INFLUENCE OF MINERAL SALTS UPON ACTIVITY OF *TRICHODERMA HARZIANUM* NON-VOLATILE METABOLITES ON *ARMILLARIA* SPP. RHIZOMORPHS

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ABSTRACT

Effect of non-volatile metabolites of *Trichoderma harzianum* together with certain salts containing Mg^{++}, Fe^{+++}, Mn^{++}, Cu^{++}, Al^{+++}, Ca^{++}, K^{++}, Na^{+}, PO_4^{---} and SO_3^{---} on the production and length of rhizomorphs of *Armillaria borealis*, *A. gallica* and *A. ostoyae* was studied. In pure medium, *T. harzianum* exhibited stimulating effect on rhizomorphs of *A. borealis* (both number and length) and *A. ostoyae* (only initiation).

Cu^{++} salt totaly inhibited the initiation of rhizomorphs of *Armillaria borealis*, *A. gallica* and *A. ostoyae*. Effect of other compounds on the activity of *T. harzianum* depended on *Armillaria* species. The majority of chemical compounds tested supressed the activity of non-volatile metabolites of *T. harzianum*. Evident stimulating effect was observed under influence of sulphate salts consisting Al^{++} and Fe^{+++} on the rhizomorph number of *A. borealis* and *A. gallica*, respectively.

KEY WORDS: rhizomorphs, *Armillaria borealis*, *Armillaria gallica*, *Armillaria ostoyae*, *Trichoderma harzianum*, mineral salts.

INTRODUCTION

Root rot caused by *Armillaria* species is a common and severe disease of coniferous and deciduous trees (Rykowski 1981; Rishbeth 1988; Termorshuizen and Arnolds 1994; Rosso and Hansen 1998; Przybył 1999; Żółciak 1999; Selochnik and Kondrashova 2002; Thomas et al. 2002).

Rhizomorphs of *Armillaria* spp. play a major role in both the infection and spread of the disease, however, spore infection and root contacts are also considered in both processes (Rishbeth 1988). Distribution of *Armillaria* spp. and growth of rhizomorphs depend on many environmental factors, from which the effect of several soil factors, e.g. pH, moisture and organic matter on rhizomorph initiation and growth were most often empasized (Rishbeth 1978; Rykowski 1981; Pritam 1983; Twery et al. 1990; Shaw and Kile 1991; Marçais and Wargo 2000).

Some authors claim that suppression or stimulation of *Armillaria* spp. and other many pathogenic fungi is connected with metabolites produced by soil microorganisms, e.g. *Trichoderma* spp. (mainly *T. harzianum* Rifai and *T. viride* Pers. et Gray), *Aureobasidium pullulans* (de Bary) Arn. and *Zygorrhynchus moelleri* Vuill. (Pentland 1965; Kelly 1976; Harrison and Stewart 1988; Anselmi et al. 1992; Fox et al. 1994; Gallet and Lung-Escarmant 1994; Kwaśna and Łakomy 1998; Languasco et al. 2001). To our knowledge there is very little information available in the literature on the effect of inorganic compounds in pathogen-microorganism interaction.

The aim of our study reported here was to establish the in vitro effect of non-volatile metabolites of *Trichoderma harzianum* grown on medium with some mineral salts on production (expressed in their number) and growth (expressed in their length) of rhizomorphs of *A. ostoyae* (Romag.) Herink, *A. borealis* Marx. et Korh. and *A. gallica* Marx. et Romag.

MATERIALS AND METHODS

Fungal material

Isolates of *Armillaria* spp. were obtained from roots showing distinct decay symptoms, of the following tree species: *Quercus robur* L. (*A. borealis* and *A. gallica*) and *Picea abies* Karst. (*A. ostoyae*). *Trichoderma harzianum* belonged to fungi which were also detected in roots affected by *Armillaria* spp. The isolation of fungi and identification

procedure of *Armillaria* spp. described by Korhonen (1978) and Przybył (1999) was used, respectively.

