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Trichoderma asperellum ameliorates phytotoxic effects of copper in onion (*Allium cepa* L.)

Jaqueline Téllez Vargas[a], Mario Rodríguez-Monroy[a], Melina López Meyer[c], Roberto Montes-Belmont[b], Gabriela Sepúlveda-Jiménez[a,*]

[a] Departamento de Biotecnología, Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Carretera Yautepec-Jojutla, Km. 6, calle CEPROBI No. 8, Col. San Isidro, Yautepec, Morelos, C.P. 62731, Mexico
[b] Departamento de Interacción Planta-Insecto, Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Carretera Yautepec-Jojutla, Km. 6, calle CEPROBI No. 8, Col. San Isidro, Yautepec, Morelos, C.P. 62731, Mexico
[c] Departamento de Interacción Microorganismos-Plantas, Centro Interdisciplinario de Investigación, Para el Desarrollo Integral Regional Unidad Sinaloa, Instituto Politécnico Nacional, Bulevar Juan de Dios Bátiz Paredes #250, Guasave, Sinaloa, C.P. 81101, Mexico

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ABSTRACT

Species in the fungal genus *Trichoderma* have been reported to modulate tolerance to heavy metal stress in plants. However, the mechanism for enhanced plant tolerance to copper (Cu) by *Trichoderma* spp., has received little attention. To evaluate the potential of the fungus *Trichoderma asperellum* (To strain) to reduce the adverse effects of Cu on plant growth, uninoculated and *T. asperellum*-inoculated onion plants were exposed to different concentrations of Cu (0, 50–250 μM $CuSO_4$). Exposure to the higher concentrations of Cu (100, 250 μM $CuSO_4$) reduced plant survival significantly. In contrast, the lower Cu concentration (50 μM $CuSO_4$) had no effect on plant survival, but dry biomass of bulbs and leaves, and the chlorophyll content were reduced and the Cu toxicity increased malondialdehyde (MDA) content; the highest MDA content was found in leaves followed by roots and bulbs. In response to this, proline content increased in leaves and bulbs. Using the bio-concentration factor (BCF) and the translocation factor (Tf), it was possible to show that Cu was accumulated in roots and bulbs as a tolerance mechanism to reduce Cu translocation into leaves. Excess Cu (100 and 250 μM $CuSO_4$) was toxic to *T. asperellum in vitro*, but at the lower Cu concentration (50 μM $CuSO_4$) mycelial growth of *T. asperellum* was not affected significantly. When onion plants were inoculated with *T. asperellum* prior to Cu exposure (50 μM $CuSO_4$), Cu accumulation and translocation in tissues was reduced and Cu toxicity was ameliorated due the increased growth and chlorophyll content, and the reduction in MDA content compared with uninoculated plants receiving the same Cu concentration. The results indicate that *T. asperellum* mediates tolerance to Cu stress in onion plants.

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1. Introduction

Copper (Cu) is an essential element for the health of all organisms because it is a cofactor in proteins and enzymes, including proteins that transport Cu (Yruela, 2009). Organisms use Cu for essential functions but avoid Cu accumulation to toxic levels using complex networks of uptake, transport and sequestration (Rubino and Franz, 2012). The structural, physiological and molecular mechanisms of uptake, transport, sequestration and detoxification of Cu and other heavy metals in herbaceous and woody plants have recently been reviewed (Luo et al., 2014, 2016). In nature, Cu is present in unpolluted soils at a concentration ranging between 20 and 100 mg kg^{-1} (Cao and Hu, 2000; Pietrzak and Uren, 2011). However, anthropogenic activities considerably increase the amounts of Cu in agricultural soils that result in Cu concentrations reaching between 200 and 1000 mg kg^{-1} (Mirlean et al., 2007; Pietrzak and Uren, 2011). The excessive application of Cu-based fungicides and bactericides to control plant disease and pests is the principal cause of Cu accumulation in agricultural soils (Scheck and Pscheidt, 1998; Mirlean et al., 2007; Pietrzak and Uren, 2011). When Cu concentrations exceed 50 mg kg^{-1} in soil, the plants and microorganisms present are exposed to the toxic effects

* Corresponding author.
E-mail addresses: polinalenina@hotmail.com (J. Téllez Vargas), mrmonroy@ipn.mx (M. Rodríguez-Monroy), mlopez@ipn.mx (M. López Meyer), rbelmont@ipn.mx (R. Montes-Belmont), gsepulvedaj@ipn.mx (G. Sepúlveda-Jiménez).

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of Cu (Borkow and Gabbay, 2005; Nagajyoti et al., 2010). Phytotoxicity due to Cu reduces crop yield and causes poor seed germination, chlorosis, necrosis, stunting of leaves, and reduced root growth (Kumar et al., 2008; Adrees et al., 2015; Ferreira et al., 2015). In fungi from the genera *Trichoderma*, Cu causes inhibition of mycelium growth (Hajieghrari, 2010). Cu damages membrane integrity, displaces essential metals, changes the conformational structure of proteins and nucleic acids, and interferes with oxidative phosphorylation and the osmotic balance of fungi (Borkow and Gabbay, 2005). In both plants and microorganisms, the redox properties of Cu catalyze production of highly reactive hydroxyl radicals that can subsequently damage lipids, proteins, DNA and other biomolecules. This injury to biomolecules is as a consequence of the oxidative stress induced by Cu (Borkow and Gabbay, 2005; Valko et al., 2005; Nagajyoti et al., 2010; Sytar et al., 2013).

The actions to mitigate the toxicity of Cu in plants involve the use of chelating agents, silicon supplementation (Habiba et al., 2015; Keller et al., 2015; Zaheer et al., 2015) and the incorporation of microorganism such as bacteria and ectomycorrhizal fungi (Sánchez-Pardo and Zornoza, 2014; Luo et al., 2014, 2016). In particular, fungi from the genus *Trichoderma* have a high tolerance to metals (Anand et al., 2006; Adams et al., 2007; Bareen et al., 2012), but, despite this, the mechanisms by which *Trichoderma* species enhance plant tolerance to Cu stress have received little attention.

