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Determination of the growth and solubilization capabilities of *Trichoderma harzianum* T1

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Abstract: The growth capability of *Trichoderma harzianum* Rifaii T1 was tested on Malt Extract and Czapeks Dox agar containing different concentrations of Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+} and Ca^{2+}. The *T. harzianum* T1 isolate was observed to produce mycelia and spores in various mineral-containing media. It showed the lowest tolerance to Ca^{2+} and the highest tolerance to Fe^{2+}. Solubilization capability of *T. harzianum* T1 for some insoluble minerals via acidification of medium has been tested on MnO_2, CuO, Fe_2O_3 and metallic Zn. *T. harzianum* T1 was able to solubilize MnO_2 and metallic Zn in a liquid medium.

Key words: *Trichoderma harzianum* T1; biosolubilization; micronutrients

Introduction

Fungi of the genus *Trichoderma* have been used to control a wide range of plant pathogenic fungi (Hjeljord & Tronsmo 1988; Küçük & Kivanç 2001) and several reasons may be accounted for its control ability including the production of volatile and nonvolatile antibiotics (Dennis & Webster 1971a,b), production of fungal cell wall degrading enzymes such as chitinases and proteases (Geremia et al. 1993; Haran et al. 1993), competion for key nutrients or elements (Shukla & Mishre 1970), and inducing resistance in plants (De Meyer et al. 1998).

Mineral nutrients play a major role in both plant growth and disease resistance in plants (Graham & Webb 1991; Jacobs et al. 2002). Particularly iron has been in the focus of studies on its solubilization by soil organisms, its availability to plants, and its effects on plant diseases (Graham & Webb 1991). Zinc and manganese are the elements required for diverse physiological functions along with their roles in plant growth and induction of disease resistance in plants. But higher oxidation state oxides of manganese are insoluble in soil (Kacar 1994). Manganese is a microelement required for several physiological functions of plants such as photosynthesis, hormones (auxins), phenols and lignin. As a consequence, manganese plays a major role in both the growth and disease resistance of plants (Huber & Wilhem 1988).

Microbial interactions with plant roots have a profound effect on the nutrient status of plants (Huber & Wilhem 1998; Abonen-Jonnarth et al. 2000; Rosling et al. 2004). Besides functioning as a biological control agent, *Trichoderma harzianum* shows plant growth promoting activity (Chet 1990; KleIfeld & Chet 1992; Inbar et al. 1994; Grondona et al. 1997; Küçük & Kivanç 2001). Furthermore, it was previously reported that a *T. harzianum* T1 strain was an effective antagonist of several soilborne plant pathogens such as *Fusarium culmorum, F. moniliforme* and *Gaeumannomyces graminis* var. *tritici* (Küçük & Kivanç 2001) and had beneficial effects on the root system of wheat (Küçük 2000).

This study was carried out in vitro to determine the growth and solubilization capabilities of *T. harzianum* T1 in the presence of different soluble or insoluble minerals.

Material and methods

Trichoderma harzianum T1

T. harzianum strain T1 was isolated from sandy loam soil surrounding wheat roots infected by a *Gaeumannomyces graminis* var. *tritici.* The soil has been taken from an experimental field in Eskisehir, Turkey. The dry soil texture consisted of sand (38%), silt (50%) and clay (12%). Soil reaction (pH) was 7.9 and organic matter content was 1.45%. The isolated culture was kept on Potato Dextrose Agar (PDA, Merck) slants at −20 °C.

Mineral medium

The growth capability of *T. harzianum* T1 was tested on Malt Extract Agar (MEA, Merck) and Czapek Dox Agar (CZA, Merck) enriched with 0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg L^{-1} concentrations of $CuSO_4$, $ZnSO_4$, $MnSO_4$, $FeSO_4$, and $CaSO_4$.

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The mineral tolerances of *T. harzianum* T1 were determined by measuring the amount of mycelial biomass. *Trichoderma harzianum* T1 was grown on PDA in petri dishes for 7 days at 25 °C. Spores were collected by washing the Petri dish contents with 0.2% (v/v) aqueous Tween 80 and this process was repeated twice. After that, the spores were freed from mycelia after centrifugation at 15 000 × *g* for 5 min at 4 °C. The spores were resuspended in sterilized water and the suspension contained approximately 1×10^7 spores mL^{-1}.

