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Improvement of copper stress tolerance in pepper plants (*Capsicum annuum* L.) by inoculation with arbuscular mycorrhizal fungi

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Abstract The effect of arbuscular mycorrhizal fungi (AMF) inoculation, on pepper plant growth and physiological parameters in response to increasing soil Cu concentrations was studied. Treatments consisted of inoculation or not with *Funneliformis mosseae* or *Rhizophagus intraradices* and the addition of Cu to soil at concentrations of 0, 2, 4 and 8 mM $CuSO_4$. The increase in copper concentration diminished the inoculation in all treatments. The highest experimental concentration of Cu (8 mM) reduced significantly the hyphae viability and ALP activity, regardless of the inocula used. The total dry weight and the leaf area were higher for mycorrhizal plants. The mycorrhizal dependence was 30 and 50% for plants inoculated with *F. mosseae* and *R. intraradices*, respectively at 8 mM $CuSO_4$. The electrolyte leakage was higher at higher Cu concentrations, in roots and leaves. Net photosynthetic rates and transpiration were lower in plants treated with Cu, regardless of the inocula. At low Cu concentration in soil no differences were observed in Cu content in the shoots and roots. At 4 mM Cu, Cu content in roots was significantly higher than in shoots. At 8 mM Cu, in non-inoculated plants accumulate more Cu than inoculated plants in the roots, leaves and fruits regardless of the inocula. Beneficial microbial inoculants such as AMF, is an attractive strategy to farmers in the context of sustainable agriculture. Pre-inoculation in transplants could be an adequate practice to alleviate the deleterious effects in stress of pepper plants. However different AMF species can differ in their ability to minimize stress effects and promote plant growth.

Keywords *Capsicum annuum* · Copper stress · Arbuscular mycorrhiza · *Funneliformis mosseae* · *Rhizophagus intraradices* · Heavy metals

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1 Introduction

Pollution of soil and water with heavy metals creates serious problems for the environment and human health. Heavy metals (HM) are one of the main sources of environmental pollution (Il'yasova and Schwartz 2005) and are responsible for several problems, associated with industrial and agricultural activities: decrease of microbial activity, soil fertility and crop yield (Yang et al. 2005). Plant tolerance to HM can be achieved through a range of physiological and morphological changes (Hall 2002).

Arbuscular mycorrhizal fungi (AMF) are important soil microorganisms that form symbiotic associations with most vascular plant families. (Göhre and Paszkowski 2006). These fungi provide their host plants an efficient supply of mineral nutrient, mainly phosphorus and some micronutrients as Cu and Zn. Similarly, some mycorrhizal fungi have been shown efficient to reduce accumulation of certain HM in shoots and it is presumed that this is due to increased retention within the root/mycorrhizal structures (Chen et al. 2004; Zhang et al. 2005). There are a number of possible explanations for the observed reductions in HM concentrations in shoots of mycorrhizal plants. These include (1) greater selectivity of metal transporters in the cell membranes of mycorrhizae, (2) compartmentation of metals in fungal vacuoles, thereby reducing transfer of the metals to the plant, and (3) increased capacity of mycorrhizal roots to immobilize metals within their cell walls. Joner and Leyval (1997) reported that the AMF *Funneliformis mosseae* had a high capacity for Cd uptake but they did not distinguish between immobilization on cell walls or in vacuoles. Despite the apparent versatility of different types of mycorrhizae to accumulate heavy metals, how this is largely achieved remains a mystery (Zhang et al. 2009). AMF colonizes the root cortex of most plant species and develop an extraradical mycelium which spreads through the plant roots and the soil surrounding. By increasing the interface between plants and the soil environment, they contribute to plant uptake of macronutrients (P and N) as well as micronutrients (Cu and Zn) (Smith and Read 2008). On the other hand, under conditions of supraoptimal levels of essential metals, or in the presence of toxic ones, AMF are able to alleviate metal toxicity in the plant (Leyval et al. 2002). Despite the significant role that AMF play in plant interactions with soil metals and the ubiquity of AMF in soil environments, only recently progress has been made towards understanding the cellular mechanisms used to control HM and to avoid their toxicity in mycorrhizal plants (Göhre and Paszkowski 2006; Hildebrandt et al. 2007; González-Guerrero et al. 2008).

Copper (Cu) is an essential element for plant growth, and plays a significant role in many physiological processes, including photosynthesis, respiration, carbohydrate distribution, nitrogen fixation, protein metabolism, and antioxidant activity. At cellular level, Cu is a structural and catalytic component inO many proteins and enzymes involved in a variety of metabolic pathways (Pilon et al. 2006). Plants usually find an ample supply of copper in soils; but at high concentrations, the metal can be a stress factor, triggering physiological negative responses. Copper concentrations in cells need to be maintained at relative low concentrations (Yruela 2005). Some HM, such as Zn, Cu, Mn, Ni and Co are essential micronutrients for plant growth, while others, such as Cd, Pb and Hg, have unknown biological function and interfere with several physiological processes, reducing plant growth, photosynthesis and consequently the biomass (Jamal et al. 2006). Though Cu is a component of both the photosynthetic (plastocyanin) and respiratory electron chains (cytochrome oxidase), and also of various proteins, excessive Cu in the growth environment causes changes in membrane permeability, protein synthesis, enzyme activity, photosynthesis and respiratory processes through its phytotoxic effect, and also in lipid peroxidation and activates senescence (Yurekli and Porgali 2006; Andrade et al. 2010). The accumulation of HM in the environment is a serious concern for agriculture, animal and human health. Among heavy metals, Cu has been classified as a non biodegradable metal pollutant which enters the environment through various anthropogenic activities such as pesticides, fungicides and municipal sewage. Also, Cu is a powerful inhibitor of chlorophyll synthesis in *Phaseolus vulgaris* (Pätsikkä et al. 1998), in wheat (Quartacci et al. 2000) in barley (Stiborová et al. 1986).

