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Contribution of arbuscular mycorrhizal fungi in attenuation of heavy metal impact on *Calendula officinalis* development

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ABSTRACT

The mycorrhizal fungi community is a significant soil rhizosphere component that benefits plant nutrition and improve plant tolerance to abiotic stresses. The present research compared the influence of three mycorrhizal strains over pot marigold (*Calendula officinalis* L.) development and their contribution to promoting the valuable secondary metabolites accumulation in the condition of heavy metal (Cd and Pb) pollution. Two *Claroideoglomus claroideum* isolates (from industrially and naturally enriched metal-contaminated sites) and *Funneliformis mosseae* (derived from a soil with a high concentration of various metals) were studied. The mycorrhizal status and acid phosphatase activity were best exposed in the roots associated with *C. claroideum* (from native metalliferous sites) and corresponded with the highest total phenols and flavonoids concentrations. Neither Pb nor Cd was detected in the marigold flowers (*Calendula* flos drug) following targeted mycorrhizal treatment. The higher Cd and Pb levels in non-mycorrhizal plants lead to the lowest flower weight, but in the aerial plant parts, we found a slight distinction. The tested strains stimulated the accumulation of important secondary metabolites (total phenols, flavonoids, carotenoids) in pot marigold flowers and, therefore, enhanced the antioxidant capacity. The carotenoid profile of mycorrhizal plants performed notable differences between the primarily defined constituents (lutein, lycopene, β-carotene). The highest β-carotene values (respectively vitamin A) and lycopene were found in pot marigold—*F. mosseae* association. The findings are essential to obtain the optimum benefits of mycorrhizal association in unfavorable conditions concerning pot marigold bioactive compounds synthesis.

1. Introduction

Soil and groundwater pollution by heavy metals (HMs), derived from industrial activities, is a severe problem. The search for alternative methods of excavation and incineration to clean polluted sites resulted in the application of bioremediation techniques. Arbuscular mycorrhizal fungi (AMF) are such as a possible tool for enhancing plant tolerance to environmental stress conditions and restoration of naturally or industrially metal-contaminated soils (Gianinazzi et al., 2010).

Heavy metals like cadmium (Cd) and lead (Pb) are tolerable to a certain extent and their excess result in biological toxicity by affecting several physiological and biochemical processes in plants. The activity of antioxidant defence system is reduced due to reactive oxygen species generated in a result of heavy metal stress (Bano and Ashfaq, 2013). Alleviation of metal toxicity can be ascribed to the impact of AMF to metal distribution at the soil–fungus–plant interface (Meier et al., 2012). Mycorrhizal structures may stabilize metals in the soil, reduce their uptake and minimise the risk of toxicity to crops growing in polluted substrates (González-Chávez et al., 2009; Azcon et al., 2010). Metal tolerant AMF isolated from polluted soils cope better with metal toxicity than those isolated from unpolluted soils (Cornejo et al., 2013). Concerning Ferrol et al. (2009), isolation of indigenous and presumably adapted AM fungi, more suitable for phytostabilisation purposes than laboratory strains, can be a potential biotechnological tool for successful restoration of degraded ecosystems.

The potential of AMF to enhance plant growth and reducing heavy metal stress is well recognized, but not exploited to the fullest extent, particularly in economically important cultures as *Calendula officinalis* L. On the other site pot marigold is well known

Abbreviations: AMF, arbuscular mycorrhizal fungi; HM, heavy metal; NM, non-mycorrhizal plants; APA, acid phosphatase activity.

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0929-1393/

for its higher tolerance and accumulation ability to Cd and Pb, without suffering phytotoxicity (Wang, 2005; Moustakas et al., 2011). Pot marigold (*C. officinalis* L.) is a valuable medicinal crop due to its biologically active compounds: volatile oil, carotenoids, flavonoids, terpenoids, coumarins, quinines, amino acids, carbohydrates, lipids and other minor constituents (Isaac, 2000; Khalid and Teixeira da Silva, 2012). *C. officinalis* stores large amounts of carotenoids in its flowers: β-carotene, lutein, lycopene, rubixanthin, flavoxanthin and γ- carotene (Pintea et al., 2003). These metabolites may have a protective role against some types of cancer, decreased the risk of cardiovascular diseases and prevented disorders related to the eye, such as macular degeneration (Khalid and Teixeira da Silva, 2012). The primary dietary source of provitamin A is plants' carotenoids, mainly β-carotene. The significant feature is their anti-oxidative activity, which protects organisms against reactive oxygenic radicals. Other components that defined the antioxidant capacity includes phenols (phenolic acids and flavonoids), water-soluble (ascorbate) and lipid-soluble (tocopherols and tocotrienols) (Geneva at al., 2010).

In order to get insight into the role of the mycorrhizal symbiosis in protecting pot marigold (*C. officinalis* L.) against the excess of metals (Cd and Pb) and its contribution to valuable secondary metabolites accumulation, we investigated plant growth responses, mycorrhizal colonization, acid phosphatase activity (root and soil), uptake and distribution of heavy metals, antioxidant capacity, non-enzymatic antioxidant components in the flowers and major carotenoids in the flowers.

