Journal of Innovative Agriculture: 8(3) : 1-10, 2021
DOI: 10.37446/jinagri/rsa/8.3.2021.1-10
Research paper

Effects of various metal ions on the growth of some phytopathogenic and biological control fungi

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Received: 20 April 2021
Accepted: 20 July 2021
Published: 30 September 2021

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Metal ions are among important environmental factors that influence various aspects of fungal biology and the knowledge on these effects can be applied in integrated plant disease management and mycotechnology. Therefore, in this study the fungal growth on potato dextrose agar, Czapek'sdox agar (CDA, containing $FeSO_4$), and its derivatives made via the replacement of $FeSO_4$ with either of $CaSO_4$, K_2SO_4, $MnSO_4$, Na_2SO_4, and $ZnSO_4$ was studied under incubation conditions of 25°C and darkness. The mycelial growth of three biological control (*Trichoderma hamatum*, *T. harzianum*, and *T. longibrachiatum*) and three plant pathogenic (*Ceratocystis radicicola*, *Fusarium oxysporum*, and *Macrophomina phaseolina*) were measured 48h and 72h after inoculation. *T. longibrachiatum* exhibited the highest mycelial growth and PDA supported the fastest and the highest mycelial growth of most tested fungi. Interestingly, $ZnSO_4$ led to the highest growth of all *Trichoderma* species, while most of the pathogenic fungi grow well on the media with K_2SO_4 or Na_2SO_4.

Key words: *Ceratocystis, Fusarium, Macrophomina, nutrient, Trichoderma*

INTRODUCTION

Fungi as a kingdom of heterotrophic eukaryotic organisms are important in plant pathology, soil microbiology, food microbiology, industry, medicine, veterinary, and biotechnology. The cultivation and mass production of fungi and fungal metabolites, as well as the management of their activity requires enough information on the effect of environmental factors. These elements may be either of nutritional value for fungi, hence known as nutrients, or they may be toxic elements and impose deleterious effects on fungi. Nutrients required at millimolar concentrations are called macronutrients. Carbon (C), nitrogen (N), oxygen (O), sulfur (S), phosphorus (P), potassium or kalium (K), and magnesium (Mg) are grouped as macronutrients. Other nutrients are required only at micromolar concentrations and therefore, are regarded as micronutrients or trace elements. Calcium (Ca), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn) are examples of micronutrients (Walker & White, 2017). The role of metal ions in the regulation of secondary as well as primary metabolism in microorganisms is well documented (Weinberg, 1969). Metal ions bind to specific sites of a group of proteins to perform catalytic, regulatory and structural functions. These proteins referred to as metalloproteins represent one of the most diverse classes of proteins which approximately comprehends one-third of all proteins. It is estimated that metalloproteins to contain at least one metal cofactor (Tristão et al., 2015). Metals functions as cofactors of enzymes to either stimulate or inhibit enzymatic reactions. They are also important in the modulation of cell wall permeability and in regulation of many pre- and post-transcriptional events

(Weinberg, 1969). With fungi, metals have been shown to play important roles in secondary metabolism. The production of various antibiotics, hormones, and mycotoxins is greatly influenced by the presence of metals and other micronutrients in the production media (Weinberg, 1969). Among the metals studied, manganese (Mn^{2+}), iron (Fe^{2+}), and zinc (Zn^{2+}) appear to be of the most importance in secondary metabolite biosynthesis. Overall, metal ions play important roles in the survival, growth, development and reproduction of fungi. Fungi defend themselves against the toxicity of metal ions through multiple lines of defense. The first line is extracellular chelation and binding to cell wall. Glutathione (GSH), oxalate, extracellular mucilaginous materials (ECMM or emulsifiers) with excellent toxic metal binding capabilities, and glycoproteins such as glomalin play important role in the first defense line of fungi. Considering the effect of metal ions on fungal growth (Cuero et al., 2003, Mwangi et al., 2014, Tkaczuk, 2005), the current study was performed.

MATERIALS AND METHODS

Fungal cultures

The cultures of three isolates of three different *Trichoderma* species, *T. hamatum*, *T. harzianum* and *T. longibrachiatum* as well as three pathogenic fungi *Ceratocystis radicicola*, *Fusarium oxysporum*, and *Macrophomina phaseolina* were grown on Czapek's dox agar (CDA) plates were used. To prepare the required fungal inoculums, the fungi were aseptically inoculated on CDA plates and incubated at 25°C in dark. Inoculations were made using a disc of 5 mm diameter taken from each culture and inversely put in the center of a CDA medium. Three 90 mm plates were grown per fungus.