Participation of mycorrhizal fungi in HM metabolism has been proposed as a mechanism to increase plant tolerance. In some cases HM can be absorbed by fungi hyphae and transported to the plant (phytoextraction), in other cases fungi contribute to HM immobilization in the soil (phytostabilization) (Khan 2005). AMF are critical for the survival of host plants growing on metal-contaminated soils because of their beneficial role in enhancing metal tolerance and nutrient acquisition (Audet and Charest 2006). In this context, associations among plants and AMF are of great importance because AMF assist in plant growth and may also contribute to a reduction in the availability of HM as a result of their immobilization in fungal structures (Gonzalez-Chavez et al. 2002; Andrade et al. 2010; Cornejo et al. 2013). Nevertheless, the overall mechanisms by which AMF alleviates metal phytotoxicity in hosts are still not completely

elucidated, with controversial results depending on the specific plant/fungal/metal species interactions (Andrade et al. 2010). Mycorrhizal roots may act as a barrier against metal transport from roots to the aerial part of the plant. This effect is attributed to metal adsorption on the hyphal walls (Joner et al. 2000). Another possible tolerance mechanism is the dilution of HM concentrations by growth increase of mycorrhizal plants. Moreover, Ruscitti et al. (2011) determined that in pepper plants mycorrhizal dependency without Cr was 18% and 33% for plants inoculated with G. *mosseae* and *G. intraradices*, respectively and 33% and 57% at the highest Cr concentration.

The hypothesis of our work was that inoculation with *Rhizophagus intraradices* or *F. mosseae* have a protective action against heavy metals disturbance, increasing pepper plant (*Capsicum annuum* L.) tolerance to Cu. The aim of this investigation was to test the hypothesis that *R. intraradices* or *F. mosseae* could enhance Cu phytoaccumulation and increase plant tolerance as determined by plant growth, stability of membranes and gas exchange of pepper plants.

2 Materials and methods

2.1 Growth conditions

Seeds of pepper (*Capsicum annuum* L. 'California Wonder 300') were sown in plastic pots previously filled with substrate composed of a mixture of soil (Argiudol vertic, pH 5.5, 12 mg kg^{-1} total P, 3.5% organic matter, 2% total C and 0.24% total N) perlite and vermiculite (2:1:1) tindalized at 100 °C for 60 min, during 3 consecutive days, for excluding native AMF propagules. *F. mosseae*, Schenck & Smith isolate GA1 and *R. intraradices* (Nicolson & Gerdemann) Gerdemann & Trappe (Banco de Glomeromycota In Vitro BGI, Buenos Aires, Argentina) were bulked-up through culture with *Trifolium repens* L. for four months in a semi-controlled growth chamber. The inoculum (10% substrate weight), a mix of soil, spores (50 spores g^{-1} inoculum) mycelium and root fragments colonized by *F. mosseae* or *R. intraradices*, was added to the substrate at sowing time. The same amount of sterilized inoculum was added to noninoculated pots in order to provide the same soil conditions. When root colonization with *R. intraradices* was approximately 50% and with *F. mosseae*, 30%, inoculated and non inoculated pepper young plants were transplanted to 500 mL pots containing the same unsterilized soil. At the same time, ten plants per treatments were transplanted to 10 litter pots, to study Cu partition and were harvested 120 days after transplanting. Before transplanting, different concentration of Cu in the form of $CuSO_4 \cdot 5H_2O$ were added to the substrate to reach concentrations of 0 mM $CuSO_4 \cdot 5H_2O$ (Cu0); 2 mM $CuSO_4 \cdot 5H_2O$ (Cu2); 4 mM $CuSO_4 \cdot 5H_2O$ (Cu4) and 8 mM $CuSO_4 \cdot 5H_2O$ (Cu8).

Pepper plants were grown in a greenhouse, under natural conditions (average temperature was: for September 11.5 °C; October 15.2 °C; November 19.0 °C and December 20.3 °C, and the fotoperiod was between 12/12 h and 14/10 h day/night).

The treatments were: (a) control (NI), the plants received no mycorrhizal inoculation: NICu0, without $CuSO_4$; NICu2, 2 mM $CuSO_4$; NICu4, 4 mM $CuSO_4$; and NICu8, 8 mM $CuSO_4$; and (b) inoculated plants (M) with: *F. mosseae* or *R. intraradices*, MmosCu0 MintraCu0 without $CuSO_4$; MmosCu2 or MintraCu2, 2 mM $CuSO_4$; MmosCu4 or MintraCu4, 4 mM $CuSO_4$; and MmosCu8 or MintraCu8, 8 mM $CuSO_4$.

At 51 days after transplanting, ten plants (500 mL pots) were randomly selected from each treatment: Dry matter *per* plant (DW) was determined, dried in a stove at 80 °C until constant weight, and leaf area (LA) were measured (Li 3000 leaf area meter, LICOR, Lincoln, NE, USA).

For assessing AMF colonization and fungal viability, an adequate amount of fresh fine roots was collected from ten plants sampled at each selected pot. Fungal colonization was assessed according to Trouvelot et al. (1986) and expressed as rate of mycorrhization (M%). Roots were cleared with 10% KOH (m/v) and stained with trypan blue in lacto-phenol (Phillips and Hayman 1970). The viability of hyphae was determined by measuring succinate dehydrogenase activity (SDH) (Schaffer and Peterson 1993). Three replicates of 10 randomly chosen root fragments were mounted on slides and examined microscopically. M% was calculated as the proportion of infected roots over total root fragments.

Alkaline phosphatase (ALP) activity in roots was determined according to Beltrano et al. (2013).