Trichoderma species are of particular interest because they have the ability to survive in a range of different environmental conditions and they also stimulate plant growth and development by increasing water and nutrient uptake (Vinale et al., 2008). The ability of strains of some *Trichoderma* species to ameliorate stress in plants by inducing physiological protection, enhancing antioxidant capacity, and delaying responses to drought are well known (Mastouri et al., 2010; Brotman et al., 2013; Contreras-Cornejo et al., 2014; Shukla et al., 2012). The fungus also has beneficial effects on plants that are under stress due to heavy metals. Generally, these studies use *Trichoderma* strains that have been isolated from metal-contaminated soil. For example, *T. reesei* (NBRI 0716) was isolated from diesel-contaminated soil; this strain ameliorates arsenic-induced stress in chickpea (*Cicer arietinum*) by promoting plant growth and restoring physiological parameters and anatomical anomalies in the stem (Tripathi et al., 2013). Likewise, *T. pseudokoningii* was isolated from solid tannery waste and subsequently found to alleviate stress in pearl millet (*Pennisetum glaucum*) due to the toxic metals commonly found in soil contaminated with tannery waste. *T. pseudokoningii* achieved this by increasing production of antioxidant enzymes as protection against oxidative damage (Bareen et al., 2012). However, studies using commercial products based on *Trichoderma* and strains isolated from food crops are scarce. It is known that *T. harzianum* strain T22, which is commercially used as a biological control agent, also promotes growth of crack willow (*Salix fragilis*) saplings in soil contaminated with toxic metals (Adams et al., 2007).

It has been demonstrated previously that *T. asperellum*, strain To, which was isolated from onion, promoted growth in onion plants and modulated the quantity of phenolic compounds and flavonoids in onion bulbs under conditions of reduced fertilizer application (Ortega-García et al., 2015). Fertilizer requirements to achieve maximum bulb biomass were reduced by up to 50% when plants were inoculated with the To strain compared with uninoculated plants. The To strain is also antagonistic to onion pathogens, such as *Sclerotia rolfsii* and *Alternaria porri* (Ortega-García et al., 2015). Here we evaluated the effect of excess Cu on mycelial growth of *T. asperellum* (To strain) *in vitro* and the potential of this strain to reduce the phytotoxic effects of Cu on growth of onion plants.

2. Materials and methods

2.1. Trichoderma asperellum

Trichoderma asperellum, strain To, was isolated from onion and preserved in the culture collection of the Phytopathology Laboratory of the Centro de Desarrollo de Productos Bióticos, Yautepec, Morelos state, Mexico. The strain was identified based on species-specific characteristics of both the ITS region and the *tef1* gene from the nuclear rDNA; the NCBI GenBank accession number for the *tef1* gene from this strain is KP059112 and KP059115 for the ITS region. The strain was maintained on potato dextrose agar (PDA; Bioxon) and not subcultured more than three times prior to experimentation.

2.2. Exposure of Trichoderma asperellum to Cu in vitro

Trichoderma asperellum strain To was subcultured onto 10 cm diameter Petri dishes (one 0.5 cm diameter plug per plate and six dishes per treatment) each with 10 mL PDA that had been supplemented with either 50, 100 or 250 μM $CuSO_4 \cdot 5H_2O$ (Sigma-Aldrich Co., St. Louis, MO) and incubated at 25 ± 2 °C, in a regime of 10 h dark: 14 h light. Controls were grown in the same way but without $CuSO_4 \cdot 5H_2O$. After eight days of incubation, the Petri dishes were photographed and the images were processed using the program Image J (version 1.48) to calculate the area of mycelial growth.

Sterile water (5 mL) was then added to each Petri dish, the surface was scraped with a spatula and the conidial suspension and mycelium collected. The conidia number per ml was determined using a Neubauer haemocytometer. The mycelium was recovered by centrifugation at 3000 × *g* for 15 min, dried at 45 °C for 24 h and the dry weight determined. The dry mycelium was ground in a mortar and used to evaluate the Cu concentration.

2.3. Inoculation of onion plants with Trichoderma asperellum

Trichoderma asperellum strain To was subcultured onto PDA in Petri dishes and incubated at 25 ± 2 °C, in a regime of 10 h dark: 14 h light for eight days. A conidial suspension was prepared in sterile distilled water using the scraping method. Specifically, sterile water (5 mL) was added to each Petri dish, the surface was scraped with a spatula and the conidial suspension and mycelium collected. The conidia number per mL was determined using a Neubauer haemocytometer and the concentration adjusted to 1×10^7 conidia mL^{-1}.

Onion (*Allium cepa* L.) seeds of the variety Crystal White (Emerald®) were acquired from the Rancho Los Molinos, Tepoztlan, Morelos state, México. Inoculation of onion plants was achieved in two stages. The first stage was achieved during the sowing of seeds in pots and the second stage was achieved three weeks after the seeds had germinated. Pots (0.1 L) were filled with a sterile substrate composed of a 1:3 (w:w) mixture of peat moss and metromix (Professional Growing Mix, Sunshine®, Proveedores Hortícolas de México). Seeds were placed on the substrate (two seeds per pot) and sprinkled with conidial suspension (1 mL). Pots were then incubated in darkness for one week and after this time, the seedlings were transferred into a greenhouse in a light regime of 12 h light: 12 h dark, 25 ± 2 °C and 90% HR; pots were watered daily. After three weeks, each pot of seedlings was sprinkled with conidial suspension (1 mL) and maintained for a further two weeks under the same conditions in the greenhouse. Uninoculated

controls were seeds and seedlings treated in exactly the same way but sprinkled only with water.

2.4. Cu treatment of plants inoculated with T. asperellum

Six-week-old inoculated and uninoculated seedlings (n = 6 per treatment) were each treated daily for 6 days with 1 mL of $CuSO_4$ (0, 50, 100 or 250 μM) added in the irrigation water, and maintained in the greenhouse in a light regime of 12 h light: 12 h dark, 25 ± 2 °C and 90% HR. After 6 days the number of living and dead plants was recorded to calculate plant survival (%). Surviving plants were removed from the pots and the roots washed to remove residual substrate. Leaves, bulbs and roots were separated and the chlorophyll, proline and MDA content determined. To evaluate the dry mass and copper content, each tissue type was dried at 45 °C for 24 h, weighed to determine dry mass and then ground in a mortar. The resulting powder from each tissue was used to evaluate the copper content.