Media preparation

The basic medium was Czapek'sdox broth amended with agar (20 gl^{-1}). Treatments included the same medium but with K_2SO_4, Na_2SO_4, $MnSO_4$, $CaSO_4$, and $ZnSO_4$ added at the rate of 10^{-2} gl^{-1} instead of the same amount of $FeSO_4$ in the commercial CD broth applied as the basic medium after agar addition. Also, potato dextrose agar (39 g l^{-1}) was applied as a standard medium to get better understanding.

Inoculation and incubation

Inoculation was performed transferring a 5 mm disc of 4 day cultures to the center of the CDA, and the cultures were incubated at 25°C in dark.

Statistical analysis

The study was performed as completely random plan-based factorial experiment with two factors (6 fungi and 7 media) tested as 42 treatments each of 6 repeats (6 plates considered per fungus-medium combination). The ANOVA of data was made using SAS software using the colony diameters 48 h and 72 h after inoculation. The comparison of means of the treatments were made using Duncan's test.

RESULTS AND DISCUSSION

The knowledge of the impact of metal ions on fungal growth and development is important from different viewpoints. Such information can be applied in the development of fungal species-specific growth media, improvement of fermentation conditions in biotechnology, proper selection and formulation of biological control agents as well as beneficial fungi, development of fungicides, and plant disease management. Therefore, the effect of calcium, iron, manganese, potassium, sodium, and zinc on fungal mycelium growth was studied in their sulfate form. Iron was studied as $FeSO_4$ provided in Czapek's dox agar (CDA) as the basic medium, and other sulfates were separately applied instead of $FeSO_4$ in CDA. Iron is known as an essential inorganic micronutrient and of low toxicity to fungi (Comensoli et al., 2017). Iron is necessary during DNA synthesis (ribonucleotide synthetase) and cleavage (endonuclease III) (Matzanke, 1994). Iron is also a redox-active element and is essential as a cofactor in the form of heme and iron-sulfur clusters in a variety of cellular processes such as respiration, amino acid metabolism, biosynthesis of sterols and DNA, peroxide detoxification and DNA replication (Nevitt, 2011, Schrettl & Haas, 2011, Netz et al., 2012). The multitude of roles of iron and its low environmental bioavailability explain its importance in fungal physiology. Therefore, it is often considered as a limiting factor for fungal growth and development (Comensoli et al., 2017). The absorption of iron in fungi may be mediated by siderophore-dependent (as in *Fusarium graminearum* and *Alternaria brassicicola*) and siderophore-independent pathways, where fungi such as some *Candida* spp. and *Cryptococcus neoformans* heavily rely on the reductive pathway for iron uptake to facilitate their growth and virulence (Gerwien et al., 2018). A study on the mutants of *Aspergillus fumigatus* unable to synthesize iron siderophore indicated that a dramatically attenuated virulence (Hissen et al., 2005). Additionally, other siderophore producing fungi such as *F. graminearum* (Greenshields et al., 2007) or *A. brassicicola* (Oide et al., 2006) cannot fully compensate the loss of siderophore-mediated iron uptake by the reductive uptake system alone (Gerwien et al., 2018). With some fungi, defects in reductive iron assimilation did not lead to any significant effect (Schrettl et al., 2004). Potato dextrose agar (PDA) was used as an organic medium to have better comparisons. Here in this study, the impact of some metal ions was studied on three *Trichoderma* spp. as the representatives of biological control fungi and three pathogenic spp. of increasing importance due to global warmth. Three *Trichoderma* species *viz*., *T. hamatum*, *T. harzianum*, and *T. longibrachiatum* were applied in order to study the impact of the metal ions on the growth and development of these biological control fungi. The pathogenic ascomycetous species included: (i) the soil inhabitant fungus, *F. oxysporum*, one of the most economically important and commonly encountered species of *Fusarium*, an omnivorous cosmopolitan fungus that infects a wide range of monocotyledonous as well as dicotyledonous, herbaceous plants as well as wooden trees. The fungus can also infect animals and humans (Lombard et al., 2019); (ii) the thermophilic generalist soil inhabitant plant pathogen, *M. phaseolina*, which is also of broad host range of at least 500 species in more than 100 plant families (Marquez et al., 2021). The fungus is also known as a human pathogen

(Babu et al., 2011); and (iii) the soilborne fungus, *C. radicicola* that infects palm dates and citrus trees (Mirzaee et al., 2009) in Iran. Due to soilborne nature of the pathogenic fungi in the study, the incubation of cultures was carried out under dark conditions. As soil is biologically a complex medium and most fungi are mesophilic with an optimal growth temperature of 25°C (), therefore incubations were made at 25°C. Additionally, *Trichoderma* fungi usually grow well at 28°C while thermophilic fungi prefer higher temperature. So, the *Trichoderma*-mediated biocontrol of such a group of fungi seems much successful at lower temperature when the inoculum of these pathogens are active but less active.