Mycorrhizal dependency (MD) was calculated using the following formula (Plenchette et al. 1983).

$$\mathrm{MD} = \frac{\text{DW inoculated plants} - \text{DW of non inoculated plants}}{\text{DW of inoculated plants}} \times 100$$

DW is the dry weight.

2.2 Carotenoids and chlorophyll contents

Carotenoids and chlorophyll contents were determined in one leaf disc (1 cm in diameter) per plant. The concentration of carotenoids and the content of chlorophyll were measured according to Wellburn (1994). All absorption spectra were recorded in a Shimadzu UV-160 spectrophotometer (Kyoto, Japan). Results were expressed as mg carotenoids cm^{-2} and mg chlorophyll cm^{-2}.

2.3 Cell membrane stability (CMS) or electrolyte leakage (EL)

This technique is based on the increase of cellular membrane permeability and concomitantly greater electrolyte diffusion out of cells when tissue is injured by a stressful condition. The EL was measured as described by Lutts et al. (1996) with a few modifications. After harvest, the uppermost fully expanded leaves of 10 plants per treatment were immediately cut into four discs of 1 cm in diameter or similar roots weight. Leaf disc or roots were washed three times in deionized water to remove solutes released during cutting. EL was determined by measuring the electrical conductivity of the vial solution, using a conductimeter and data were expressed as mS cm^{-1}. Relative electrical conductivity (EL) was calculated as follows:

$$\mathrm{EL}(\%) = (\mathrm{ELi}/\mathrm{ELf}) \times 100$$

ELi: initial and ELf: final.

2.4 Leaf gas exchange

Net photosynthetic rate (Pn), stomatal conductance (gs) and transpiration (E) were measured on the second fully expanded leave of each plant using a LI-COR 6400 system (LI-COR Inc., Lincoln, NB, USA), between 09:00 h and 13:00 h. Average leaf temperature, leaf-to-air difference in water mole fraction, ambient CO_2 mol fraction and photon flux density were, respectively: 25 °C, 11 mmol mol^{-1}, 360 µmol mol^{-1} and 1500 µmol m^{-2} s^{-1}. Other ten plants (10 L pots) were randomly selected from each treatment, for determining metal accumulation 120 days after transplanting. Plants were separately washed to remove adhering soil particles, and separated into root, shoot, lower leaves (leaves of the inferior half) and top leaves (leaves of the superior half), and fruits. Then, samples were oven-dried at 80 °C till constant weight. The plant parts were then weighed and pounded separately (for metal concentration analysis).

2.5 Metal analysis

One gram of each plant sample (each in triplicate) was digested overnight with a mixture of HNO_3:$HClO_4$ (4:1, v/v). Samples were slowly digested on the hot plate until a clear solution was obtained (Barman et al. 2000). Digestion of plant parts were made by atomic absorption spectrophotometry (AAS) (Varian Spectra AA-250 Plus) and use of blanks to exclude introduction of impurity through reagents. The AAS value of blank (without sample) of metal was deducted from the sample value for final calculations.

Uptake efficiency (UE): was determined as Cu uptake by plants/roots dry weight (µg Cu/g root DW).

Translocation efficiency (TE) was determined as the ratio of Cu content in shoots ((both stem and leaves)/Cu content in roots (µg Cu g shoot DW/µg Cu g root DW)).

Phytoextraction efficiency (PE) was determined as the ratio of Cu uptake by shoots ((both stem and leaves)/roots dry weight (µg Cu/g root DW)).

The copper dependency (DCu) was determined as the ratio: Total DW (Cu0) − Total DW (Cu)/Total DW (Cu0) × 100.

2.6 Data analysis

The experiment was a 3 × 4 factorial, in a completely randomized design with three mycorrhizal levels (NI, Mmos, Mintra) and four levels of Cu (Cu0, Cu2, Cu4, Cu8). Data were analyzed by ANOVA, and comparison were made using LSD ($p < 0.05$). For the statistical analysis all inoculation percentage values were arcsine transformed to improve homogeneity. The number of replicates was: for growth data (n = 10), and for mycorrhizal observations (n = 3 replicate of 30 roots fragments).

3 RESULTS

3.1 Mycorrhizal analysis

Non-inoculated plants were colonized by native fungi. Both inoculation and Cu addition had a significant effect, and the interaction was also significant. Without Cu, the colonization level was 48% in non-inoculated plants, meanwhile with *F. mosseae* was 46% and with *R. intraradices* it was 73%. The percentage of arbuscules and vesicles was higher in plants inoculated with *R. intraradices* than in plants colonized with *F. mosseae* or with native inoculum (data not shown). The increasing Cu concentration in soil diminished the mycorrhizal colonization in all treatments. In the Cu8mM treatment, the colonization was 4% in non-inoculated plants, while in Mmos and Mintra, it was colonized by 25% and 58%, respectively. These data showed a greater sensitivity of the native inoculum to high Cu concentration in substrate (Table 1).

3.2 Fungal viability (SDH) and alkaline phosphatase (ALP)

Viability of hyphae, expressed by SDH activity, in plants grown on substrate contaminated by Cu and inoculated or not inoculated with *F. mosseae* and *R. intraradices* was 43, 42 and 60%, respectively, and the viability was not affected by 2 mM of Cu. Viability of hyphae was higher in plants inoculated with *R. intraradices* than in those inoculated with *F. mosseae*. The highest concentration of Cu (8 mM) reduced significantly the viability and was 2.21% and 53% in non inoculated plants and those inoculated with *F. mosseae* and *R. intraradices* respectively. Both inoculation and Cu addition had a significant effect, and the interaction was also significant (Table 1). The ALP activity was lower in non inoculated plants compared with inoculated plants, regardless of Cu availability and the inocula ($p < 0.001$). The ALP activity decreased with the increasing of Cu in soil, regardless of the inocula used. When comparing Cu0 with Cu8 in non-inoculated plants, the activity of ALP diminished by 86%, while it was reduced by 62% and 47% in Mmos and in Mintra, respectively (Table 1).