The mineral media were prepared by adding the above listed concentrations of copper, zinc, manganese, calcium, and iron sulphates to 20 mL of sucrose yeast extracts (SY) (30 g of sucrose, 1 g of yeast extract/L of distilled water) into 150 mL Erlenmayer flasks (Haran et al. 1993). After inoculation of 1 mL of the spore suspensions of *T. harzianum* T1 (10^7 spore mL^{-1}) onto these mineral media, the cultures were incubated for 72 h at 28 °C using an orbital shaker at 160 rpm (Küçük 2000). Then, the mycelia produced were filtered using a filter paper with a pore size 0.45–0.22 μm, dried overnight at 70 °C and weighed. Three replications of all assays were performed.

Solubilization of minerals in liquid media

Insoluble or sparingly soluble minerals MnO_2, Fe_2O_3, CuO, and granular metallic Zn were used for solubilization experiments. These minerals (50 mg) were added to 100 mL of sucrose yeast extract (SY) in a 250 mL Erlenmayer flask and they were sterilized in an autoclave. The pH values of the flask contents were 6.5. The Erlenmayer flask contents were inoculated with 1mL of the spore suspension of *T. harzianum* T1 and the cultures were incubated on an orbital shaker (160 rpm) at 25 °C for 8 days. The flasks were sampled each day and the cultures were filtered through a No. 5 filter paper (Whatman) and 0.45–0.22 μm pore size of cellulose filters. At the same time the uninoculated flasks, used as controls, were processed the same way as the inoculated ones. Then the pH values of the clear inoculated and uninoculated culture filtrates were measured again. After that, a 10 mL aliquot from each filtrate was taken and acidified with a few drops of concentrated HCl to prevent the loss of soluble ions and microbial growth until the analyses were completed. Three parallel assays were performed. The acidified filtrate, or dilutions were analyzed for the amounts of metal ions with an atomic absorption spectrometry (A.A.S) (Altomare et al. 1999).

Solubilization of minerals by cell-free culture filtrates

The sterilized 90 mL cultures of T1 on the SY medium remaining from previous section were divided into three nearly equal volumes (about 30 mL each) and filtered. One of these three filtrates was autoclaved for 20 min at 121 °C, the second one was digested with 50 μg mL^{-1} of protease K (Sigma) per mL for 4 h at 37 °C and the third one was used as a control. The same process was applied to the uninoculated filtrates. Then 5 mL of the aliquot from each of the treatments was dispensed into oven-sterilized test tubes containing 5 mg of MnO_2, Fe_2O_3, CuO and granular metallic Zn. After that, the tubes were incubated at room temperature for 48 h on an orbital shaker. After the broths were filtered through 0.45–0.22 μm pore size filters, the filtrates were analyzed for metal content by A.A.S (Altomare et al. 1999) and for the presence of malic acid, oxalic acid, citric acid, succinic acid and fumaric acid by thin layer chromatography (TLC). TLC was carried out on SIL G-25 plates using a diethyl ether/formic acid/water (60:30:10) mixture as the solvent. The detection was 10 μg mL^{-1} for all the acids analyzed.

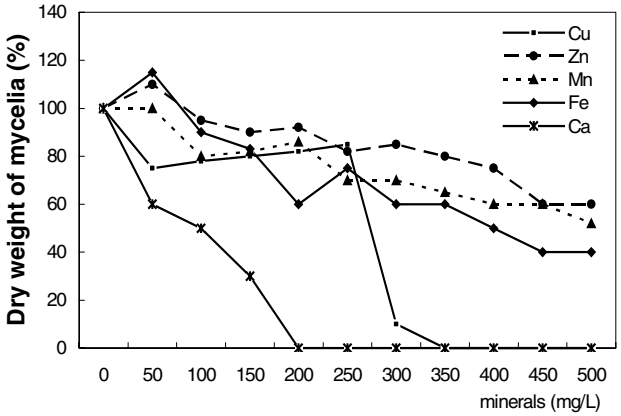

Fig. 1. Effects of copper, zinc, manganese, calcium, and iron on the growth of *T. harzianum* T1 after 72 h incubation.

Results

It was observed that *T. harzianum* T1 had the ability to grow at different concentrations of $CuSO_4$, $ZnSO_4$, $MnSO_4$, $FeSO_4$ and $CaSO_4$. The concentrations of each mineral in the liquid medium varied from 0 to 500 mg L^{-1}. Figure 1 gives the dry weight percentage of mycelia versus the concentration of the minerals. These data correspond to the average value of four measurements and confirm that *T. harzianum* T1 shows a high degree tolerance to copper, zinc, manganese and iron ions *in vitro* (Fig. 1). In the case of copper, *T. harzianum* T1 isolate survived at concentrations of Cu^{2+} between 0 and 250 mg L^{-1} and produced almost constant levels of biomass. But its growth decreased by about 70% at 300 mg L^{-1} and no growth was detected at 350 mg L^{-1} of Cu^{2+} . The isolate was capable to survive at higher concentrations, even at 500 mg L^{-1} of zinc, manganese and iron. Calcium was the least tolerated mineral (Fig. 1) and no detectable growth at a concentration of 200 mg L^{-1} and beyond was observed.