Table 1. The mean of overall colony growth rate of the tested fungal species on the tested agar media† at 25°C in dark 48 h after inoculation compared by Duncan's test (α= 0.01)

Fungus	Mean Colony Diameter (mm)
Trichoderma longibrachiatum	82.67[a]
Ceratocystis radicicola	71.02[b]
Macrophomina phaseolina	63.26[c]
Trichoderma harzianum	62.48[c]
Trichoderma hamatum	62.40[c]
Fusarium oxysporum	33.81[d]

†The tested agar media were potato dextrose agar (PDA), Czapek's dox agar (CDA) and CDA modified via the replacement of $FeSO_4$ with either of $ZnSO_4$, $MnSO_4$, $CaSO_4$, K_2SO_4, and Na_2SO_4 applied at the same concentration (0.01 g L^{-1})

Growth rates were statistically very different among various combinations of the studied fungal species and agar media 48h after inoculation ($F_{41, 210}$ = 89.04***; CV = 7.19). Also, the rate of mycelial growth was highly significantly affected by the species of the tested fungi ($F_{5, 210}$ = 540.09***; CV = 7.19), the applied metal ion applied via addition of particular sulfate salt ($F_{6, 210}$ = 60.67***; CV = 7.19) and their interactions ($F_{30, 210}$ = 19.54***; CV = 7.19). Comparison of the means growth rates of the fungi through Duncan's multiple range test indicated that based on their growth rates in the second day of incubation, *Trichoderma* species ranked in two different groups (α= 0.01) and *T. longibrachiatum* was statistically of higher growth compared to other species, while *F. oxysporum* was of the least growth 48h after inoculation (Table 1).*T. longibrachiatum* was more competitive than all three pathogenic fungi under experimental conditions. The pathogens are usually encountered in warm seasons or in sub-tropical regions of the world. Comparison of the means growth rates in seven agar media using Duncan's multiple range test indicated that based on their growth rates measured 48h after inoculation, the agar media were of highly significant impact on fungal growth rate (α= 0.01) and PDA supported the highest growth rate compared to other media, while the least growth of fungi was measured on C(Na)A, CDA, and CKA (Table 2).The difference is expectable because of the richness of PDA in organic carbon and other growth factors supplied through potato extract in PDA.

Table 2. Comparison of different agar media from the view point of their overall favorability for the mycelial growth of the tested filamentous fungi at 25°C in dark 48h after inoculation as revealed by Duncan's test (α= 0.01)

Agar Medium†	Mean Colony Diameter (mm)
PDA	73.11[a]
C(Zn)A	66.64[b]
C(Mn)A	64.17[bc]
C(Ca)A	61.44[c]
C(K)A	57.89[d]
CDA	57.75[d]
C(Na)A	57.25[d]

†**PDA:** potato dextrose agar, **CDA:** Czapek's dox agar**, C(X)A:** CDA modified via the replacement of $FeSO_4$ with $ZnSO_4$, $MnSO_4$, $CaSO_4$, K_2SO_4, and Na_2SO_4 applied at the same concentration (0.01 g L^{-1})

Comparison of the interactive impact of each fungal species-agar medium has been presented 48h after inoculation (Table 3). While all of *Trichoderma* species grew well on PDA as well as on the medium amended with Zn, they were generally of the least growth rates in the presence of monovalent ions Na, and K. However, the reactions of *Trichoderma* species to the monovalent ions were not similar. For instance, *T. longibrachiatum* indicated the most tolerance, while *T. harzianum* and *T. hamatum* were rather sensitive to both monovalent ions. Each *Trichoderma* species behaved equally toward both tested monovalent ions. Interestingly, the growth rate of two latter species on CDA plates was not significantly different of their growth rates on the media with either of monovalent ions. Among the tested divalent metal ions, zinc ions led to the highest rate of *Trichoderma* species.