2.5. Determination of chlorophyll, proline and MDA content

Chlorophyll: Fresh leaf tissue (0.025 g) from each sample was ground using a motor-driven grinder (Sigma- Aldrich Co., St. Louis, MO) in an Eppendorf tube and the chlorophyll extracted in 1 mL of 80% acetone. The extract was centrifuged at 15,000 × *g* for 15 min and the absorbance of the supernatant measured at 646.8 and 663.2 nm using a Genesys 2 spectrophotometer (Thermo Spectronic, Madison, WI). Total chlorophyll content was calculated according to the equations of Lichtenthaler (1987).

Proline: The proline content was evaluated according to Bates et al. (1973), based on the reaction of ninhydrin with proline. Briefly, fresh tissue (0.5 g) was homogenized in a mortar with liquid nitrogen and 10 mL of 3% sulfosalicylic acid was added. The extract was centrifuged at 9, 500 × *g* at 15 °C for 10 min and the supernatant recovered. The reaction mixture for each sample was composed of 2 mL of supernatant, 2 mL of ninhydrin and 2 mL of glacial acetic acid and was incubated in glass tubes at 100 °C for 1 h in a water bath. The reaction tubes were then immersed in an ice bath for 10 min, 4 mL of toluene were added to each and the suspensions were mixed vigorously. The upper phase was separated and the absorbance was read at 520 nm in a spectrophotometer. The proline content was calculated using a standard curve prepared using known concentrations of proline (Sigma-Aldrich Co., St. Louis, MO) and the results were expressed as μmoles proline g^{-1} of fresh weight.

MDA: From each sample of fresh tissue 0.2 g were finely ground in a mortar with 1 mL of trichloroacetic acid 5% (w/v), centrifuged at 15,000 × *g*, and 4 °C for 10 min and the supernatant recovered. 1 mL of thiobarbituric acid (TBA) 0.5% in 20% trichloroacetic acid was added to the supernatant and the mixture incubated at 95 °C in a water bath for 30 min and then centrifuged at 9500 × *g* at 15 °C for 10 min. The absorbance of the supernatant was measured at 532 and 600 nm in a spectrophotometer (Shimadzu UV–VIS 1601 model, Tokyo Japan). The absorbance values were used to calculate the MDA equivalents using a standard curve prepared using known concentrations of MDA and the results were expressed as nmol equivalents of MDA g^{-1} fresh weight.

2.6. Analysis of Cu content

The dry powdered onion tissues and mycelium were sieved through a 60 μm mesh. For each sample 0.25 g of the resulting powder was digested with a mixture of 1:1 (v/v) HNO_3 and H_2O_2 in a microwave (CEM Corporation, MARSXP15000 plus, Matthews, NC) at 150 °C for 15 min. The Cu concentration was determined by atomic absorption spectroscopy using a Varian SpectrAA 55 B flame spectrometer (Varian, Inc., 55B, Palo Alto, CA) at a wavelength of 324.8 nm. All evaluations were performed at the Chemical Laboratory of the Science Institute of the Benemérita Universidad Autónoma de Puebla, Mexico.

2.7. Calculation of the Cu bio-concentration factor (BCF) and the translocation factor (Tf)

BCF is defined as the ratio of the concentration of the metal in plant roots or aerial tissues relative to its concentration in the soil or nutrient solution. Tf indicates the ability of plants to translocate metals from the roots to the aerial tissues (He et al., 2013). BCF and Tf were calculated using the formulae of Shi et al. (2010). Specifically:

$$\mathrm{BCF} = \frac{[\mathrm{Cu}]_{leaves,\ bulbs\ or\ roots}}{total[\mathrm{Cu}]_{soil}}$$

$$\mathrm{Tf} = 100 \times \frac{[\mathrm{Cu}]_{leaves\ or\ bulbs\ roots}}{[\mathrm{Cu}]_{roots}}$$

2.8. Statistical analysis

Data were subjected to ANOVA followed by Tukey's tests (Sigma Stat 11, San Jose, CA, USA) for comparison of treatments within the same tissue and amongst the Cu concentrations within the same tissue and for each treatment.

3. Results

3.1. Cu concentration, mycelial growth and sporulation of T. asperellum in vitro

The Cu concentration was 78.5 and 272.3 times higher in the mycelium cultured on media supplemented with 50 and 100 μM $CuSO_4$, respectively, compared with the control grown in the absence of copper (Table 1). In the control and the 50 μM $CuSO_4$ treatment, the appearance of the mycelial growth was typical. However, in the 100 μM $CuSO_4$ treatment, mycelial growth was irregular and in the 250 μM $CuSO_4$ treatment there was no mycelial growth at all (Fig. 1). When *T. asperellum* was cultured in the absence of copper (control) or with 50 μM $CuSO_4$, the area of mycelial growth and dry mass were similar, however, when it was

Table 1
Copper content, mycelium growth and sporulation of *Trichoderma asperellum* when cultured *in vitro* for eight days on potato dextrose agar (PDA) supplemented with different concentrations of $CuSO_4$.

$CuSO_4$ concentration (μM)	Copper content in mycelium (μg/g DM*)	Mycelial area (mm^2)	Dry mass of mycelium (mg)	Number of conidia produced by mycelium (1×10^8)
0	22 ± 12[a]	60.0 ± 1.2 [a]	40.7 ± 4.60 [a]	8.40 ± 0.98 [a]
50	1728 ± 411[b]	59.4 ± 1.5 [a]	39.7 ± 6.74 [a]	4.04 ± 0.93 [b]
100	5990 ± 1,211[c]	42.5 ± 1.7 [b]	13.2 ± 4.14 [b]	1.53 ± 0.01 [c]

* DM, dry mass. Values are mean ± SD (n = 6). Values followed by different superscript letters are statistically different to each other according to Tukey test ($p < 0.001$).