For solubilization experiments, cultures were sampled daily for 8 days during the growth of *T. harzianum* T1. It was found that T1 was able to solubilize MnO_2 and Zn in culture medium (Fig. 2). We also determined the ability of cell-free culture filtrates of *T. harzianum* T1 to solubilize various materials. The solubilization efficiencies of the autoclaved or proteinase K treated culture filtrates were assayed in comparison with the crude culture filtrate (untreated) (Fig. 3). With the exceptions of CuO and MnO_2, no difference in solubilizing capability of cell free culture filtrates of T1 was found among the untreated and treated filtrates. Furtheremore, citric acid, malic acid, succinic acid and fumaric acid were not found in culture filtrates at the detection limit of 10 μg mL^{-1}. Only oxalic acid was detected in manganese containing culture filtrates (data not shown).

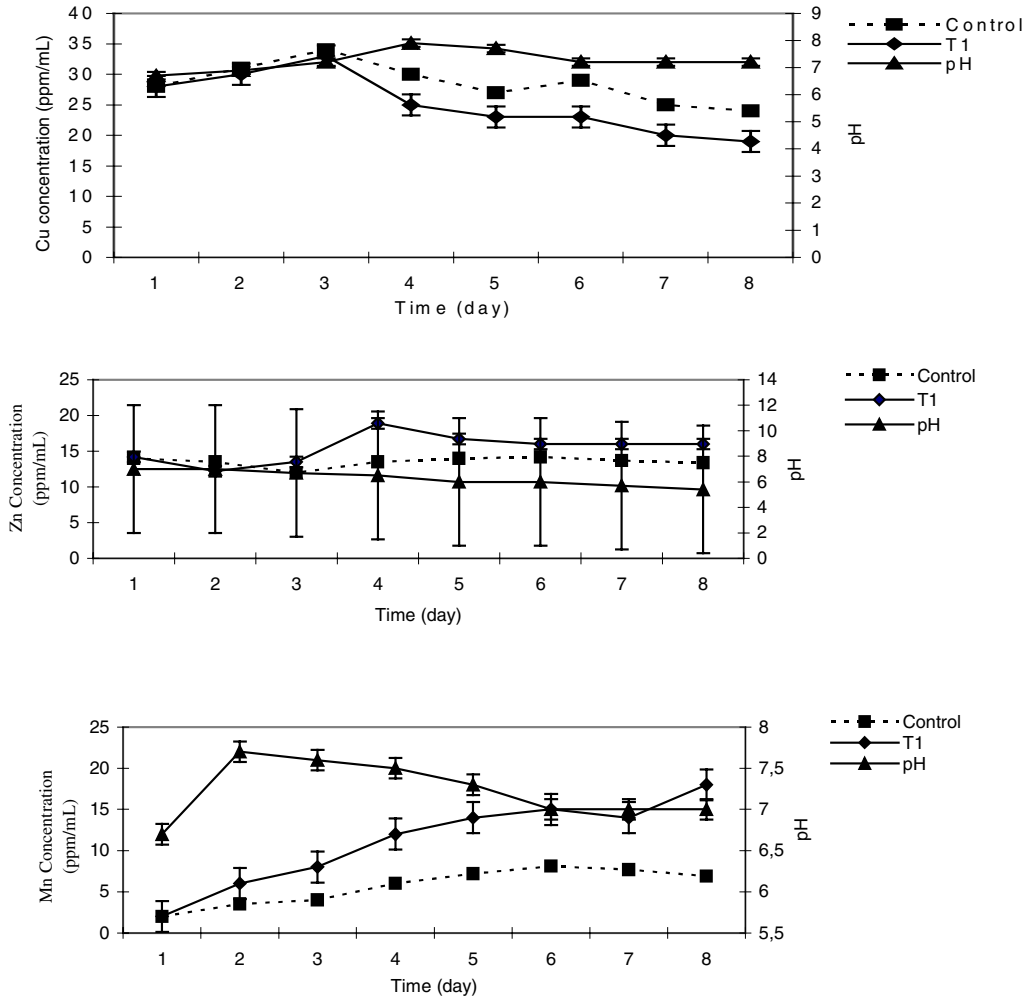

Fig. 2. Solubilization of minerals in liquid culture. Results are the mean of three parallel determinations. Error bars indicate standard deviation of three parallel.