T. hamatum and *T. harzianum* were of the least growth rates on CDA, while *T. longibrachiatum* exhibited its least rate of growth on the medium with calcium ions. Calcium involves in the production of mitochondrial proteins (Walker & White, 2017). The replacement of iron with either of the ions Zn or Mn led to better growth of fungi. This superiority is well explained considering the versatile roles of these two ions in fungal physiology and biochemistry. Zinc exists in the structure of at least four enzymes, alcohol dehydrogenase, Cu-Zn superoxide dismutase, and RNA polymerase. Other enzymes that need zinc for their activities are important enzymes as dehydrogenase, aldolase, isomerase, transphosphorylase, RNA polymerases and DNA polymerases (Marschner, 1995). The first sign of zinc deficiency is the reduction of RNA and ribosomes that leads to the inhibition of protein biosynthesis and as a result to the accumulation of the amino acids, while levels of glucose, non-protein nitrogen and DNA increase. Zinc constitutes the catalytic and/or structural core of many proteins involved, among other functions, in transcriptional control, reactive oxygen species (ROS) detoxification, carbohydrate oxidation and alcoholic fermentation (Murakami & Hirano, 2008, Wilson et al., 2012). Zinc, like iron, is a constant subject of competition during infections, and its sequestration is another aspect of the vertebrate 'nutritional immunity' (Corbin et al., 2008). A similar situation may occur *in planta*. The near-neutral pH lowers the solubility of zinc and restricts its accessibility for microorganisms (Gerwien et al., 2018). The uptake of zinc

from the extracellular environment takes place mainly through two ZRT-IRT-like protein (ZIP) transporters in *Saccharomyces cerevisiae*, the high-affinity Zrt1 (Zhao and Eide 1996a) and low-affinity Zrt2 membrane transporters (Zhao and Eide, 1996b). There are two known classes of eukaryotic zinc transporters: ZIP and the cation diffusion facilitators (Gerwien et al., 2018). The pathogenic fungi express high-affinity membrane zinc importers and specialized secreted zinc uptake proteins known as zincophores, to acquire zinc from the host environment (Citiulo et al., 2012). The negative impact of excessive levels of zinc may be due to its competition with other metals for metal-binding sites in enzymes (McDevitt et al., 2011, Gu & Imlay, 2013) because zinc does not participate in Fenton chemistry (Gerwien et al., 2018). At least some fungi express the low-affinity metal transporters that import several metals into the cell. For instance, S. cerevisiae expresses Fet4 involved in import of zinc, iron, and copper into the yeast cell (Li & Kaplan, 1998). Additionally, there is a phosphate/H+ symporter family member Pho84, a known phosphate transporter, able to import zinc-phosphate complex (Jensen et al., 2003). Zinc exerts its toxic effects on filamentous fungi via three mechanism (Robinson et al., 2021): (i) increased chitin deposition within the cell wall, preventing hyphal extension (He et al., 2011, Lanfranco et al., 2002); (ii) increased hyphal branching and apical swelling (He et al., 2011); interruption of conidia and conidiophore development (Lanfranco et al., 2002).

Table 3. The mean colony growth rate of the tested fungal species on the tested agar media† at 25°C in dark 48h after inoculation compared by Duncan's test (α = 0.01)

Fungus	Agar Medium	Mean Colony Diameter (mm)
Trichoderma longibrachiatum	PDA	90.00^{a}
Ceratocystis radicicola	PDA	89.17^{ab}
Trichoderma hamatum	PDA	88.33^{ab}
Trichodermal ongibrachiatum	C(Zn)A	88.00^{ab}
Trichoderma harzianum	PDA	83.83^{abcd}
Trichoderma longibrachiatum	CDA	83.17^{abcd}
Macrophomina phaseolina	C(Mn)A	81.67^{bcd}
Trichoderma longibrachiatum	C(Mn)A	81.50^{bcd}
Trichoderma longibrachiatum	C(Ca)A	79.33^{cde}
Trichoderma longibrachiatum	C(K)A	78.67^{def}
Trichoderma longibrachiatum	C(Na)A	78.00^{def}
Trichoderma harzianum	C(Zn)A	73.33^{efg}
Ceratocystis radicicola	C(K)A	70.67^{fgh}
Ceratocystis radicicola	C(Na)A	69.83^{gh}
Macrophomina phaseolina	C(Zn)A	69.33^{gh}
Ceratocystis radicicola	C(Mn)A	68.83^{ghi}
Trichoderma hamatum	C(Zn)A	68.17^{ghi}
Ceratocystis radicicola	C(Zn)A	67.50^{ghi}
Ceratocystis radicicola	C(Ca)A	67.50^{ghi}
Trichoderma hamatum	C(Mn)A	65.17^{ghij}
Trichoderma harzianum	C(Ca)A	64.00^{hij}
Ceratocystis radicicola	CDA	63.67^{hijk}
Macrophomina phaseolina	C(Ca)A	63.67^{hijk}
Macrophomina phaseolina	C(Na)A	61.00^{ijkl}
Trichoderma hamatum	C(Ca)A	59.00^{jklm}
Macrophomina phaseolina	C(K)A	58.50^{jklm}
Trichoderma harzianum	CDA	56.83^{klmn}
Trichoderma hamatum	CDA	55.83^{klmn}
Macrophomina phaseolina	PDA	55.67^{klmn}
Trichoderma harzianum	C(K)A	54.67^{lmn}
Trichoderma harzianum	C(Mn)A	54.50^{lmn}
Macrophomina phaseolina	CDA	51.00^{mn}
Trichoderma hamatum	C(Na)A	51.00^{mn}
Trichoderma harzianum	C(Na)A	50.17^{n}
Trichoderma hamatum	C(K)A	49.33^{n}
Fusarium oxysporum	CDA	36.00^{o}
Fusarium oxysporum	C(K)A	35.50^{o}
Fusarium oxysporum	C(Ca)A	35.17^{o}
Fusarium oxysporum	C(Zn)A	33.50^{o}
Fusarium oxysporum	C(Na)A	33.50^{o}
Fusarium oxysporum	PDA	31.67^{o}
Fusarium oxysporum	C(Mn)A	31.33^{o}

