Copper compartmentalization in spores as a survival strategy of arbuscular mycorrhizal fungi in Cu-polluted environments

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Arbuscular mycorrhizal (AM) fungi are present in Cu-polluted soils. By using in vivo cultures of Claroideoglomus claroideum in association with Imperata condensata and monoxenic cultures of Rhizophagus irregularis in association with carrot roots we show for the first time the presence of AM fungal spores of a green–blue colour in Cu-polluted environments. In both experiments, the number of green–blue spores increased with Cu concentration in the soil or in the culture medium. The green–blue colour was associated with an accumulation of Cu in the spore cytoplasm. These spores were metabolically inactive. These data suggest that a fungal strategy to survive in Cu-polluted environments is to compartmentalize the excess metal in some spores.

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Copper (Cu) is an essential micronutrient required for normal growth of plants and soil microorganisms, but toxic when it is present in excess (Cuillel, 2009). Copper toxicity results from the formation of reactive oxygen species or from the interaction with proteins impairing key cellular processes, inactivating enzymes and disturbing protein structure (Yruela, 2009). Although Cu is a trace element, owing to anthropogenic activities, Cu toxicity has become an agricultural and environmental problem in recent years.

Arbuscular mycorrhizal fungi (AMF), obligate biotrophs of higher plants, colonize the root cortex of many plant species and develop an extraradical mycelium that radiates into the soil surrounding plant roots. They expand the interface between plants and soil contributing to plant uptake of low mobility mineral nutrients, such as P, N, Cu or Zn (Smith and Read, 2008). However, under conditions of supraoptimal levels of essential metals, AMF are able to alleviate metal toxicity in the plant. Alleviation of metal toxicity can be attributed not only to AM-mediated nutritional effects, but also to the impact of AMF on metal distribution at the soil–fungus–plant interface (Leyval et al., 1997; Gonzalez-Chavez et al., 2002; Göhr and Paszkowski, 2006; Hildebrant et al., 2007; Meier et al., 2012).

Metal tolerant AMF have been isolated from polluted soils and these indigenous populations cope better with metal-toxicity than those isolated from unpolluted soils (del Val et al., 1999; Meier et al., 2011). To persist in environments with high metal content, AMF must have evolved a series of strategies to avoid the damage produced by the metal (Ferrol et al., 2009). The aim of the present work was to determine if compartmentalization of the excess metal in the AMF resting spores could be one of those strategies. For this purpose, spore formation and vitality in Cu-polluted soils and in monoxenic cultures in response to toxic Cu levels were monitored.

For the in vivo experiment, a Cu-polluted soil was used. This soil is an Alfisol, from the Chilcauquén soil series, with fine sandy loam texture, 1.25 g mL−1 density and 387 mg kg−1 total Cu. It was collected from Puchuncavi Valley (Chile, 32°46′30″S; 71°28′17″W) at 0–20 cm depth, sterilized by tyndallisation for three consecutive days, spiked with 0, 150, 300 or 450 mg kg−1 Cu (CuCl2) and stabilized for 6 weeks. The initial DTPA extractable Cu was 67.7, 145.1, 172.8 and 225.8 mg kg−1 for the soils added with 0, 150, 300 or 450 mg Cu kg−1, respectively. Plantlets of Imperata condensata inoculated with Claroideoglomus claroideum were grown for
1 month in a seedbed, transplanted to the contaminated soils and grown for 5 months in a greenhouse (25 ± 3 °C/15 ± 3 °C day/night temperature; 16/8 h light/dark photoperiod). Plants were watered as required to maintain 80% field capacity (determined by pot weight). At harvest, DTPA extractable Cu was 38.7, 66.4, 84.7 and 176.1 mg kg⁻¹ for the soils added with 0, 150, 300 or 450 mg kg⁻¹ Cu, respectively. AMF spores were separated from the soil by wet sieving and sucrose centrifugation (Johnson et al., 1999), rinsed thoroughly with milli-Q H₂O and analysed under a dissecting microscope.

The in vitro experiment was carried out using Rhizophagus irregularis, synonym of Glomus intraradices DAOM 197198 (Krüger et al., 2012), with carrot roots. Liquid monoxenic cultures were established in bi-compartmental Petri plates to allow separating the root compartment (RC) from the hyphal compartment (HC) (St-Arnaud et al., 1996), as described by Pérez-Tienda et al. (2011). After 2 weeks of growth in the HC, the medium was replaced by fresh medium supplemented with 0, 5, 50 or 500 μM CuSO₄. The fungus was monitored under dissecting and optical microscopes 1, 2, 5, 7 and 14 days after Cu supply. Spores from both experiments were rinsed thoroughly with milli-Q H₂O before further analyses. Spore vitality was estimated by staining for succinate dehydrogenase (SDH) activity using iodonitrotetrazolium as oxidizing agent and checking for the formation of an iodonitrotetrazolium violet-formazan precipitate (Smith and Gianinazzi-Pearson, 1990; Vierheilig et al., 2005). Copper was detected on crushed spores by the formation of a red copper ferrocyanide precipitate after the addition of acetic acid and potassium ferrocyanide (Pritz et al., 1913). Data were analysed by one-way ANOVA after arcsin transformation when needed using SPSS v.10.0 software.

C. claroideum spore number in the rhizosphere of I. condensata decreased as the Cu concentration in the soil increased (260 vs. 150 spores/100 g soil in the soil supplemented with 0 and 450 mg Cu kg⁻¹). Some spores of green-blue colour were observed in the Cu-spiked soils (Fig. 1A–C) and the proportion of green-blue to light yellow (Fig. 1D) spores increased with the soil Cu content (Fig. 2A). Since many Cu bearing compounds are green-blue, it is likely that the green-blue spores isolated from the Cu-polluted soils have a high Cu content. Accumulation of metals in both extra- and intraradical AMF structures has been shown in different studies (Kaldorf et al., 1999; Joner et al., 2000; González-Chávez et al., 2002; González-Guerrero et al., 2008; Aguilera et al., 2011).