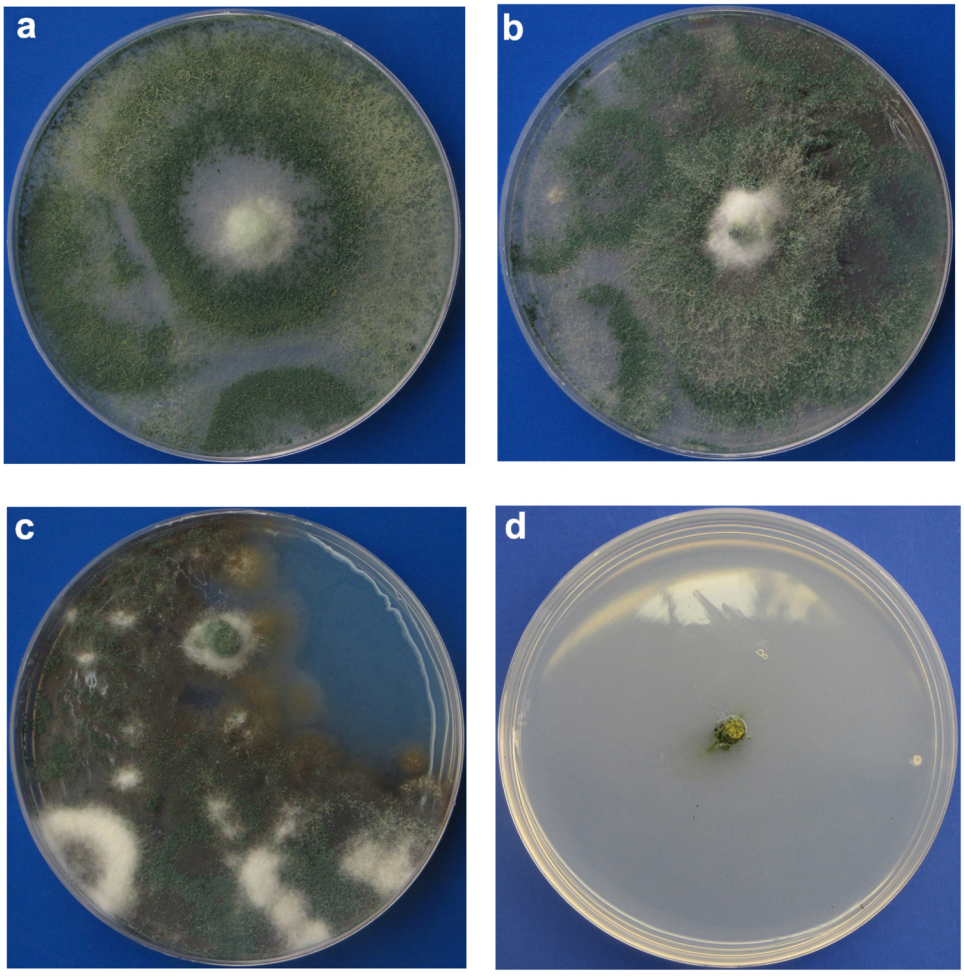

Fig. 1. *In vitro* mycelial growth of *Trichoderma asperellum* after eight days on culture medium without copper (a) or with 50 (b), 100 (c) or 250 μM $CuSO_4$ (d).

cultured with 100 μM $CuSO_4$, the area of mycelial growth and dry mass had diminished by 29 and 32%, respectively compared with the control in the absence of copper. Sporulation was reduced to 18.2% with an incremental increase in Cu concentration (Table 1).

3.2. Plant survival and biomass following Cu treatment

Plant survival was 100% for both *T. asperellum*-inoculated and uninoculated onion plants in the absence of Cu treatment and when exposed to 50 μM $CuSO_4$. However, only 81% and 46% of uninoculated plants survived following treatment with 100 and 250 μM $CuSO_4$, respectively. In contrast, when *T. asperellum*-inoculated plants were treated with the same Cu concentrations, plant survival was 100 and 56%, respectively (Fig. 2).

When uninoculated plants were treated with Cu, the dry mass of all the evaluated tissues was reduced and the degree of reduction was directly related to Cu concentration (Fig. 3). The smallest dry mass of leaves and bulbs was recorded for plants treated with 50 μM $CuSO_4$ while the smallest dry mass of roots was recorded in the 100 μM $CuSO_4$ treatment. In the absence of $CuSO_4$, inoculation with *T. asperellum* promoted growth in all the tissues evaluated compared with uninoculated plants. Generally, following Cu treatment, the dry mass of the three tissues evaluated was always greater for *T. asperellum*-inoculated plants than for uninoculated plants (Fig. 3).

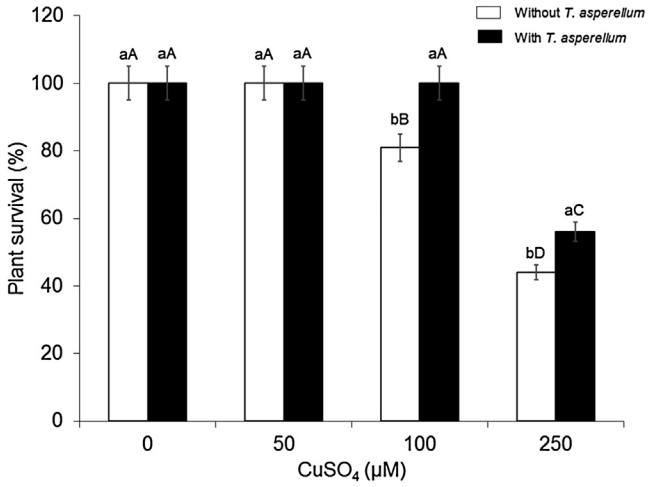

Fig. 2. Survival of onion plants inoculated with *T. asperellum* following treatment with different concentrations of $CuSO_4$ (0, 50, 100, 250 μM). Bars indicate mean ± SD (n = 8). Bars with different lowercase superscript letters indicate that the values are significantly different between inoculated and uninoculated plants at the same $CuSO_4$ concentration; bars with different uppercase superscript letters indicate that the values are significantly different between different concentrations of $CuSO_4$ for either *T. asperellum*-inoculated or uninoculated plants according to Tukey test ($p < 0.05$).