The cultures were stored ca. 12 months on 3.5% malt extract agar (MEA; Merck, pH 5.3) at + 3°C before the study. They were transferred onto fresh MEA and then incubated in the dark at 23-24°C. All studied isolates of *Armillaria* species were able to form rhizomorphs under these conditions.

In the preliminary study, the non-volatile metabolites of three *T. harzianum* isolates were tested on MEA. They exhibited significant stimulating effect on rhizomorphs of *A. borealis* (both number and length) and *A. ostoyae* (number). In the case of *A. gallica* the effect was not visible in comparison with control data (results are included in Tables 1, 2 and 3). The difference in effect of the *Trichoderma* isolates on the formation and length of rhizomorphs was not significant. Therefore only one isolate was randomly selected for further studies.

Effect of chemical compounds – experiment 1 (MEA + C)

The following chemical compounds were applied: Mg – $MgSO_4 \times 7H_2O$, Fe – $Fe_2(SO_4)_3 \times 7H_2O$, Mn – $Mn\,SO_4 \times 5H_2O$, Cu – $Cu\,SO_4 \times 5H_2O$, Al – $Al_2(SO_4)_3$, Ca – $CaCl_2$, K – KCl, P – KH_2PO_4, Na – NaCl, S – Na_2SO_3 and K_2SO_3 (Ważny 1963; Sierota 1983). Mineral salts solutions at concentration 100 ppm of examined compounds were passed individually through 0.22 µm Millipore (Bedford, MA) filter and then separately added into steriled MEA. The pH of media were adjusted to 5.3 with 0.1 N NaOH or HCl using the Microcomputer pH-meter Cp-315 (Dhingara and Sinclair 1986).

Medium plugs of three isolates of each *Armillaria* species measuring approximately 5 mm by 5 mm were placed in the centre of a Petri dishes containing MEA with individual mineral salts. Seven replications were made for each isolate. Cultures were then incubated at 23°C. The average number of rhizomorphs and their mean length were measured after 26 days. In the second case the length of all rhizomorphs was calculated per one rhizomorph for each culture.

Effect of non-volatile metabolites of T. harzianum – experiment 2

Non-volatile metabolites produced by *T. harzianum* were tested using the procedure described by Dennis and Webster (1971) and Rudawska et al. (1993). The fungus was grown from its inoculum disk over the surface of cellophane membrane laid on pure MEA (MEA + T) and on MEA containing separately added salts (MEA + T + C).

Metabolites produced by *T. harzianum* were allowed to diffuse through the cellophane into medium. Plug mycelium (ca 5 × 5 mm) of *Armillaria* sp. was placed onto middle of each medium 6 days after the removal of the cellophane with *T. harzianum*. Mycelium of the *Armillaria* species was cut from colonies after 24 days of incubation on MEA at 23-24°C. The activity of metabolites was assessed by measuring the number and length of rhizomorphs after 26 days in the same way as in experiment 1. Seven replications for each isolate were performed.

TABLE 1. Effect of some chemical compounds and *Trichoderma harzianum* on production and length of *Armillaria borealis* rhizomorphs.