3.3 Growth parameter

Total dry weight and LA were higher for inoculated plants than for non-inoculated ones, regardless of the inocula used and Cu treatments. The enhancement in dry weight due to inoculation with *F. mosseae* and *R. intraradices* was more than 30% and 50%, respectively under a high Cu concentration (8 mM CuSO4), compared to NI plants (Fig. 1). The M × Cu interaction was significant ($p < 0.05$ %).

Mycorrhizal dependency without Cu was 14.8% and 21.65% for plants inoculated with *F. mosseae* and

Table 1 Mycorrhizal colonization (MC), mycorrhizal dependence (MD), percentage of viable hyphae (SDH) and alkaline phosphatase activity (ALP) in *Capsicum annuum* L. plants non-inoculated (NI) or inoculated with *Funneliformis mosseae* (FM) and *Rhizophagus intraradices* (RI), grown in soil with increasing Cu concentrations

Cu (mM)	%MC			%MD			%SDH			ALP (UI mg^{-1})		
	NI	FM	RI	NI	FM	RI	NI	FM	RI	NI	FM	RI
0	48.0aB	46.0aB	73.3aA		14.8bA	21.6bA	43.0aB	42.0aB	60.0aA	1.5aB	2.9aA	3.2aA
2	44.0aB	42.0aB	66.2aA		39.5aA	36.5aA	43.0aB	41.0aB	61.0aA	1.3aB	2.3aA	2.7aA
4	36.0aB	36.0aB	61.0aA		18.9bA	15.1bA	33.0aB	30.0aB	57.0abA	1.3aB	1.7bA	1.9bA
8	4.0bC	25.0bB	58.0bA		30.0aB	49.8aA	2.0bC	21.0bB	53.0bA	0.2bC	1.1cB	1.7bA
M		<0.001			<0.05			<0.001			<0.001	
Cu		<0.001			<0.001			<0.001			<0.001	
M × Cu		<0.001			ns			<0.001			<0.05	

M mycorrhization, *ns* non significant

For each Cu concentration (on each line), same uppercase letters are not significantly different between NI, FM and RI by LSD test ($P < 0.05$). For NI, FM and RI (on each column) same lowercase letters are not significantly different between Cu concentrations by LSD test ($P < 0.05$)

R. intraradices, respectively, and 30% and 49.8% for plants ionoculated with *F. mosseae* and *R. intraradices,* respectively, at the highest Cu concentration (Table 1). The M × Cu interaction was not significant ($p < 0.05$ %).

Leaf area was higher in inoculated plants than in non inoculated ones, regardless of Cu treatments. Copper reduced LA in all treatments, regardless of mycorrhizal treatments. Compared to the control without Cu, the plants inoculated with *F. mosseae* and with *R. intraradices* showed a 25% higher LA, in relation to NI plants. In comparison to NI plants, the ones exposed to 8 mM Cu showed a 36% higher LA when inoculated with *F. mosseae* and 30% higher LA when inoculated with *R. intraradices* (Fig. 2). In addition, M × Cu interaction was not observed (Fig. 2).

3.4 Chlorophyll and carotenoids contents of leaves

Total chlorophyll, chlorophyll *a* and *b*, and carotenoids contents were not affected by the treatments, regardless of the inocula and Cu (data not shown).

3.5 Determination of electrolyte leakage

Cell membrane stability (CMS) or EL was affected by Cu and inocula, and showed significant differences in roots and leaves of inoculated and non inoculated plants. EL was higher with the increase of Cu concentrations, in roots and leaves, regardless of the inocula. Electrolyte leakage increased when Cu concentration increased and showed significant differences in roots of non inoculated plants and, in those inoculated with *R. intraradices*. Significant differences were observed in leaves, in non inoculated and inoculated plants, regardless of the inocula used. Leakage of root solutes increased due to high Cu concentrations and due to mycorrhization. In non inoculated plants the highest EL was observed in Cu8mM. Compared to Cu0mM, such increase was of 46% and 37% in root and leaves, respectively. In roots of plants inoculated with *F. mosseae* EL did not show differences in response to the increase of Cu. In plants inoculated with *R. intraradices*, EL showed an increase of 46% and 19% in roots and leaves, respectively between Cu0mM and Cu8mM respectively (Table 2). The interaction between M × Cu was significant for EL in roots. On the other hand, the interaction between M × Cu was not significant for EL in leaves.

3.6 Leaf gas exchange

Plants grown on contaminated soil showed significant reductions in gs (data not shown) and photosynthetic rate at increasing Cu, as compared to those grown in uncontaminated soil.

Net photosynthetic rates were lower in Cu treated plants compared to the control plants, regardless of the inoculum. The reduction of net photosynthesis was higher in NI (29%) than in plants contaminated with *F. mosseae* (23%) and *R. intraradices* (20%), when comparing Cu0mM versus Cu8mM. Differences in

