1971) and Mohammadi Goltapeh and Danesh (2000). The major volatile compound in *Trichoderma* species is 6-pentyl- α -pyrone (6-PAP), but *T. virens* produces a different spec-

trum of metabolites. All strains of this species seem to produce viridin and viridol, but some strains also produce gliovirin and heptelidic acid, whereas others produce gliotoxin (Howell *et al.* 1993).

Some of the interaction mechanisms of the *Trichoderma* hyphae with *A. mellea* rhizomorphs in microscopically features include penetration of antagonist hyphae in rhizomorphs and disintegration of rhizomorph content were similar to work of Dumas and Boyonoski (1992).

Trichoderma species produce various enzymes such as cellulases, proteases, chitinases, cellobiases, exo- and endo-glucanases. These compounds digest the components of the hyphal wall in fungi but about *Armillaria* rhizomorphs, considering to melanin content in outer cortex of rhizomorphs, antagonist fungi used other compounds for direct penetration, degradation and lysis.

Considering the *in vitro* significant results of biocontrol, application of antagonist fungi requires further understanding of pathogenic behavior of *Armillaria* species, ecological relationships of pathogen, antagonist, and soil micro flora and practical field tests. Biocontrol projects on fruit orchards may require monitoring to evaluate field treatment fully, so to develop an effective biological method in the future.

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POLISH SUMMARY

ANTAGONIZM GRZYBÓW Z RODZAJU *TRICHODERMA* W BIOLOGICZNYM ZWALCZANIU *ARMILLARIA MELLEA* NA DRZEWACH OWOCOWYCH W IRANIE

Zgnilizna korzeni powodowana przez gatunki rodzaju *Armillaria* jest jednym z najważniejszych schorzeń drzew owocowych i leśnych w Iranie. W przedstawianej pracy badano efekt antagonistyczny grzybów z rodzaju *Trichoderma* w biologicznym zwalczaniu grzybów z rodzaju *Armillaria*. *Armillaria mellea* izolowano z zainfekowanych korzeni drzew wiśniowych i migdałowych i identyfikowano metodą „pairing tests”. Gatunki *Trichoderma* pozyskiwano z ryzomorfów i z gleby otaczającej korzenie zainfekowane przez *Armillaria*. Zidentyfikowano następujące gatunki *Trichoderma*: *T. virens* (dziewięć izolatów) i *T. harzianum* (trzy izolaty). Krążki *Trichoderma* kładziono na kultury opieńki miodowej w celu zbadania efektu antagonistycznego. Wszystkie izolaty *Trichoderma* kolonizowały opieńki w ciągu 5–7 dni. Lotne związki wydzielane przez *Trichoderma* hamowały wzrost kolonii opieniek i tworzenie się ryzomorfów. Mechanizmy biologicznego zwalczania badano metodą mikroskopii optycznej i elektronicznej skaningowej. Mechanizmy te obejmowały wnikanie strzępek *Trichoderma* do ryzomorfów, kolonizację ryzomorfów przez grzybnię *Trichoderma*, kolonizację wierzchołków merystemu oraz pąków ryzomorfów, zarodnikowanie *Trichoderma* na zewnętrznej i wewnętrznej powierzchni ryzomorfów, degenerację i lizę tkanek ryzomorfów a także wydzielanie ich zawartości.