†**PDA:** potato dextrose agar, **CDA:** Czapek's dox agar, **C(X)A:** CDA modified via the replacement of $FeSO_4$ with $ZnSO_4$, $MnSO_4$, $CaSO_4$, K_2SO_4, and Na_2SO_4 applied at the same concentration (0.01 g L^{-1}).

Manganese is required in the function of polymerases, sugar transferases of the Golgi and of course for the Mn-Superoxide dismutases especially of the mitochondria (Reddi et al., 2009). The intracellular concentration of manganese varies significantly over nearly two orders of magnitude (Reddi et al., 2009) due to its activity as an antioxidant at higher concentrations, where Mn-complexes can compensate for the deletion of SOD in yeast mutants (Reddi et al., 2009). However, excessive rates of Mn are toxic to yeasts and lead to the induction of apoptosis (Liang & Zhou, 2007). Manganese activates enzymes involved in citric acid cycle. Manganese plays a catalytic role in the activation of several reactions as phosphorylation, decarboxylation, hydrolysis, and other reactions. Therefore, it influences on respiration, and amino acid biosynthesis. Also, non-specific enzymes like phosphikinases, phosphotransferases and nucleotidases are activated by manganese. Manganese is absorbed in *S. cerevisiae* via two Nramp transporters, Smf1 responsible for keeping up the intracellular levels of Mn required for its antioxidant action, and Smf2 that imports Mn for Mn-requiring enzymes (Gerwien et al., 2018). Like Zn, high extracellular manganese can be imported as Mn-phosphate complexes by the yeast Pho84 transmembrane transporter (Jensen et al., 2003). The intracellular manganese can be transported by the Golgi P-type Ca^{2+}/Mn^{2+} ATPase, Pmr1 to

serve as a cofactor in the secretory pathway (Dürr et al., 1998).

Table 4. The mean colony growth rate of the tested fungal species on the tested agar media† at 25°C in dark 72h after inoculation compared by Duncan's test (α= 0.01)

Fungus	Mean Colony Diameter (mm)
Trichoderma longibrachiatum	90.00[a]
Ceratocystis radicicola	90.00[a]
Trichoderma harzianum	87.67[b]
Trichoderma hamatum	86.67[b]
Macrophomina phaseolina	80.98[c]
Fusarium oxysporum	45.67[d]

†The tested agar media were potato dextrose agar (PDA), Czapek's dox agar (CDA) and CDA modified via the replacement of $FeSO_4$ with either of $ZnSO_4$, $MnSO_4$, $CaSO_4$, K_2SO_4, and Na_2SO_4 applied at the same concentration (0.01 g L^{-1})

Table 5. Comparison of different agar media from the view point of their favourability for the mycelial growth of the tested filamentous fungi at 25°C in dark 72h after inoculation as revealed by Duncan's test (α = 0.01)

Agar Medium	Mean Colony Diameter (mm)
C(Ca)A	81.22[a]
C(Zn)A	81.19[a]
C(Mn)A	81.06[a]
C(Na)A	79.75[a]
C(K)A	79.64[ab]
CDA	78.83[ab]
PDA	77.33[b]

†**PDA:** potato dextrose agar, **CDA:** Czapek's dox agar**, C(X)A:** CDA modified via the replacement of $FeSO_4$ with $ZnSO_4$, $MnSO_4$, $CaSO_4$, K_2SO_4, and Na_2SO_4 applied at the same concentration (0.01 g L^{-1})