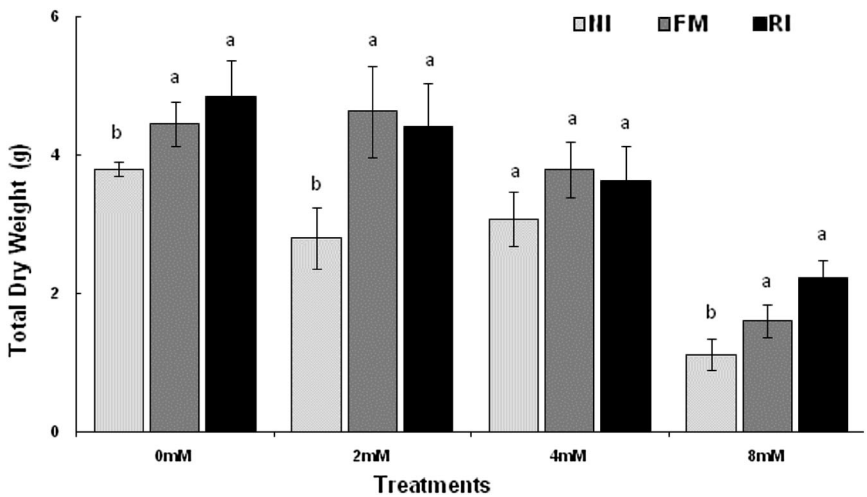

Fig. 1 Total dry weight of *Capsicum annum* L. not inoculated and inoculated with *Funneliformis mosseae* (FM) and *Rhizophagus intraradices* (RI), grown on soil with increasing Cu concentrations. Columns are mean values (n = 10) and *bars* are SE. For each Cu concentration, *different letters* represent significant differences between NI, FM and RI by LSD ($P < 0.05$)

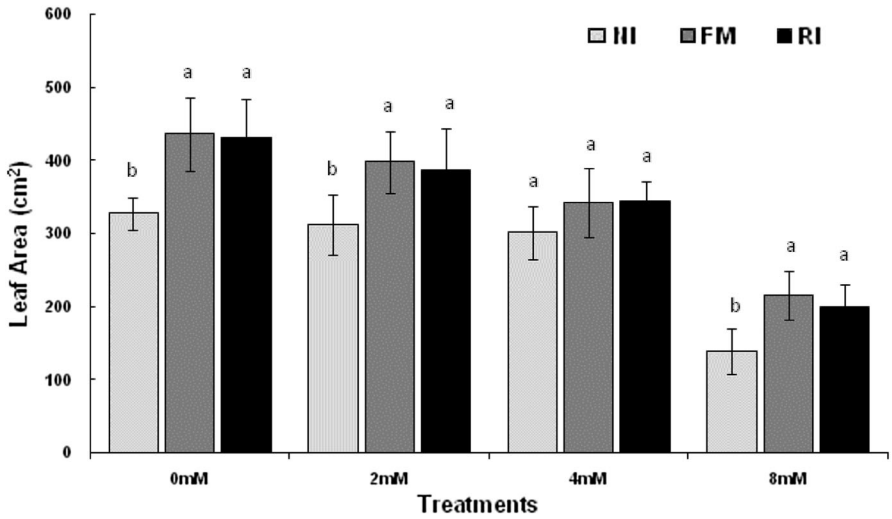

Fig. 2 Leaf area of *Capsicum annum* L. not inoculated and inoculated with *Funneliformis mosseae* (FM) and *Rhizophagus intraradices* (RI), grown on soil with increasing Cu concentrations. Columns are mean values (n = 10) and *bars* are SE. For each Cu concentration, *different letters* represent significant differences between NI, FM and RI by LSD ($P < 0.05$)

Table 2 Electrolyte leakage (EL) from root and leaf tissues in *Capsicum annuum* L. plants not inoculated (NI) and inoculated with *Funneliformis mosseae* (FM) and *Rhizophagus intraradices* (RI), and grown on soil with increasing Cu concentrations

Cu (mM)	EL roots (%)			EL leaf (%)		
	NI	FM	RI	NI	FM	RI
0	34.00bA	31.20aAB	28.21 dB	29.11bA	31.73bA	30.4bA
2	37.56bA	30.50aB	32.54cB	33.36bA	32.03abA	33.57abA
4	37.12bA	30.23aA	35.43bA	33.48bA	34.59aA	35.27aA
8	49.6aA	40.56aC	43.80aB	40aA	38.00aA	36.28aA
M		<0.001			ns	
Cu		<0.001			<0.001	
M × Cu		<0.05			ns	

M mycorrhization, *ns* non significant

For each Cu concentration (on each line), same uppercase letters are not significantly different between NI, FM and RI by LSD test ($P < 0.05$). For NI, FM and RI (on each column) same lowercase letters are not significantly different between Cu concentrations by LSD test ($P < 0.05$)

transpiration rates between Cu0mM and Cu8mM was similar to those in photosynthesis between these same treatments. It was reduced by 17%, 34% and 28% in NI plants and in those inoculated with *F. mosseae* and *R. intraradices,* respectively. However, WUE was not affected in inoculated plants and it was only reduced in non inoculated plants at the highest Cu concentration (data not shown). The interaction M × Cu was not significant (Table 3).

3.7 Copper uptake

Copper content was at the basal level (<10 μg g^{-1} DW) in all untreated Cu (Cu0) plant fraction. No significant differences in Cu concentrations were detected in pepper plants not exposed to Cu (Cu0mM), regardless of mycorrhizal treatments, either in shoots (stem + lower leaves + top leaves) fruits and roots. The positive effects of AMF on Cu nutrition was also evident in different tissue types. In general, the Cu concentration in roots, shoots (stem + lower leaves + top leaves) and fruits tended to increase with the increasing of Cu in the substrate. The copper dependency (DCu) was higher in inoculated plants. In non inoculated plants, at low Cu concentration Cu dependence was negative. The Cu content in plants increased with the increase of Cu in the soil. At Cu0mM and Cu1mM no significant differences were observed in Cu content in shoots (stem + lower leaves + top

Table 3 Net photosynthetic rate (Pn), and transpiration (*E*) in leaves of *Capsicum annuum* L. plants not inoculated (NI) and inoculated with *Funneliformis mosseae* (FM) and *Rhizophagus intraradices* (RI), grown on soil with increasing Cu concentrations