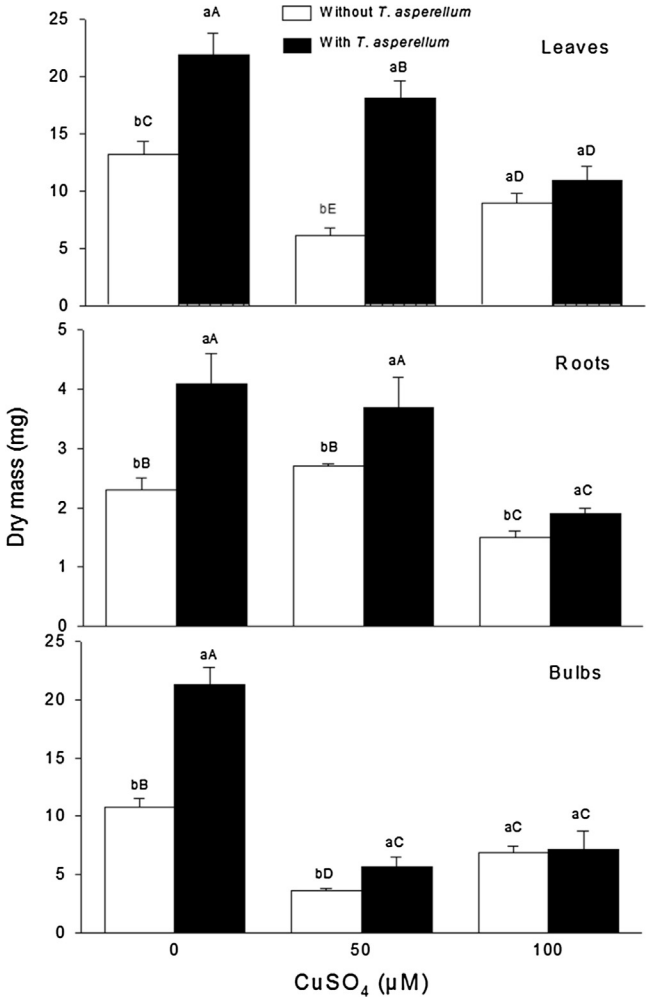

Fig. 3. Dry mass of different tissues from *T. asperellum*-inoculated and uninoculated onion plants following treatment with different concentrations of $CuSO_4$ (0, 50, 100 μM). Bars indicate mean ± SD (n = 6). Bars with different lowercase superscript letters indicate that the values are significantly different between inoculated and uninoculated plants at the same $CuSO_4$ concentration; bars with different uppercase superscript letters indicate that the values are significantly different between different concentrations of $CuSO_4$ for either *T. asperellum*-inoculated or uninoculated plants according to Tukey test ($p < 0.05$).

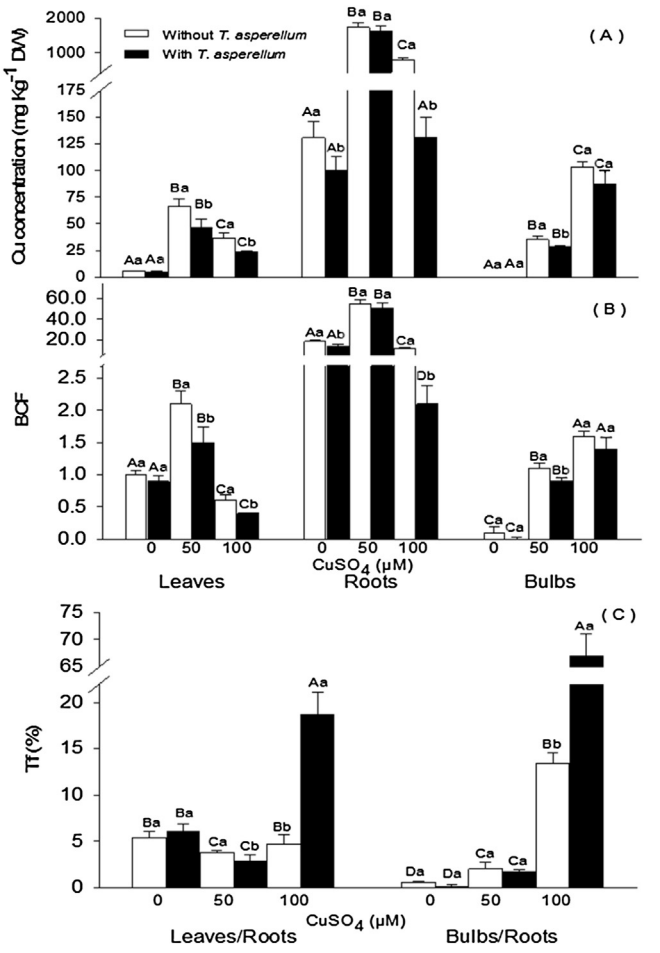

Fig. 4. Cu concentration, bio-concentration factor (BCF) and traslocation factor (TF) in different tissues of *T. asperellum*-inoculated and uninoculated onion plants following treatment with different concentrations of $CuSO_4$ (0, 50, 100 μM). Bars indicate mean ± SD (n = 6). Different capital letters on the bars for the same tissue indicate significant difference between $CuSO_4$ concentration and different uppercase letter on the bars for the same $CuSO_4$ concentration indicate significant difference between *T. asperellum*-inoculated or uninoculated plants according to Tukey test ($p < 0.001$).

3.3. Concentration, accumulation and translocation of Cu in plants

Cu concentration and BCF values for leaves and roots of uninoculated and inoculated plants both increased when treated with 50 μM $CuSO_4$ compared with the control. However, when the plants were treated with 100 μM $CuSO_4$ the Cu concentration and BCF values for leaves and roots decreased compared with those of the plants treated with 50 μM $CuSO_4$. In bulbs, Cu concentration and BCF values increased with the Cu concentration applied. However, the Cu concentration and BCF values in roots were always higher than those in leaves and bulbs. Generally, the tissues of inoculated plants had lower Cu concentrations and BCF values than the same tissues from uninoculated plants (Fig. 4a and b).

The Tf values for translocation from roots to leaves in uninoculated plants decreased following treatment with 50 μM $CuSO_4$ but did not decrease further following treatment with 100 μM $CuSO_4$. In contrast, the Tf values for translocation from roots to bulbs in uninoculated plants increased in direct relation to the increasing Cu concentration applied. In *T. asperellum*-inoculated plants subsequently treated with 50 μM $CuSO_4$, the Tf value for translocation from roots to leaves also decreased, but increased following treatment with 100 μM $CuSO_4$. Likewise, the Tf values for translocation from roots to bulbs increased following treatment with 100 μM $CuSO_4$, and these Tf values were higher than those for uninoculated plants receiving the same Cu treatment (Fig. 4c).