Compounds	Number of rhizomorphs (averages)	Length of rhizomorphs (averages; mm)
MEA	5.0 b	5.1 efg
MEA + T	18.6 cd	18.4 i
(1) Cupric sulfate ($CuSO_4\,5H_2O$)	0.0 a	0.0 a
Cupric sulfate + T	0.0 a	0.0 a
(2) Potassium sulphite (K_2SO_3)	9.1 bcd	2.0 ab
Potassium sulphite + T	9.3 bcd	6.8 cde
(3) Manganese sulphate ($MnSO_4\,5H_2$)	8.5 bcd	2.3 abc
Manganese sulphate + T	10.3 bcd	3.0 bcdef
(4) Magnesium sulphate ($MgSO_4\,5H_2O$)	10.1 bcd	2.9 bcdef
Magnesium sulhate + T	21.6 d	10.2 h
(5) Aluminium sulphate Al_2SO_4	8.1 bc	3.0 bcdef
Aluminium sulphate + T	33.3 e	3.1 bcdef
(6) Ferrous sulphate ($Fe_2(SO_4)_3\,7H_2O$)	13.9 cd	3.6 bcdef
Ferrous sulphate + T	19.6 cd	4.9 cdefg
(7) Potassium hypophosphite (KH_2PO_4)	7.9 bc	2.4 abc
Potassium hypophosphite + T	16.6 cd	2.8 bcde
(8) Potassium chloride (KCl)	7.5 bc	2.7 bcde
Potassium chloride + T	16.6 cd	3.2 bcdef
(9) Sodium sulphite (Na_2SO_3)	9.6 bcd	2.2 ab
Sodium sulphite + T	16.6 cd	7.3 gh
(10) Sodium chloride (NaCl)	7.1 bc	3.0 bcdef
Sodium chloride + T	11.0 bcd	4.6 cdefg
(11) Calcium chloride ($CaCl_2$)	17.1 cd	2.5 a bcd
Calcium chloride + T	16.0 cd	4.6 cdefg

Data followed by the same letters do not differ significantly (p=0.05) with Duncan test

MEA – Malt extract agar; T – *Trichoderma harzianum*

Mineral salts were composed of: (1) – Cu, (2) – S, (3) – Mn, (4) – Mg, (5) – Al, (6) – Fe, (7) – P, (8) – K, (9) – S, (10) – Na, (11) – Ca

TABLE 2. Effect of some chemical compounds and *Trichoderma harzianum* on production and length of *Armillaria gallica* rhizomorphs.

Compounds	Number of rhizomorphs (averages)	Length of rhizomorphs (averages; mm)
MEA	21.0 bcde	21.2 hi
MEA + T	23.4 bcde	25.4 i
(1) Cupric sulphate ($CuSO_4 \times 5H_2O$)	0.0 a	0.0 a
Cupric sulphate + T	0.0 a	0.0 a
(2) Potassium sulphite (K_2SO_3)	30.2 cdef	5.0 abcd
Potassium sulphite + T	28.3 cdef	11.3 defg
(3) Manganese sulphate ($MnSO_4 \times 5H_2O$)	23.3 cde	12.2 efg
Manganese sulphate + T	29.6 cdef	18.3 gh
(4) Magnesium sulphate ($MgSO_4 \times 5H_2O$)	14.6 abc	4.9 abcd
Magnesium sulphate + T	15.1 abc	24.6 i
(5) Aluminium sulphate (Al_2SO_4)	28.0 cdef	3.9 abc
Aluminium sulphate + T	20.6 bcde	13.0 efg
(6) Ferrous sulphate ($Fe_2(SO_4)_3 \times 7H_2O$)	27.5 cdef	4.8 abcd
Ferrous sulphate + T	45.6 g	14.0 efgh
(7) Potassium hypophosphite (KH_2PO_4)	26.1 cdef	4.8 abcd
Potassium hypophosphite + T	20.3 bcde	16.9 fgh
(8) Potassium chloride (KCl)	33.6 def	2.8 ab
Potassium chloride + T	27.6 cdef	14.7 efgh
(9) Sodium sulphite (Na_2SO_3)	24.2 cde	5.1 abcd
Sodium sulphite + T	26.6 cdef	13.3 efg
(10) Sodium chloride (NaCl)	23.0 cde	3.9 abc
Sodium chloride + T	18.6 bcde	18.3 gh
(11) Calcium chloride ($CaCl_2$)	18.6 bcdef	4.5 abcd
Calcium chloride + T	28.1 cdef	18.2 gh

Data followed by the same letters do not differ significantly (p=0.05) with Duncan test

MEA – Malt extract agar; T – *Trichoderma harzianum*

Mineral salts were composed of: (1) – Cu, (2) – S, (3) – Mn, (4) – Mg, (5) – Al, (6) – Fe, (7) – P, (8) – K, (9) – S, (10) – Na, (11) – Ca

TABLE 3. Effect of some chemical compounds and *Trichoderma harzianum* on production and length of *Armillaria ostoyae* rhizomorphs.