Cu (mM)	Pn (μmol m^{-2} s^{-1})			E (mmol m^{-2} s^{-1})		
	NI	FM	RI	NI	FM	RI
0	7.01aA	7.67aA	7.62aA	2.18aA	2.8aA	2.88aA
2	6.32abA	6.32bA	7.20abA	1.95aB	2.35abB	2.92aA
4	5.43bcA	6.10bA	6.32bA	1.93aA	2.27bcA	2.18bA
8	4.98cB	5.90bA	6.13bA	1.82aB	1.85cAB	2.08bA
M		<0.05			<0.001	
Cu		<0.001			<0.05	
M × Cu		ns			ns	

M mycorrhization, *ns* non significant

For each Cu concentration (on each line), same uppercase letters are not significantly different between NI, FM and RI by LSD test ($P < 0.05$). For NI, FM and RI (on each column) same lowercase letters are not significantly different between Cu concentrations by LSD test ($P < 0.05$)

leaves) and fruits and in roots of the non inoculated or inoculated plants. At Cu2mM, Cu content in roots was significantly higher than in shoots, regardless of the inocula used and, in inoculated plants it was higher than non-inoculated plants. At the highest Cu concentration, Cu content was significantly higher compared with Cu2mM, and non inoculated plants accumulated more Cu in the roots, top leaves and fruits than inoculated plants. No differences were observed between *F. mosseae* (data not shown) and *R. intraradices* (Fig. 3).

We analyzed the dry weight relationship between plants grown on soil contaminated with Cu (DWCu) versus the controlled plants grown without Cu (DW). Copper Uptake efficiency, Translocation efficiency and phytoextraction efficiency, increased with increasing amount of Cu added. Copper affected more inoculated plants than non-inoculated ones because when not exposed to Cu (Cu0mM) inoculated plants showed a more vigorous growth when compared to non-inoculated plants. The DWCu was higher in mycorrhizal plants. Copper translocation from root to shoot was measured by translocation efficiency (TE). The non inoculated or inoculated pepper plants exhibited significant differences in Cu absorption and translocation, assessed by TE values. When TE > 1, it means that translocation of metals effectivelly occurred from root to the shoot (Table 4). The efficiency of Cu uptake, Cu translocation efficiency and phytoextraction efficiency was higher in inoculated plants grown in 2 mM Cu, while there were no differences in 0, 1 and 4 mM Cu (Table 4).

4 Discussion

According to our results, the hypothesis proposed is accepted, the inoculation with mycorrhizal fungi increased the tolerance to Cu, the plant growth, stability of membranes and photosynthetic activity. The excess of Cu in plant tissues may affect several physiological and biochemical processes, including photosynthesis (Göhre and Paszkowski 2006; Kabata-Pendias and Pendias 2001). The tolerance to heavy metal in mycorrhizal plants could be associated with a reduction of metal transfer into shoots; in certain cases, mycorrhiza improves plant growth but also increases the accumulation of HM in plant organs (Leyval et al. 2002; Jamal et al. 2002; Chen et al. 2004; Zhang et al. 2005; Khan 2005; Pilon et al. 2006; Hall 2002; Jamal et al. 2006). Heavy metal disturbances reduces the dry matter in shoots and roots, and also leaf expansion (Minnich et al. 1987; Chen et al. 2004; Zhang et al. 2005; Ruscitti et al. 2011; Santana et al. 2015). In the present study, the concentration of Cu was higher in roots than in shoots. Plants grown on soil contaminated with Cu showed significant reductions in stomatal conductance (data not shown) compared with those grown in non-contaminated soil. The content of chlorophyll and carotenoids were not