3.4. Chlorophyll, proline and MDA content of plants

In uninoculated plants, the chlorophyll content was reduced by 30 and 48% following treatment with 50 and 100 μM $CuSO_4$, respectively, compared with plants not receiving Cu. In contrast, *T. asperellum*-inoculated plants always had higher chlorophyll content than uninoculated plants receiving the same Cu concentrations (Fig. 5).

The MDA content of all tissues from uninoculated plants increased with increasing $CuSO_4$ concentration. The highest MDA content was found in leaves followed by roots and finally bulbs. However, MDA content was reduced in *T. asperellum*-inoculated plants compared with uninoculated plants receiving the same $CuSO_4$ concentration. In *T. asperellum*-inoculated plants treated with 50 and 100 μM $CuSO_4$ the MDA content was reduced by 42 and 25%, respectively, in leaves; 90 and 11%, respectively, in roots; and 15 and 17.4%, respectively in bulbs (Table 2).

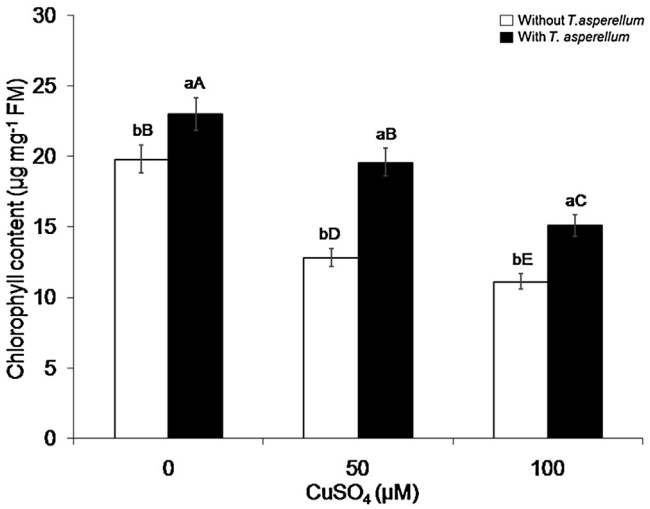

Fig. 5. Chlorophyll content in *T. asperellum*-inoculated and uninoculated onion plants following treatment with different concentrations of $CuSO_4$ (0, 50, 100 µM). The contents are expressed in relation to fresh mass (FM). Bars indicate mean ± SD (n = 6). Bars with different lowercase superscript letters indicate that the values are significantly different between inoculated and uninoculated plants at the same $CuSO_4$ concentration; bars with different uppercase superscript letters indicate that the values are significantly different between different concentrations of $CuSO_4$ for either *T. asperellum*-inoculated or uninoculated plants according to Tukey test ($p < 0.05$).

The proline content of leaves and roots was always higher than in bulbs; in leaves and bulbs only the proline content increased with increasing Cu concentration. The proline content of leaves and roots of *T. asperellum*-inoculated plants was always higher than the same tissues from uninoculated plants receiving the same $CuSO_4$ concentration (Fig. 6a, b). In contrast, the proline content of bulbs was similar in uninoculated and inoculated plants.

4. Discussion

4.1. Toxic effect of copper on survival, biomass and physiology of plants

Growth parameters such as biomass, stem elongation and the number of roots and shoots have been used to evaluate metal toxicity in plants (Ait Ali et al., 2002; Shi et al., 2010). In onion plants, root elongation has been proposed as the most sensitive parameter for evaluation of toxicity caused by copper, other metals and polycyclic aromatic hydrocarbons (Geremias et al., 2010, 2011). However, because different tissues have different responsiveness to metals, chemical and biological analysis in a range of tissues provides a more robust representation of the overall toxic effects of metals in the complete organism and was subject of this study.

The survival and biomass of onion plants depended of the $CuSO_4$ concentration applied. Plant survival was not affected by exposure to 50 µM $CuSO_4$, but dry biomass was reduced in the leaves and bulbs of the plants that survived, indicating that this concentration of $CuSO_4$ is still toxic to these onion tissues. Root growth was not affected by application of 50 µM $CuSO_4$, but was reduced when 100 µM $CuSO_4$ was applied. This differential effect on tissue growth in onion plants exposed to Cu and other metals has been reported previously (Geremias et al., 2011). Furthermore, growth inhibition is the principal toxic effect of Cu reported in several other crops such as wheat, rice, maize, sunflower and cucumber (Adrees et al., 2015). In cucumber (*Cucumis sativus*) plants a reduction in leaf expansion is the first symptom of copper toxicity and photosynthesis is inhibited by Cu-induced accumulation of carbohydrates in leaves (Alaoui-Sossé et al., 2004). Accumulation of Cu in leaves of *Phaseolus vulgaris* L. causes severe symptoms such as chlorosis and necrosis, and the subsequent associated changes in nutrient balance are responsible for a reduction in leaf growth (Bouazizi et al., 2010). In onion, the reduction in root development caused by Cu has been attributed to a decrease in mitotic index and the formation of chromosome aberrations (Liu et al., 2009; Yıldız et al., 2009).

It is known that the mechanisms for absorption of Cu in plants involve the apoplastic and symplastic metabolic pathways, and that the metal is accumulated mainly in the central cylinder of the roots (Keller et al., 2015). The bio-concentration factor (BCF) is used to quantify the accumulation of Cu (and other metals) in plant roots and aerial tissues (Ait Ali et al., 2002; Shi et al., 2010). In this study the Cu concentration and BCF values in the roots were always higher than those found in leaves and bulbs, which indicates that the roots accumulated more Cu than other onion tissues. Preferential accumulation of Cu in roots has been reported previously in onion plants (Geremias et al., 2010) and other plants such as cucumber, *Arabidopsis thaliana* and wheat (*Triticum turgidum*) (Alaoui-Sossé et al., 2004; Lequeux et al., 2010; Keller et al., 2015). However, Cu accumulation decreased in both roots and leaves of plants treated with 100 µM $CuSO_4$, while it increased in bulbs. This could be due to the negative effects of Cu toxicity on root growth, because the dry biomass of the roots was also reduced in this treatment compared with the other treatments; to compensate, Cu was accumulated in other tissues, such as the bulbs.