A homolog of Pmr1 is required for full virulence of Candida albicans due to its cofactor role in glycosylation (Bates et al., 2005). Manganese exerts its toxic impact on filamentous fungi via a mechanism potentially associated to the reduced functioning of manganese peroxidase (Xu et al., 2017). Replacement with calcium resulted in fungal growth rate almost similar to that supported with manganese. Growth rates were statistically very different among various combinations of the studied fungal species and agar media 72h after inoculation ($F_{41, 205}$ = 122.52***, CV = 4.53). Also, the fungi ($F_{5, 205}$ = 942.30***; CV = 4.53), the media ($F_{6, 205}$ = 2.67*; CV = 4.53) and their interactions ($F_{29, 205}$ = 5.98***; CV = 4.53) were of significant effects on the growth. Comparison of the means of growth rates 72h after inoculation of the tested fungi led to their placement in four groups through Duncan's multiple range test (Table 4). Among the tested pathogens, only *C. radicicola* grew well on PDA, and the medium was not so favourable for *M. phaseolina* and *F. oxysporum*. Interestingly, while monovalent Na and K ions reduce growth of *Trichoderma* fungi, they are generally of more favourable impact on the growth of pathogenic fungi, *C. radicicola* and *Fusarium oxysporum*. Potassium is known as a macronutrient in fungal physiology of nutrition (Walker & White, 2017), however, since the replacement of $FeSO_4$ with K_2SO_4 resulted in insignificantly better growth, this contradiction cannot be explained based on the changes in the pH value of the medium. Potassium is involved in ionic balance, osmoregulation, and enzyme activity (Walker & White, 2017). While *C. radicicola* and *T. longibrachiatum* were of the largest colonies, *F. oxysporum* developed the smallest colonies (α= 0.01).Based on table 4, *T. longibrachiatum* was the most fast-growing biological control fungus tested that might compete well with *C. radicicola*, and especially with *F. oxysporum* and *M. phaseolina*. *T. harzianum* and *T. hamatum* might be more competitive than *F. oxysporum* and *M. phaseolina*, however, they could not be expected to compete well with *C. radicicola*. There are other mechanisms of biological control in addition to competition for food and ecological niches exhibited by *Trichoderma* spp. These are antibiosis, parasitism, and induction of plant resistance (Hjeljord & Tronsmo, 1998). Also, considering the information from tables 1 and 4, it is revealed that inoculation of plant organ is important due to late acceleration of growth of some *Trichoderma* spp.Comparison of the means of growth rates in seven agar media using Duncan's multiple range test indicated that based on their growth rates measured 72h after inoculation, the agar media were of highly significant impact on fungal growth rate (α= 0.01) and PDA supported the least growth rate compared to other media (Table 5). Based on table 5, PDA supported the least growth rate of the tested fungi. This may be because of primary fast growth of the fungi that can lead to fast depletion of the medium and accumulation of toxic metabolites excreted by the fungi. Also, some factors in an organic medium such as PDA may act as signals for the induction of secondary metabolite production and secretion, and these can lead to reduced primary metabolism and growth rate after primary induction of a competitive growth for food and energy sources. With CDA and its derivatives, sucrose is the only carbon source and there is no any additional and effective organic matter in the medium. Agar is not considered as a nutritionally valuable matter. Interestingly, calcium and sodium ions increased shifted up fungal growth rate 72h after inoculation, while other metal ions exhibited similar trends during incubation (compare tables 2 and 5). These changes can be explained based on the differences in the rate of growth, so the growth on more favorable media like PDA enters stationary phase faster than other media not much favorable. Comparison of the interactive impact of each fungal species-agar medium has been presented 72h after inoculation (Table 6). Based on the data presented in table 6, different fungal species exhibited different growth rates in the response to the metal ions under study. These differences may reflect the inter-specific differences in metal ion absorption and assimilation, as well as the discrepancies in their tolerance of metal ions. Three lines of fungal defence have been identified (Pócsi, 2011): (i) Extracellular chelation and binding to cell wall constituents. Fungi may secret small molecular mass metal chelators (known as siderophores) as a non-ignorably crucial part of almost all metal/ metalloid detoxification processes (Tamás et al., 2005; Wysocki & Tamás, 2010). Secretion of an average 12-14 siderophores has been reported in *Trichoderma* species including *T. hamatum*, *T. harzianum*, and *T. reesei*, a species

from *Longibrachiatum* clade of *Trichoderma* genus. These siderophores are dimerum acid, coprogen, fusigen, fusarinine A, and the intracellular siderophore ferricrocin (Lehner et al., 2013). Secretion of glutathione (GSH) (Perrone et al., 2005), oxalate (Jarosz-Wilkołazka and Gadd, 2003), extra-cellular mucilaginous materials (ECMMs or emulsifiers) (Vesentini et al., 2006) such as pullulan (Čertik et al., 2005), and glomalin (González-Chávez et al., 2004), and increased synthesis and deposition of chitin and melanin (González-Guerrero et al., 2009) are various reactions of fungi recorded in response to metal ions.