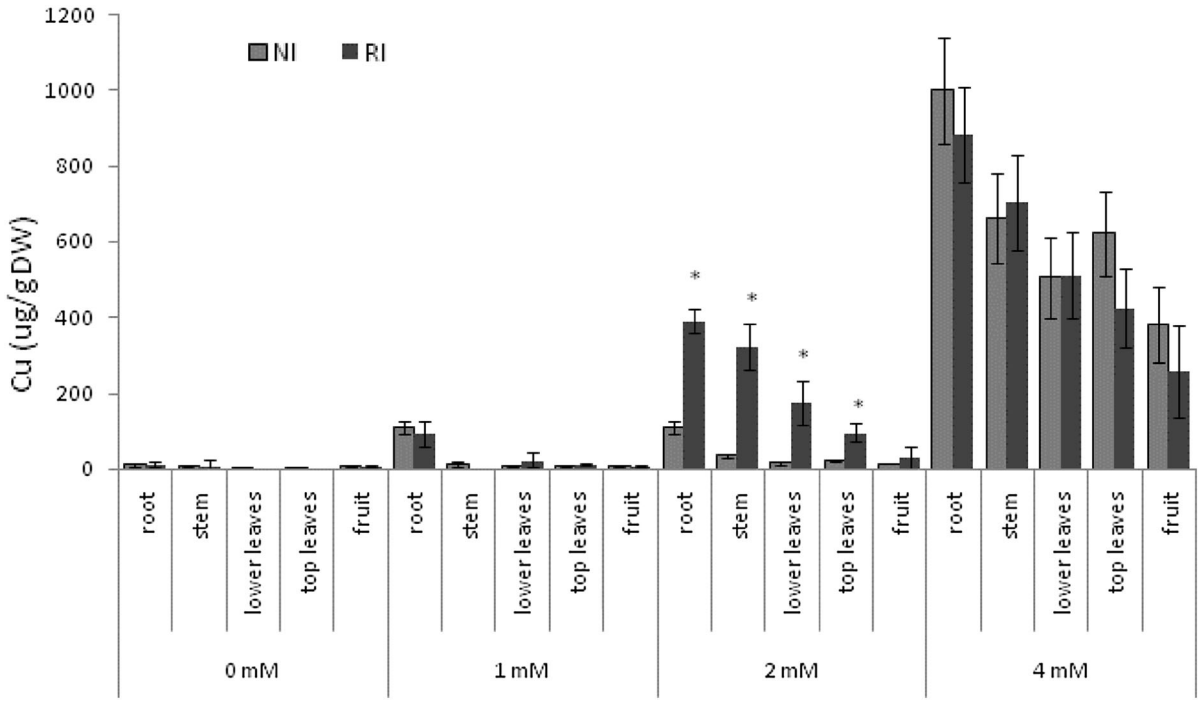

Fig. 3 Copper content (μg Cu/g DW) in *Capsicum annuum* L. plants not inoculated (NI) and inoculated with *Rhizophagus intraradices* (RI), when grown on soil with 0, 1, 2 and 4 mM Cu. Columns are mean values (n = 10) and *bars* are SE. *Asterisks* represent differences by LSD ($P < 0.05$) between NI and RI

Table 4 Uptake efficiency (UE), translocation efficiency (TE), phytoextraction efficiency (PE), dependency by copper (DCu) in *Capsicum annuum* L. plants not inoculated (NI) and inoculated with *Rhizophagus intraradices* (RI), grown on soil with increasing Cu concentrations

Cu (mM)	Non inoculated (NI)				*Rhizophagus intraradices* (RI)			
	UE (μg g^{-1})	TE	PE (μg g^{-1})	DCu (%)	UE (μg g^{-1})	TE	PE (μg g^{-1})	DCu (%)
0	25.1cA	–	1.7cA	–	18.9dA	–	6.9cA	–
1	128.3bA	0.18cA	19.3bA	−3.5bB	105.1cA	0.14cA	12.6cA	25.9cA
2	163.3bB	0.47bB	52.3bB	28.3aB	828.6bA	1.12bA	438.1bA	49.2bA
4	2426.4aA	1.42aA	1424.9aA	17.8aB	2428.8aA	1.75aA	1545.8aA	70.7aA
M	ns	ns	ns	<0.05				
Cu	<0.05	<0.05	<0.05	<0.05				
M × Cu	ns	ns	ns	<0.05				

M mycorrhization, *ns* non significant

For each Cu concentration (on each line), same uppercase letters are not significantly different between UE, TE, PE and DCu by LSD test ($P < 0.05$). For UE, TE, PE and DCu (on each column) same lowercase letters are not significantly different between Cu concentrations by LSD test ($P < 0.05$)

affected by the high Cu content, although different results were obtained by Pätsikkä et al. (1998), Quartacci et al. (2000), Stiborová et al. (1986) and Ferreira et al. (2015). Meanwhile, photosynthesis was diminished by the high Cu content in the substrate, in accordance to Ouzounidou et al. (1992), Santana et al. (2015), Luna et al. (1994), and Ouzounidou et al. (1994). Moreover, the HM could produce damage in macromolecules, resulting in loss of membrane integrity, and accelerate senescence processes. The

cell membranes are the first structures to be affected by stresses. Our data show that the effect of Cu on the integrity of cell membranes, assessed by the EL, was significantly higher in NI compared to inoculated plants and in the roots compared to the leaves, confirming findings by other authors (Kaya et al. 2009; Beltrano et al. 2013). The inoculation maintained the integrity of cell membranes, and may contribute to stress tolerance, in agreement with Beltrano and Ronco (2008), Evelin et al. (2012) and Beltrano et al. (2013).

It is well known that the association of plants with soil microorganisms, including mycorrhizal fungi, modifies the responses to stress by metal, and increases tolerance in contaminated soils (Göhre and Paszkowski 2006). When soil was Cu-free, the mycorrhization was high, confirming that pepper is a mycotrophic species, in accordance with Ronco et al. (2008). The uptake of HM by mycorrhizal plants depends on several factors such as the physicochemical properties of the soil (Wang and Chao 1992), the host plants (Griffioen and Ernst 1989), the fungi involved (Gildon and Tinker 1981) and the concentration of the metals in the soil. One of the questions addressed in this study is whether different species of AMF can be affected differently by the presence of heavy metals in the soil. In the present study, *R. intraradices* showed a higher level of root colonization compared with *F. mosseae*, and maintains similar levels of root colonization at 0, 2 and 4 mM Cu, being these colonizations higher than those reported by Kaya et al. (2009) for *Glomus clarum,* Ruscitti et al. (2011) and Cekic et al. (2012) for *F. mosseae* and R. *intraradices*, and Joner and Leyval (1997) and Ronco et al. (2008) for *G. mosseae*. Although, the mycorrhization in pepper roots was significantly reduced at highest Cu concentration (8 mM), this response is in accordance with Andrade et al. (2008). This depends of AMF strain, the plant species involved, as well as soil properties. Our results agree with previous studies showing that addition of HM reduced the hyphal growth and the mycorrhizal colonization (Ruscitti et al. 2011). The activity and the viability of hyphae were higher without heavy metals and diminish with the increase of HM, in accordance with Ruscitti et al. (2011) and Beltrano et al. (2013). The sensitivity of hyphal to Cu was demonstrated by the reduction in viability (SDH) and activity (ALP), that was higher in mycorrhized by *F. mosseae* and *R. intraradices*, that were not affected by Cu at 2 and 4 mM, but were affected at 8 mM. At 8 mM the natives strains were significantly affected, in accordance with Yruela (2005). The results obtained can lead to an important progress in land use planning and phytoremediation because crops could be cultivated in highly Cu contaminated soils. A number of mechanisms have been proposed for explaining the mycorrhizal action in metal accumulation in plants (Meier et al. 2011). Nevertheless, some of these mechanisms are still a matter of investigation. Several studies have shown that AMF have developed mechanisms, which avoid metal transference to the shoots, thus promoting the phytostabilization process (Audet and Charest 2006). In contrast, other reports have shown that AMF promotes the phytoextraction, enhancing Cu translocation to shoots (Trotta et al. 2006). Although the protection mechanisms are not clear, alternatives could emerge as the possible retention of heavy metals by the fungal mycelium, or Cu retention by cell walls with later fixation by cellular polyphosphate granules (Galli et al. 1994). It can be assumed that inoculation with AMF could protect plants from the potential toxicity caused by Cu, but the degree of protection varies according to the fungus–plant interaction. In our study, Cu concentration in plants was related not only to the Cu presence, but also with the mycorrhizal interactions in the rhizosphere. Moreover, AMF produces changes in Cu distribution within plants. For instance, at 2 mM Cu, plants colonized by Mintra increased Cu concentration in roots and shoots (in comparison with non inoculated plants), supporting the hypothesis that AMF increases the metal translocation from roots to shoots, as proposed by Trotta et al. (2006). Nevertheless, AMF responses were not uniform and presented high variability, as observed in other studies. Similarly, some mycorrhizal fungi reduced accumulation of HM in shoots and it is presumed that this is due to increased Cu retention in the root and/or mycorrhizal structures (Brunner and Frey 2000; Chen et al. 2004; Zhang et al. 2005). Based on evidence of other HM, Ruscitti et al. (2011) proposed that the mycorrhizal association with pepper could potentially reduce the deleterious effects of Cu uptake. Our results do not agree with those of Lin et al. (2007) and Zhang et al. (2009), which show a