Plants have several tolerance mechanisms to maintain adequate concentrations of Cu in different organs and avoid Cu accumulation in sensitive tissues. The immobilization of excess Cu in roots and the ability to exclude Cu from the shoots are mechanisms for copper tolerance (Yruela, 2009; Adrees et al., 2015; Luo et al., 2016). The translocation factor (Tf) provides a measure of the ability of plants to translocate metals, including Cu, from roots to other aerial tissues (Ait Ali et al., 2002; He et al., 2015). The Tf was calculated and used to compare Cu translocation between roots and other onion tissues in different treatments. In uninoculated plants, the Tf values indicated that Cu translocation from roots to leaves was limited but that Cu was translocated from roots to bulbs. Together, the BCF and Tf values for uninoculated

Table 2
MDA content of different tissues from *Trichoderma asperellum*-inoculated and uninoculated onion plants following treatment with different concentrations of $CuSO_4$.

$CuSO_4$ (µM)	MDA content (nmol g^{-1} fresh weight)					
	Leaves		Roots		Bulbs	
	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
0	0.021 ± 0.001^{aA}	0.024 ± 0.001^{aA}	0.02 ± 0.003^{aA}	0.01 ± 0.001^{aA}	0.03 ± 0.001^{aA}	0.02 ± 0.002^{aA}
50	73.80 ± 8.1^{aB}	43.20 ± 4.1^{bB}	4.00 ± 0.2^{aB}	0.40 ± 0.01^{bB}	1.30 ± 0.1^{aB}	1.10 ± 0.08^{bB}
100	88.10 ± 9.6^{aC}	66.20 ± 9.7^{bC}	3.60 ± 0.1^{aC}	3.20 ± 0.7^{bC}	1.70 ± 0.2^{aC}	1.40 ± 0.2^{bC}

Values are mean ± SD (n = 6). Values followed by different lowercase superscript letters in the same tissue are significantly different between uninoculated and inoculated plants when treated with the same concentration of $CuSO_4$. Values followed by different uppercase superscript letters in the same tissue indicate are significantly different between plants that were either treated or untreated with $CuSO_4$ according to Tukey test ($p < 0.001$).

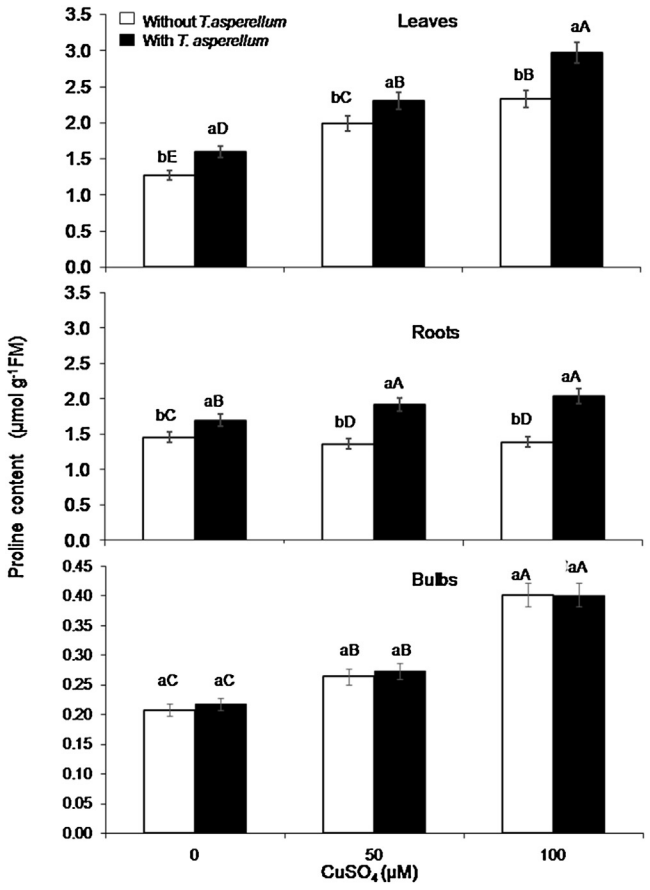

Fig. 6. Proline content in tissues from *T. asperellum*-inoculated and uninoculated onion plants following treatment with different concentrations of $CuSO_4$ (0, 50, 100 μM). Bars indicate mean ± SD (n = 6). Bars with different lowercase superscript letters indicate that the values are significantly different between inoculated and uninoculated plants at the same $CuSO_4$ concentration; bars with different uppercase superscript letters indicate that the values are significantly different between different concentrations of $CuSO_4$ for either *T. asperellum*-inoculated or uninoculated plants according to Tukey test ($p < 0.05$).

plants indicate that Cu accumulation in roots and bulbs is the main mechanism responsible for limiting Cu translocation into leaves in onion plants. This was also the case in *Phragmites australis* (reed plants) treated with Cu, where the BCF values were also higher in roots than in shoots. It was suggested that while this plant accumulated Cu effectively in roots, the low translocation of Cu into the shoots limited its use for phytoextraction of Cu from contaminated soils (Ait Ali et al., 2002). BCF and Tf have also been used in the selection of plant species with greater bio-accumulation capability and therefore tolerance to heavy metals (Shi et al., 2010; He et al., 2013). According to Luo et al. (2016) plants that accumulated Cu concentrations above 1000 $\mu g\, g^{-1}$ in their aerial tissues could be considered as hyperaccumulating plants. Thus, our results indicate that onion plants are accumulators of Cu in roots, but would have limited use in Cu phytoextraction.