Compounds	Number of rhizomorphs (averages)	Length of rhizomorphs (averages; mm)
MEA	3.6 abc	2.2 abc
MEA + T	10.4 de	9.6 c
(1) Cupric sulphate ($CuSO_4 \times 5H_2O$)	0.0 a	0.0 a
Cupric sulphate + T	0.0 a	0.0 a
(2) Potassium sulphite (K_2SO_3)	5.1 bcd	4.6 abc
Potassium sulphite + T	2.6 abc	9.6 c
(3) Manganese sulphate ($MnSO_4 \times 5H_2O$)	2.0 abc	4.0 abc
Manganese sulphate + T	4.0 abcd	2.5 abc
(4) Magnesium sulphate ($MgSO_4 \times 5H_2O$)	1.7 abc	3.5 abc
Magnesium sulphate + T	12.0 e	11.8 c
(5) Aluminium sulphate (Al_2SO_4)	3.6 abc	3.6 abc
Aluminium sulphate + T	11.0 de	4.5 abc
(6) Ferrous sulphate ($Fe_2(SO_4)_3 \times 7H_2O$)	0.5 ab	1.0 ab
Ferrous sulphate + T	1.3 abc	3.6 abc
(7) Potassium hypophosphite (KH_2PO_4)	3.2 abc	2.2 abc
Potassium hypophosphite + T	4.6 bcd	2.7 abc
(8) Potassium chloride (KCl)	6.2 cde	5.4 abc
Potassium chloride + T	9.6 de	4.6 abc
(9) Sodium sulphite (Na_2SO_3)	6.7 cde	6.1 abc
Sodium sulphite + T	2.7 abc	6.0 abc
(10) Sodium chloride (NaCl)	3.1 abc	2.2 abc
Sodium chloride + T	2.6 abc	2.7 abc
(11) Calcium chloride ($CaCl_2$)	3.6 abcd	6.9 bc
Calcium chloride + T	5.0 bcd	4.7 abc

Data followed by the same letters do not differ significantly (p=0.05) with Duncan test

MEA – Malt extract agar; T – *Trichoderma harzianum*

Mineral salts were composed of: (1) – Cu, (2) – S, (3) – Mn, (4) – Mg, (5) – Al, (6) – Fe, (7) – P, (8) – K, (9) – S, (10) – Na, (11) – Ca

Duncan's multiple range test (at a 5% of significance level) was used to compare the means for all final data obtained in both experiments.

RESULTS

Number of rhizomorphs

Full inhibition of rhizomorphs initiation of all *Armillaria* species were observed in the medium containing Cu^{++} ($CuSO_4$) both without and with effect of *T. harzianum* metabolites.

Significantly greater number of *A. borealis* and *A. gallica* rhizomorphs occurred in the presence of *T. harzianum* together with Al^{+++} and Fe^{+++} (MEA + T + C; 33.3 and 45.6, respectively) in comparison with the number produced both on control media MEA + T and at MEA + C (Tables 1 and 2). The strong stimulating effect of *T. harzianum* metabolites together with the tested chemical compounds was not observed in the case of *A. ostoyae* rhizomorphs (Table 3). In this case the highest number of rhizomorphs (12.0) was found on medium containing *Trichoderma* metabolites together with Mg^{++} ($MgSO_4$), but was not significantly differed from MEA+T. Whereas data relating to Mg^{++} effect, and also to Al^{+++} (MEA + C + T) differed significantly from data obtained for cultures growing on MEA + C. Moreover statistic analysis revealed a significant inhibiting effect on rhizomorph number on media (MEA + T + C) containing Fe^{+++} (1.3), Na^{+} (2.6) and SO_3^{---} in the form of both K_2SO_3 (2.6) and Na_2SO_3 (2.7) in comparison with the data obtained for MEA + T (Table 3).