To get further insights into the features of these green-blue spores, R. irregularis extraradical mycelia grown in the HC of the monoxenic cultures were exposed to increasing Cu concentrations. Twenty-four hours after 500 μM Cu supplementation, a few (<10%) R. irregularis spores of the fungal colony became green-blue and the number of coloured spores increased with time (9, 15 and 32% after 1, 2 and 7 d Cu exposure, respectively). In the 50 μM Cu plates, the green-blue spores were not detected until 5 d after Cu addition. As in the pot cultures, the proportion of spores of green-blue colour (Fig. 1E–H) in the fungal colony increased with Cu concentration (Fig. 2B). Retraction of the cytoplasm was frequently observed in the green-blue spores, being the colour mainly associated with the cytoplasm (Fig. 1F). This feature suggests that the green-blue colour was due to an accumulation of Cu inside the spore rather than on the cell wall, which was further evidenced by the release of a bluish content from the crushed spores (Fig. 1G–H).

The presence of Cu ions in the spore cytoplasm was revealed by the formation of a red precipitate after the addition of acetic acid.

**Fig. 1.** AMF spores from the Cu addition experiments. C. claroideum spores from the rhizosphere of I. condensata growing for 6 months in a 450 mg Cu kg⁻¹ spiked-soil (A–C) or in a non-spiked soil (D). R. irregularis spores monoxenically-grown in the hyphal (E–H) or in the root (I) compartment of a Petri dish 1 (E–G, I) or 2 (H) weeks after the addition of 500 μM Cu to the hyphal compartment. Mycorrhizal carrot root from the R. irregularis monoxenic culture 2 weeks after the addition of 500 μM Cu to the hyphal compartment (J); R. irregularis spores (K) and a mycorrhizal carrot root (L) from a control plate.
Green-blue spores (Fig. 2). The green culture medium led to a significant phenomenon that occurs in other AM fungal species. Families, suggests that spore Cu compartmentalization should be and C. claroideum and potassium ferrocyanide to the crushed blue spores (Fig. 3). Copper detection in the spore is consistent with previous observations of preferential accumulation of this metal in the spores of G. intraradices (González-Guerrero et al., 2008). Since a direct incorporation of metals through the spore is unlikely, our observations predict an as-yet unknown mechanism of metal-sensing in the fungus that would translocate metal-accumulating organelles or compounds from hyphae to spores. This hypothesis is supported by the observation of some green-blue spores in the extra- and intraradical mycelium growing in the RC of the plates supplemented with Cu in the HC (Fig. 1C). These data altogether indicate that in AMF, like in all organisms that operate an intracellular compartmentalization strategy to detoxify pollutants (Gadd, 2007), the excess Cu is translocated to subcellular compartments where it can be stored away from the cytosol and to specific fungal structures with limited core metabolic functions, such as spores and vesicles, where the metal would cause less damage. Detection in Cu polluted environments of green-blue coloured spores of C. claroideum and R. irregularis, two AMF belonging to different families, suggests that spore Cu compartmentalization should be a common phenomenon that occurs in other AM fungal species.

Vital staining of the spores collected from the in vivo and in vitro cultures showed that increasing Cu levels in the soil or in the culture medium led to a significant decrease in spore vitality (Fig. 2). The green-blue spores showed a faint purple colour indicating that these spores were dead (not shown). The finding that, even at the highest Cu doses used, some spores remained viable both in the pot and in the monocot cultures suggests that one of the strategies evolved by AMF to survive in Cu-polluted environments is to accumulate the excess metal in some spores, perhaps protecting in this way the rest of the fungal colony.

While this report describes for the first time green-blue AMF spores in Cu-polluted environments, lichens have been previously reported to display a green colour or contain blue-green inclusions in cupriferous habitats due to an accumulation of Cu (Chisholm et al., 1987; Purvis et al., 2008). Although our data indicate that the green-blue colour of the spores is due to an accumulation of Cu, the identity of the compound involved is unknown. Given the central role played by polyphosphates in phosphorus translocation in the symbiosis (Cox et al., 1980), the high polyphosphate concentrations harboured by AMF (Solaiman et al., 1999) and the role polyphosphate plays in metal detoxification and tolerance in other organisms (Orell et al., 2010), it is tempting to speculate that the blue-green spores might have a high content of Cu-bound polyphosphates. Alternatively, Cu might be bound to triacylglycerols, the major carbon storage compound in AMF spores (Bago et al., 2000). Finally, since oxalic acid has been implicated in Cu tolerance in brown-rot fungi and lichens (Green and Clausen, 2003; Gadd et al., 2012), Cu accumulation in the spores might be also linked to oxalic acid production, which would precipitate Cu into oxalate rendering Cu inert. This suggestion is supported by the increased viscosity of the R. irregularis spore content over Cu exposure time (Fig. 1G vs. H), the observed displacement of the green-blue colour of the spore content by ferrocyanide (Fig. 3B) and the crystalline content of the C. claroideum spores collected from Cu-polluted soils (Fig. 1C). However, further experiments are needed to identify the compounds involved in Cu accumulation.

In conclusion, this report describes for the first time visual detection of Cu accumulation in AMF spores and that the Cu-accumulating spores are metabolically inactive. These data suggest that one of the fungal strategies to survive in Cu-polluted environments is to compartmentalize the excess metal in some spores.
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