It is widely known that toxic levels of Cu reduce growth of food crops due to: growth inhibition; oxidative damage; alterations in mineral nutrition, photosynthesis and enzyme activities; and a decrease in chlorophyll biosynthesis (Adrees et al., 2015). In relation to oxidative damage, the MDA content is a measure of lipid peroxidation status and can be used as an indicator of plant oxidative damage in Cu toxicity bioassays (Hartley-Whitaker et al., 2001; Choudhary et al., 2007). An increase in MDA content has been reported in several plant species following exposure to Cu, including *A. thaliana* (Ann et al., 2011), duckweed (*Lemna minor*) (Kanoun-Boulé et al., 2009) and cabbage (*Brassica oleracea*) (Posmyk et al., 2009). In this study, Cu toxicity in onion leaves was also related to a reduction in growth and chlorophyll content, and an increase in MDA content. An increase in MDA content was also found in roots and bulbs, although the highest MDA content was found in leaves; the MDA contents were twenty and seventy times lower in roots and bulbs compared with leaves. In an experiment with oilseed rape (*Brassica napus*), using similar Cu concentrations to those evaluated in our study, an increase in MDA content was also observed and it was higher in leaves than roots; this oxidative stress reduced plant growth, plant biomass and the content of photosynthetic pigments (Habiba et al., 2015; Zaheer et al., 2015). Low chlorophyll content is related to alterations in chlorophyll biosynthesis and changes in chloroplast structure and thylakoid composition (Fernandes and Henriques 1991; Yruela, 2005).

Proline content remained unchanged in roots, whereas in bulbs, the level of this amino acid was three to six times lower than in roots and leaves. Proline is an amino acid involved in plant responses to heavy metals such as cadmium, arsenic and Cu (Chen et al., 2004; He et al., 2013; Tripathi et al., 2013) and is achieved *via* a number of different mechanisms including osmoregulation, metal chelation and free radical scavenging (Sharma and Dietz, 2006). Our results suggest that the involvement of proline in response to excess Cu is by a mechanism of free radical scavenging, and that its purpose is to reduce injury in leaves. Furthermore, Cu accumulation in roots and bulbs reduces Cu translocation to leaves. In the case of onion bulbs, it is known that this tissue is rich in phenolic compounds with an antioxidant capacity that may also contribute to reducing the oxidative stress caused by Cu (Prakash et al., 2007; Ortega-García et al., 2015).

4.2. Trichoderma asperellum *ameliorates the toxic effect caused by Cu*

Trichoderma species isolated from metal-polluted sites exhibit tolerance to Cu and the potential for copper bioaccumulation (López Errasquin and Vázquez, 2003; Anand et al., 2006; Ting and Choong, 2009). Mycelial growth of a *T. asperellum* strain isolated from metal-contaminated sediment was not affected by 100 ppm of Cu (Iskandar et al., 2011). The *T. asperellum* isolate selected for our study, which was obtained from an onion crop, was able to grow at 50 μM $CuSO_4$ (equivalent to 120 ppm Cu). However, concentrations above 50 μM $CuSO_4$ inhibited mycelial growth and there was also a negative relationship between sporulation and Cu concentration. It is known that metals such as Cu, cobalt and zinc cause inhibition of mycelial growth, and that cadmium and nickel reduce conidial germination in *Trichoderma* isolates (Hajieghrari, 2010; Cacciola et al., 2015; Nongmaithem et al., 2016).

The fact that the *in vitro* mycelial growth of the *T. asperellum* strain used in our study was not affected to 50 μM $CuSO_4$ suggested the potential for Cu accumulation in the mycelium. It was for this reason that we evaluated the potential of *T. asperellum* to ameliorate the toxic effects of Cu in onion plants. Our results show that the interaction between *T. asperellum* and onion plants both promoted growth and ameliorated the toxic effects of Cu. Growth promotion in onion plants under low mineral fertilizer conditions had been demonstrated previously in response to inoculation with *T. asperellum* strain To (Ortega-García et al., 2015). In the current study we also found that plant survival and the growth of leaves and roots were increased in *T. asperellum*-inoculated onion plants exposed to 50 μM $CuSO_4$ compared with uninoculated plants.

The Cu concentration and BCF of leaves and roots, and the Tf values for translocation of Cu from roots to leaves were all lower in *T. asperellum*-inoculated plants that were treated with 50 μM

$CuSO_4$ compared with the same tissues from uninoculated plants. This indicates that the presence of *T. asperellum* reduced Cu accumulation and translocation to leaves in onion plants, thereby reducing the negative effects of Cu toxicity on the dry biomass of these tissues. These beneficial effects of *T. asperellum* are related to increases in chlorophyll content and a reduction in the MDA content and associated lipid peroxidation. This is similar to the effects of *T. reesei* (NBRI 0716) which restored the chlorophyll content of chickpea plants exposed to arsenic stress (Tripathi et al., 2013). Also, in pearl millet, inoculation with *T. pseudokoningii* in combination with plant growth regulators increased plant growth under toxic concentrations of metals found in soil contaminated with tannery waste (Bareen et al., 2012).

In plants treated with 100 μM $CuSO_4$, the dry biomass of all three tissues in surviving plants was reduced, even when inoculation with *T. asperellum* had reduced the Cu concentration and BCF values in tissues. Tf values also increased in the 100 μM $CuSO_4$ treatment indicating that toxic levels of Cu were being translocated from roots to leaves and bulbs. *In vitro* mycelial growth and survival of *T. asperellum* decreased significantly at concentrations of 100 μM $CuSO_4$; it is therefore possible that *T. asperellum* was inhibited/absent in the rhizosphere of onion plants in the 100 μM $CuSO_4$ treatment, thus limiting the beneficial effect of the fungus on the onion plants.

Studies of the effect of *Trichoderma* species on plants under Cu stress are scare and this study has demonstrated that the interaction between *T. asperellum* and onion plants also ameliorates stress due to excess Cu. Future studies to understand the physiological and molecular mechanisms involved in the interaction between *T. asperellum* and onion plants under conditions of Cu stress would aid the development of integrated management strategies for onion crops.

5. Conclusions

Excess copper is toxic to *T. asperellum* and onion plants resulting in a reduction in dry biomass and chlorophyll content. This Cu toxicity increased the malondialdehyde (MDA) content of onion tissues with the highest concentration being found in leaves followed by roots and bulbs. However, *T. asperellum* ameliorated the toxic effects of excess Cu in onion plants. These beneficial effects of *T. asperellum* were related to growth promotion, increases in chlorophyll content and a reduction in MDA level in onion plants.

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