Discussion

The high tolerance to zinc, manganese and iron were observed in biocontrol fungus *Trichoderma harzianum* T1. Several authors have demonstrated in vitro adaptation of fungi to minerals (Graham & Webb 1991; Jacobs et al. 2000; Rosling et al. 2004). Minerals, especially copper, mercury, manganese and zinc are increasingly released into the environment from fertilisers and other human activities. Copper, iron, manganese and zinc are essential micronutrients for most, if not all, living organisms. The response of microorganisms to exposure to multiple minerals may differ from their response to individual minerals and additive synergistic or antagonistic interactions may occur between minerals (Babich & Stotzky 1983). Many micronutrients are crucial for plant defense capabilities (Graham & Webb 1991) and they play a major role in the growth and disease resistance of plants (Huber & Wilhem 1998).

In the present work, the growth capability of *T. harzianum* isolate T1 was tested in media containing different concentrations of copper, zinc, manganese, iron and calcium. The tested *Trichoderma harzianum* T1 grew in mineral media without any problem. Growth and sporulation of *T. harzianum* T1 was slower when $CuSO_4$ or $CaSO_4$ were added to mineral media, and furthermore high copper concentrations caused poor germination of *T. harzianum* spores (data not shown). The inhibitory effect of copper sulfate on the growth of *T. harzianum* was reported by Grondona et al. (1997). In our study, we observed that copper sulfate in the agar based assay accumulated on the edge of the petri dishes as reported by Grondona et al. (1997). Some researchers have reported tolerance levels of *Aspergillus flavipes* that are similar to those reported here. According to Babich & Stotzky (1983), *Aspergillus flavipes* was not resistant to 200 mg L^{-1} of copper. Also, Detroy & Ciegler (1971) observed that it had yeast like morphology and stopped growing when a concentration of 150 mg L^{-1} of manganese was added to culture medium.

Altomare et al. (1999) reported the solubilization of Zn, MnO_2 and phosphorous in medium by *T. harzianum* T22 and found that the pH values never fell below 5 in their cultures. In this study, we also observed that the pH values in all cultures did not fall below 5.4. Our study has also been conducted to determine the ability of cell-free culture filtrates to solubilize various materials. Data on the solubilization of

CuO in liquid culture were not conclusive. SY medium alone solubilized significantly more copper from CuO than did the cell-free culture filtrates. The filtrates released significantly more soluble materials than did the SY medium. The *T. harzianum* T1 solubilized Mn in MnO_2 regardless of the medium (Fig. 3) and solubilization was not affected by pH changes of the culture of the *T. harzianum* T1. Under the experimental conditions, solubilization of MnO_2, Fe_2O_3 and Zn occured at slightly acidic or alkaline pH values. The availability of manganese in soil mainly depends on pH and soil microflora that can either oxidize or reduce manganese. The *T. harzianum* T1 appears to be able to solubilize Mn^{+4} by reducing and/or chelating it.

Metallic zinc and Fe_2O_3 were solubilized by the cell-free culture filtrates. Differences in solubilizing capability of MnO_2 and metallic zinc were found among untreated and treated filtrates. Solubilizing substances are heat stable and probably not proteinaceous.

It has also been shown that *Rhizoctonia solani, Cortinarius glaucopus*, *Aspergillus* spp. and *Penicillium* spp. are able to solubilize mineral nutrients (Detroy & Ciegler 1971; Jacobs et al. 2000; Rosling et al. 2004). Solubilization of metallic zinc depends on its oxidation to Zn^{+2}. T1 proved to have the ability in culture to accelerate the oxidative dissolution of metallic zinc to Zn^{+2}.

Production and exudation of low molecular weight organic acids, oxalic acid in particular, is common in fungi (Detroy & Ciegler 1971; Ahonen-Jonnart et al. 2000). Oxalic acid production in *Aspergillus* spp. is largely dependent on pH of the substrate with production drastically decreasing at substrate pH below 3.5 (Detroy & Ciegler 1971). In our experiment, oxalic acid was detected in manganese containing culture filtrates. Further experiments are being carried out to analyse organic acid produced by the *T. harzianum* T1 by HPLC. In conclusion *Trichoderma harzianum* T1 isolated in Turkey was able to solubilize MnO_2 and metallic zinc *in vitro*. These features of *T. harzianum* T1 could provide new opportunities to study the interactions between *T. harzianum* and plants or its biocontrol effects.

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References

Ahonen-Jonnart U., Van Hees P.A.W., Lundstrom U.S. & Finlay R.D. 2000. Organic acids produced by mycorrhizal *Pinus sylvestris* exposed to elevated aluminium and heavy metal concentrations. New Phytol. **146:** 557–567.