Length of rhizomorphs

It should be emphasised that Mn^{++}, PO_4^{---} and SO_3^{---} added to the medium alone, (without *Trichoderma*; MEA + C), significantly inhibited length of *A. borealis* rhizomorphs in pure medium (MEA; Table 1). A similar effect was obtained for *A. gallica* rhizomorphs being under influence of all individually tested compounds (Table 2).

Trichoderma effect on *A. borealis* rhizomorph length was inhibited by all individually investigated compounds (media MEA + C + T) when the data were compared with those obtained for cultures growing in MEA + T. On the other hand metabolites activity significantly increased under influence of Mg^{++} (10.2) and SO_3^{---} (K_2SO_3 – 6.8. Na_2SO_3 – 7.3) (media MEA + T + C) in comparison with the activity in MEA + C (Table 1). In the *A. gallica*, inhibition of the metabolites activity was not noted in medium containing Mg^{++} (MEA + T + C – 24.6 mm) in comparison with MEA + T (25.4 mm). For majority of other compounds the significant effect of *Trichoderma* (MEA + T + C) occurred when it was compared with data obtained on medium MEA + C (Table 2). In the case of *A. ostoyae* no clear effect on the length of rhizomorphs was observed (Table 3).

DISCUSSION

In the opinion of Morrison (1982) larger number of initials and a faster growth rate of rhizomorphs of *A. mellea* is due to organic carbon and nitrogen present in the soil. Whereas Przybył (1998) found that sterilized soil rich in N, P, K, Ca and Mg more positively effected the initiation and length of rhizomorphs of *A. ostoyae* in comparison with *A. borealis* and *A. gallica*.

The results presented show, that $CuSO_4$ (containing 100 ppm Cu^{++}) inhibited the initiation of rhizomorphs of *Armillaria borealis*, *A. gallica* and *A. ostoyae*. The effect of other tested compounds on the activity of *T. harzianum* expressed in number and length of rhizomoprhs was dependent on *Armillaria* species. Generally, the majority of tested chemical compounds supressed activity of non-volatile metabolites of *T harzianum* in comparison with results obtained in pure MEA + T. Stimulation of *T harzianum* activity was observed under influence of sulphate salts consisting Al^{++} and Fe^{+++} on the rhizomorph number of *A. borealis* and *A. gallica*, respectively. On the other hand Fe^{+++} inhibited effect of *T. harzianum* on formation of *A. ostoyae* rhizomorphs. Fe^{+++} salt, used in the study, is able to inhibit antagonistic ativity of certain fungi, e.g. *Phlebiopsis gigantea* toward *Heterobasidion annosum* (Negrutskiy et al. 1998). It is also proper to add that Mg, Mn and Zn ions at a concentration of 100 ppm increased the inhibitory effect of filtrates from *T. viride* cultures on *H. annosum* (Sierota 1983).

The authors are aware that the investigations presented, regarding only non-volatile metabolites, do not present an total effect of *T. harzianum* on rhizomorphs of tested *Armillaria* spp. In general, *T. harzianum* and other *Trichoderma* spp. can act by activity of their non-volatile and volatile metabolites. Variation within *T. harzianum* isolates with

regard to production of these metabolites was found by Dennis and Webster (1971); Harrison and Stewart (1988) and Rudawska et al. (1993). Moreover, mycoparasitism of *T. harzianum* on rhizomorphs of *A. gallica* was reported by Dumas and Boyonoski (1992). Undoubtedly, the activity of *Trichoderma* spp. by all the possible means can explain the antagonistic effect of *T. harzianum* against *Armillaria* species which was proved by Fox et al. (1994) and Languasco et al. (2001). On the other hand, Mughogo (1967, 1968, after Denis and Webster 1971) observed that isolates of *T. harzianum* did not affect the growth of *A. mellea in vitro* (in this case the complex of *A. mellea* was studied).

The results obtained for interaction of certain inorganic compouns with the pathogen and other microorganism (*T. harzianum*) provide encouragement for further studies on this subject. Different concentrations of inorganic compounds should also be analysed.

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