Melanins contain various functional groups which provide an array of multiple nonequivalent binding sites for metal ions (Fogarty & Tobin, 1996). Metal binding depends on pH, type of melanin and metal ion and involves interaction with carboxyl (for example, Mg^{2+}, Ca^{2+}, and Zn^{2+}), amine (for instance, Fe^{2+}), imine (for instance, Fe^{2+}), acetate (for instance, Fe^{2+}) and hydroxyl (for instance, Fe^{2+}, and Cu^{2+}) functional groups of the pigment (Cordero & Casadevall, 2017). In this study, *M. phaseolina*, as a filamentous fungus with highly melanised hyphae was found susceptible to the tested monovalent metal ions. This indicates the limited value of melanin in the tolerance of metal ions, in particular monovalent ions. (ii) Transport, intracellular chelation and compartmentalization. The heavy metal influx through micronutrient metal transporters can be blocked with high concentrations of the essential ions like Zn^{2+}, which leads to the elimination of the zinc-transporter Zrt1p (Gitan et al., 1998, 2003). Also, the overexpression of the transporters which pump toxic metals/ metalloids and/ or their chelates out of the cells or into subcellular organelles (importantly into vacuoles) represents additional mechanism of fungal tolerance. For example, superfluous Zn^{2+} ions can be pumped into vacuoles by Cot1p vacuolar Zn^{2+} transporter as well as Zrc1p vacuolar membrane Zn^{2+} transporter (Conklin et al., 1994), Fe^{2+} and Mn^{2+} by Ccc1p vacuolar transporter (Li et al., 2001). Furthermore, ATP-binding cassette transporters (ABC transporters) are involved in the excretion of GSH and phytochelatin complexes of ions into vacuoles (Song et al., 2003; Ortiz et al., 1995) and out of the cells (Nagy et al., 2006). Phytochelatins and metalloproteins are important intracellular chelators that are respectively involved in the chelation of metal/ metalloid ions, and of metal ions such as Zn^{2+}, and confer tolerance to fungi (Pócsi, 2011). Cu, Zn-superoxide dismutase (Cu/ Zn-SOD) as one of the important parts of the antioxidative defense system of fungi is of high affinity to the intracellular Cu^{2+} and Zn^{2+} ions under both aerobic and anaerobic conditions (Culotta et al., 1995; Avery, 2001). In filamentous fungi, intracellular siderophores like ferricrocin and hydroxyferricrocin keep excess iron in a thermodynamically inert state (Eisendle et al., 2006; Schrettl et al., 2007). The iron regulon and the included siderophore biosynthetic pathways are tightly and negatively regulated by the GATA factor SreA in *Aspergillus fumigatus* (Schrettl et al., 2008). (iii) The antioxidative defense system. Fungi exposed to toxic metal/ metalloid stress commonly face oxidative cell injuries caused by reactive oxygen species (Avery, 2001). Fungal cells possess a wide range of antioxidants to cope with different types of oxidative stresses. For instance, GSH-dependent and –independent enzymes are able to effectively neutralize reactive oxygen species (Pócsi et al., 2004). Cu/ Zn–SOD dismutases buffer redox active Cu^{2+} levels within cytosol (Culotta et al., 1995) but also catalyse the conversion of superoxide to peroxide and oxygen molecule.

Table 6. The mean colony growth rate of the tested fungal species on the tested agar media† at 25°C in dark 72h after inoculation compared by Duncan's test (α = 0.01)