Altomare C., Norvell W.A., Bjorkman G. & Harman G. 1999. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *T. harzianum* Rifaii 1295–22. Appl. Environ. Microbiol. **65:** 2926–2933.

Babich H. & Stotzky G. 1983. Synergism between nickel and copper and their toxicity to microbes: mediation by pH. Ecotoxicol. Environ. Safety **7:** 576–587.

Chet I. 1990. Biological control of soil borne plant pathogens with fungal antagonists in combination with soil treatments, pp.15–25. In: Hornby D. (ed.), Biological Control of Soilborne Plant Pathogens. CAB Internations, Wallingford.

De Meyer G., Bigirimana J., Elad Y. & Hofte M. 1998. Induced system resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. Eur. J. Plant Pathol. **104:** 279–286.

Dennis C. & Webster J. 1971a. Antagonistic properties of spesific groups of *Trichoderma*. I. Production of non-volatile antibiotics. Trans Br Mycol. Soc. **57:** 25–39.

Dennis C. & Webster J. 1971b. Antagonistic properties of spesific groups of *Trichoderma*. II. Production of volatile antibiotics. Trans Br Mycol. Soc. **57:** 41–48.

Detroy R.W. & Ciegler A. 1971. Induction of yeast like development in *Aspergillus parasiticus*. J. Gen. Microbiol. **65:** 259–264.

Geremia R., Goldman G.H., Jacobs D., Ardiles W., Vila S.B., Van Montagu M. & Herrera-Estrella A. 1993. Molecular characterization of the proteinase-encoding gene, prb 1, related to mycoparasitism by *Trichoderma harzianum*. Mol. Microbiol. **8:** 603–613.

Graham R.D. & Webb M.J. 1991. Micronutrients and disease resistance and tolerance in plants, pp. 329–370. In: Welch R.M. (ed.), Micronutrients in agriculture. Soil Sci. Soc. of America, Madison, Wis.

Grondona I., Hermosa R., Tejada M., Gomis M.D., Mateos P.F., BrIdge P.D., Monte E. & Garcia-Acha I. 1997. Physiological and biochemical characterization of *Trichoderma harzianum,* a biological control agent against soilborne fungal plant pathogens. Appl. Environ. Microbiol. **63:** 3189–3198.

Haran S., Schickler H., Oppenheim A. & Chet, I. 1993. New components of the chitinolytic system of *Trichoderma harzianum*. Mycol. Res. **99:** 441–446.

Hjeljord L. & Tronsmo A. 1988. *Trichoderma* and *Gliocladium* in biocontrol: an overview, pp.135–151. In: Kubicek C.P. & Harman G.E. (ed.), *Trichoderma* and *Gliocladium*. Taylor & Francis, Ltd, London, U.K.

Huber D.M. & Wilhem N.S. 1998. The role of manganase in resistance to plant diseases, pp.155–173. In Graham R.D., Hannam R.J. & Uren N.C. (ed.), Manganase in Soils and Plants. Kluwer Academic Publishers, Dardrecht, The Netherlands.

Inbar J., Abramsky M., Cohen D. & Chet I. 1994. Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. Eur. J. Plant Pathol. **100:** 337–346.

Jacobs H., Boswell G.P., Ritz K., Davidson F.A. & Gadd G.M. 2002. Solubilization of calcium phosphate as a consequence of carbon translocation by *Rhizoctonia solani*. FEMS Microbiol. Ecol. **40:** 65–71.

Kaçar B. 1994. Bitki Besleme ve Toprak Verimliliği, Ankara Üniversitesi, Ziraat Fak. No: 1361, Ankara, pp. 259–297.

Kleifeld O. & Chet I. 1992. *Trichoderma harzianum*-interaction with plants and effect on growth response. Plant Soil. **144:** 267–272.

Küçük Ç. 2000. *Trichoderma harzianum* ile toprak kökenli bazı bitki patojenlerinin kontrolü. A.Ü. Fen Bilimleri Ens. Eskişehir, MSc Thesis, 80 pp.

Küçük Ç., & Kivanç M. 2001. Sera ve laboratuvar koşullarında *Trichoderma harzianum*'un toprak kökenli bazı fungal bitki patojenleri üzerine etkisi. Biyoteknoloji. **25:** 85–92.

Rosling A., Lindahl B.D., Taylor A.F.S. & Finlay R.D. 2004. Mycelial growth and substrate acidification of ectomycorrhizal fungi in response to different minerals. FEMS Microbiol. Ecol. **47:** 31–37.

Shukla D.D. & Mishre A. 1970. Effects of salts on growth of *Trichoderma viride*. Friesia. **9:** 299–301.

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