reduction in Cu accumulation in roots and shoots of the mycorrhizal plants under high Cu conditions. In our experiments mycorrhizal plants with 2 mM Cu were more efficient in absorbing and transporting Cu, and Cu was accumulated in all fractions tested, including fruits. Meanwhile, mycorrhizal and non-mycorrhizal plants with 4mM Cu accumulate more Cu in all plant fractions including the fruit, regardless of the inoculum. However, total dry weight and leaf area was higher in inoculated plants compared to non-inoculated plants when exposed to the highest concentrations of Cu, indicating an efficient symbiosis activity, in agreement with Andrade et al. (2010). In the absence of Cu, our results showed that the highest growth was determined in plants inoculated with *R. intraradices*. At the highest Cu concentration, toxicity was high for both mycorrhizal and non-mycorrhizal plants. In addition, the detrimental effects produced by the presence of Cu was lower in inoculated plants compared to non inoculated ones, and in those inoculated with *R. intraradices* when compared to those inoculated with *F. mosseae*. These results clearly indicated that excessive Cu in the soil represents a disturbance for the environment, which reduced the plant growth and biomass accumulation, as also evidenced by Singh and Sinha (2004), Singh and Agrawal (2007) for palak (*Beta vulgaris*) and Andrade et al. (2010) for Jack bean (*Canavalia ensiformis*). Copper significantly influences mycorrhizal dependency. Inoculated plants not exposed to Cu were less dependent to mycorrhiza, while plants exposed to 8 mM Cu were more dependent to mycorrhiza, which demonstrates the favorable relationship between pepper plants and AMF. This shows the ecological importance of AMF for the plants survival and growth under HM stress, however there is still a lack of knowledge of the control mechanism. We agree with Zhang et al. (2009), who reported that the roots of mycorrhizal plants are a composite structure of two organisms, and their abilities to absorb nutrient or heavy metals are not necessarily the same. In accordance with results reported by Ruscitti et al. (2011), we observed that plant resistance to HM can be associated with one or more mechanisms, such as (i) metal retention in roots preventing its translocation to the shoots (Patra et al. 2004); (ii) metal immobilization in the cell wall (Cosio et al. 2005); (iii) homeostatic cellular mechanisms to regulate the concentration of metal ions inside the cell (Benavides et al. 2005); (iv) increase of tolerance to mineral deficiency or low nutritional requirements; (v) increase in absorption of certain macronutrients or development of the capacity to absorb and use minerals in the presence of HM (Meda et al. 2007). Consistent differences between mycorrhizal and non-mycorrhizal plants in response to the increasing Cu concentrations in the soil were already reported (Andrade et al. 2010). Ability of plants to translocate HM from roots to shoots is measured by calculating the translocation efficiency (TE) (Singh and Agrawal 2007). In the present study, at high Cu additions inoculated pepper plants exhibited lower Cu concentrations in roots and shoots and those inoculated with *R. intraradices* exhibited an increased growth. The inoculation increased the tolerance of pepper plants to high concentrations of Cu in the soil, the dry weight, LA and net photosynthesis were higher, supporting our hypothesis.

In conclusion, increase in Cu concentration in the soil reduces the root colonization, LA, dry weight, and CMS. The colonization by *R. intraradices* and *F. mosseae* can alleviate the deleterious effects of Cu disturbance and attenuate the senescence syndrome in pepper plants. The two fungal species we tested behaved differently. Indeed, *R. intraradices* appears to have increased activity than *F. mosseae*. The mechanisms by which mycorrhization protects plants from Cu are unclear. The use of mycorrhizal fungi that are tolerant to disturbances such as excessive Cu may be a promising strategy to develop tools for soil remediation and improvement. Beneficial microbial inoculants, such as AMF are attractive to farmers in the context of sustainable agriculture. According to these results and those of other authors, it is possible to recommend mycorrhizal inoculation to obtain reasonable growth of pepper plants under contaminated soils. Pre-inoculating seedlings could be an economically feasible means of growing crops in intensive agricultural systems. Further studies regarding toxic metal accumulation in soils of agricultural interest are needed, and new investigations on metals transfer from soil to plant must be pursued in order to better understand the processes involved.

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