2. Materials and methods

2.1. Biological material and growth conditions

C. officinalis L. plants (var. Plamen) were grown from seeds in the glasshouse for 90 days (from April to July): without air conditioning; under natural sunlight (with a photoperiod of around 15 h). The glasshouse temperature (night to day) was between 15 °C and 30 °C, and the relative humidity ranged from 40% to 65%. The plants were grown in 1.2 kg plastic pots (2 plants per pot) on a unsterilised soil/sand substrate (3:1). Four replicates per treatment were prepared. The soil (type *Chromic Luvisols* (FAO, 1998), 30–40 cm depth) was collected from the field near a waste depository of a ferrous metallurgical plant and have the following agrochemical characteristics: pH 7.8; 9.0 mg kg^{-1} soil total mobile nitrogen ($N–NO_3^- + N–NH_4^+$), 26.0 mg kg^{-1} soil P_2O_5, 310 mg kg^{-1}soil K_2O; organic matter (3%), clay content (60%), sand content (13%) and silt content (26%). The concentrations of heavy metals (HMs) (mg kg^{-1} DW) in the start soil were measured: Cd—6.7, Pb—230 and Zn—199.5. According to the Bulgarian legislation, the permissible limit concentrations (at pH 7.8) are Cd < 3.0, Pb < 120 and Zn < 400 mg kg^{-1} DW).

The mycorrhizal isolates were kindly provided from the AMF collection of Estación Experimental del Zaidín (CSIC Granada, Spain):

a *C. claroideum* (N.C. Schenk & G. S. Sm., Schüβler and Walker, 2010), (syn. *Glomus claroideum* N. C. Schenk & G. S. Sm.), isolated from the rhizosphere of *Zea mays* (Braunschweig, Germany), growing in a parcel contaminated by the repeated addition of sludge's containing a low amount of metals (ref. EEZ 35).
b *C. claroideum* (N.C. Schenk & G. S. Sm., Schüβler and Walker, 2010), (syn. *Glomus claroideum* N. C. Schenk & G. S. Sm.), isolated from the rhizosphere of *Lavandula stoechas* (Rio Tinto, Spain) growing in a soil naturally rich in heavy metals (ref. EEZ 54).
c *F. mosseae* (Nicol. & Gerd., Schüβler and Walker, 2010), (syn. *Glomus mosseae* (Nicol. & Gerd.) Gerd & Trappe), isolated from the rhizosphere of *Lavandula stoechas* (Rio Tinto, Spain) growing in a soil naturally rich in heavy metals (ref. EEZ 55).

Mycorrhizal inoculation was done by placing the seeds over a thin layer of the AMF inoculum (2 g kg^{-1} soil substrate) following the layering method (Jackson et al., 1972). The inoculum consisted of colonized roots and soil from 4 months old oat pot cultures.

Four treatments were compared: 1-control (non-mycorrhizal plants - NM), 2- inoculated with strain EEZ 35, 3- inoculated with strain EEZ 54 and 4- inoculated with strain EEZ 55.

2.2. Determination of root colonization

The extent of mycorrhizal root colonization was determined using the grid line intersect method (Giovanetti and Mosse, 1980). To visualize the AMF colonization, roots were cleared in 10% KOH and staining with 0.05% Trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970).

2.3. Acid phosphatase activity

Acid phosphatase activity (APA, EC 3.1.3.2) was measured according to the method of Schneider et al. (2000), based on the original one of Tabatabai and Bremner (1969). Root tissue was homogenized with 0.1 M sodium acetate buffer (pH 5.0). After centrifugation, the supernatant was assayed for the enzyme activity by incubation in 5 mM *p*-nitrophenyl phosphate and 0.1 M sodium acetate buffer (pH 5.0). The reaction was stopped by the addition of 0.2 M NaOH, and absorbance measured at 405 nm. Soil phosphatase activity was assayed by colorimetric estimation of the *p*-nitrophenol released by phosphatase activity when the soil was incubated with buffered (pH 6.5) sodium *p*-nitrophenyl phosphate solution and toluene at 37° C for 1 h.

2.4. Heavy metal content

Soil samples were air dried and ground using a mortar and pestle and then were sieved through a 0.149 mm sieve. Both the plant and soil samples were digested in a solution containing 3:1 (v/v) HNO_3: $HClO_4$ solution. The samples were heated on a heating block at 200 °C to evaporate the samples to dryness. The residue was taken up in 25 ml of 1 N HCl (Doumett et al., 2008). Heavy metal concentrations were determined using an atomic absorption spectrophotometer (AAS, PerkinElmer 2100, USA).

2.5. Antioxidant capacity assays

2.5.1. Total antioxidant capacity

Free radical scavenging activity was measured from the bleaching of the purple methanol solution (2,2-diphenylpycrilhydrazyl, DPPH•), according to Tepe et al. (2006). DPPH• is a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant. The percent inhibition of the DPPH• radical (*I*%) was calculated by the following equation:

$$I\% = \left(\frac{A_{blank} - A_{sample}}{A_{blank}}\right) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound, i.e. calendula extracts.

2.5.2. Ferric reducing power (FRAP assay)

The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl), and $FeCl_3$–$6H_2O$ (20 mM) in a ratio of 10:1:1 (Benzie et al., 1996). To