Fungus	Agar Medium	Mean Colony Diameter (mm)
Ceratocystis radicicola	C(Zn)A	90.00^{a}
Ceratocystis radicicola	C(K)A	90.00^{a}
Ceratocystis radicicola	C(Na)A	90.00^{a}
Ceratocystis radicicola	C(Ca)A	90.00^{a}
Ceratocystis radicicola	C(Mn)A	90.00^{a}
Ceratocystis radicicola	CDA	90.00^{a}
Ceratocystis radicicola	PDA	90.00^{a}
Macrophomina phaseolina	C(Mn)A	90.00^{a}
Trichoderma hamatum	C(Zn)A	90.00^{a}
Trichoderma hamatum	C(Mn)A	90.00^{a}
Trichoderma hamatum	PDA	90.00^{a}
Trichodermalongibrachiatum	C(Zn)A	90.00^{a}
Trichoderma longibrachiatum	C(K)A	90.00^{a}
Trichoderma longibrachiatum	C(Na)A	90.00^{a}
Trichoderma longibrachiatum	C(Ca)A	90.00^{a}
Trichoderma longibrachiatum	C(Mn)A	90.00^{a}
Trichoderma longibrachiatum	CDA	90.00^{a}
Trichoderma harzianum	C(Zn)A	90.00^{a}
Trichoderma harzianum	C(Ca)A	90.00^{a}
Trichoderma harzianum	PDA	90.00^{a}
Trichoderma harzianum	CDA	89.33^{ab}
Trichoderma hamatum	C(Ca)A	87.17^{abc}
Trichoderma harzianum	C(K)A	86.67^{abcd}
Trichoderma hamatum	C(Na)A	85.00^{abcd}
Trichoderma hamatum	CDA	84.33^{abcd}
Trichoderma harzianum	C(Mn)A	84.33^{abcd}
Macrophomina phaseolina	C(Na)A	83.67^{abcd}
Trichoderma harzianum	C(Na)A	83.33^{bcd}
Macrophomina phaseolina	C(Ca)A	82.17^{cde}
Macrophomina phaseolina	C(K)A	82.00^{cde}
Macrophomina phaseolina	C(Zn)A	81.83^{cde}
Trichoderma hamatum	C(K)A	80.17^{de}
Macrophomina phaseolina	PDA	76.17^{ef}
Macrophomina phaseolina	CDA	71.00^{f}
Fusarium oxysporum	C(K)A	49.00^{g}
Fusarium oxysporum	CDA	48.33^{gh}
Fusarium oxysporum	C(Ca)A	48.00^{gh}
Fusarium oxysporum	C(Na)A	46.50^{ghi}
Fusarium oxysporum	C(Zn)A	45.33^{ghi}
Fusarium oxysporum	C(Mn)A	42.00^{hi}
Fusarium oxysporum	PDA	40.50^{i}

†**PDA:** potato dextrose agar, **CDA:** Czapek's dox agar**, C(X)A:** CDA modified via the replacement of $FeSO_4$ with $ZnSO_4$, $MnSO_4$, $CaSO_4$, K_2SO_4, and Na_2SO_4 applied at the same concentration (0.01 g L^{-1}).

Additionally, the enzyme is secreted to the environment by Zn^{2+}-tolerant ericoid mycorrhizal fungus Oidiodendronmaius and may be involved in in the metal tolerance (Vallino et al., 2009). Based on the findings from this study, *T. longibrachiatum* seems a superior biological control agent against summer crop fungal pathogens. Also, it seems that the addition of zinc sulfate can improve biological control of plant pathogens such as *C. radicicola*, *Fusariumoxysporum*, *M. phaseolina*. This is important to remind that zinc sulfide minerals are certainly common enough in the biosphere, but in this form Zn is not very usable. Only few organisms are able to mobilize Zn from such a source (Lyons & Eide, 2014).

The biological control potential of *T. longibrachiatum* against *M. phaseolina* has recently indicated (Sridharan et al., 2021). Anyway, these differences can be of practical applications in fungal biotechnology and fermentation, the facilitated isolation of fungal species on agar media, in the development of more efficient formulations for biological control fungi (here *Trichoderma* spp.), in the integrated management of soil-born plant diseases through the improvement of soil biology through rational application of metal ions that are also of importance in plant nutrition, and in the predictions of soil-born plant disease incidence in special soil chemical (as well as physical) conditions. The information in the tables 4 and 6 give clues to comprehensive studies on soil incorporation impact of starchy plant materials and metal sulfates on the biological control of charcoal rot and fusarium vascular wilt diseases by *Trichoderma* species.

CONCLUSION

The study indicates that while biological control fungi from three different sections of the genus *Trichoderma* grow well on the rich medium, potato dextrose agar and in the modified synthetic medium made via the replacement of its content of $FeSO_4$ with $ZnSO_4$ [Here shown as C(Zn)A], phytopathogenic fungi such as *F. oxysporum* and *M.phaseolina* grow more on other media. The results imply to the positive effect of organic matter incorporation in soil, as well as the use of zinc sulfate in the management of fungal soil-borne diseases caused by the tested pathogens i.e. *C. radicicola*, *F. oxysporum*, and *M. phaseolina.* Synchronous use of zinc sulfide and effective *Trichoderma* isolates seems useful in the control the tested pathogens, however, further studies are still required.

AUTHOR CONTRIBUTIONS

All theoretical and practical steps of the current paper except the statistical analyses of the data have been performed by the author. Also, the results of statistical analyses were interpreted and discussed by the author.

ACKNOWLEDGMENTS

The current paper was prepared based on the results of the research project no. 971.42 financially supported by Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Khuzestan, Iran. The author thanks Mr. Dr. Mohamad Farkhari for his contribution to the statistical analysis of the data.

COMPETING INTERESTS

Not applicable

ETHICS APPROVAL

Not applicable

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