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ORIGINAL ARTICLE

# Symbiotic association between golden berry (*Physalis peruviana*) and arbuscular mycorrhizal fungi in heavy metal-contaminated soil

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**Abstract**

*Physalis peruviana* is one of the most promising tropical fruit plants because of its rapid growth, high yield, and nutritional quality. This study was designed to investigate plant development under heavy metal contamination (Cd, Pb) and responsiveness to arbuscular mycorrhizal fungi (AMF) colonization by *Rhizophagus clarum* and *Claroideoglomus claroideum*. The antioxidant capacity, total lipid content and fatty acid profile in fruits, accumulation of Cd and Pb in different plant parts, plant dry biomass, and mycorrhizal colonization were determined. As a result of inoculation, a considerable reduction in Cd and Pb in the fruits was observed, compared with non-inoculated plants. The fruit number and dry weight increased in plants associated with *C. claroideum*. These plants also showed higher acid phosphatase activity, root protein accumulation and glomalin production. The type of antioxidant defense was AMF strain-dependent. Antioxidant activity and $H_2O_2$ neutralization were enzymatic rather than non-enzymatic processes in the fruits of *C. claroideum* plants compared with those forming an association with *R. clarum*. Mycorrhizal establishment changed the composition and concentration of fruits' fatty acids. The ratio of unsaturated fatty acids was increased. With respect to the accumulation of bioactive compounds in golden berry the present findings are important for obtaining the optimum benefits of mycorrhizal association under unfavorable conditions.

**Key words:** arbuscular mycorrhizal fungi, Cd and Pb, fruit quality, *Physalis peruviana*

## Introduction

Arbuscular mycorrhizal (AM) symbioses occur in most habitats and about 95% of all terrestrial plants belong to characteristically mycorrhizal families (Liang *et al.* 2009). Mycorrhizal symbiosis provides a direct physical linkage between the soil and plant roots. The mycorrhizal role in some extremely contaminated environments such as smelting and mining tailings containing abundant toxic heavy metals (HMs) involves facilitating water and nutrition uptake, improving plant resistance to stressful environments and suppressing plant pathogen infections (Liang *et al.* 2009; Ziedan *et al.* 2011). The search for tools to enhance plant tolerance to environmental stress and to restore naturally or industrially metal-contaminated soils has led to extensive studies on the soil-fungus-plant interface (Meier *et al.* 2012). Mycorrhizal fungi can affect the transformation of trace metals in the rhizosphere in several ways, such as acidification, immobilization, and modification of root exudates, hyphal sequestration, and chemical precipitation in the soil (Upadhyaya *et al.* 2010). Metal-tolerant arbuscular mycorrhizal fungi (AMF) isolated from contaminated areas cope better with metal toxicity than those isolated from unpolluted soils (Cornejo *et al.* 2013). Ferrol *et al.* (2009) suggested that isolation of indigenous, and presumably adapted, AM fungi was more suitable for phytostabilization purposes than laboratory strains, and can serve as a potential biotechnological tool for successful restoration of degraded ecosystems.

Heavy metals, like Pb and Cd, affect different physiological and biochemical processes in plants. Their presence induces an accumulation of reactive oxygen species (ROS) and methylglyoxyl, which results in lipid peroxidation, DNA damage, oxidation of proteins, and inactivation of enzymes (Bano and Ashfaq 2013). Plants possess very efficient enzymatic (superoxide dismutase – SOD; catalase – CAT; different specific peroxidases and enzymes of the ascorbate-glutathione cycle) and non-enzymatic (ascorbic acid, glutathione, alkaloids, phenolic compounds, α-tocopherols, non-protein amino acids, etc.) antioxidant defense systems, which work together to manage uncontrolled oxidation and protect plant cells from oxidative damage by scavenging ROS (Gill and Tuteja 2010).

Golden berry (*Physalis peruviana*) is a short-lived perennial, native to the Andes region (Fischer 2000). Recently, *P. peruviana* has become popular as a source of antioxidants and trace elements of natural origin (Eken *et al.* 2016). The presence of biologically active compounds, such as phytosterols, tocopherols, vitamins, essential minerals, different groups of phenols and flavonoids in the fruits, makes it a valuable food supplement with medicinal qualities (Puente *et al.* 2011). It is orange due to the presence of β-carotene, the main active component of vitamin A. The β-carotene and vitamin C content of *P. peruviana* provides its anticancerous function, associated with preventing accumulation of free radicals in tissues. In particular, withanolides and physalins are very important, biologically active components for their anti-inflammatory, antimicrobial, antitumor, immunomodulatory and antiparasitic properties (Demir *et al.* 2014). Golden berry can develop in a wide range of soils and climates, and it is classified as a highly tolerant species (Ramadan and Mörsel 2003). Ecological factors affect the composition of golden berry and lead to differences in chemical characteristics (Yıldız *et al.* 2015). Arbuscular mycorrhizae have been shown to enhance golden berry growth rates under saline conditions (Miranda *et al.* 2011). However, there are no studies concerning the effect of AMF on the development of *P. peruviana* grown in the presence of HM.

To understand the role of AM symbiosis in protecting *P. peruviana* plants against the excess of toxic metals, we investigated plant growth responses, metal accumulation, and the total content of lipids in fruits, fatty acids and the enzymatic and non-enzymatic antioxidant components of plants grown in soil polluted with Cd and Pb.

## Materials and Methods

### Biological materials and growth conditions

*Physalis peruviana* (golden berry, goose berry) plants were grown from seeds in a greenhouse under natural sunlight for 150 days, from April to September. The 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 3 kg plastic pots (2 plants per pot) on an unsterilized soil/sand substrate (3 : 1, v/v). Four replicates per treatment were prepared. The fruits for analyses were visually selected and harvested at three degrees of ripeness (4 – light orange; 5 – orange; 6 – deep orange), based on the color specification given in the Columbian Technical Standard NTC 4580 (Icontec 1999).

The soil (type – Chromic Luvisols, 30–40 cm depth) was collected from a field near a waste depository of a ferrous metallurgical plant and had the following agrochemical characteristics: pH = 7.8; 9.0 mg · $kg^{-1}$ soil total mobile nitrogen ($N\text{-}NO_3^-$ + $N\text{-}NH_4^+$), 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 HMs (mg · $kg^{-1}$ dry weight «DW») in the soil were measured: Cd – 6.7, Pb – 230, Zn – 199.5. According to the Bulgarian legislation (State newspaper 2008), the permissible limits (at pH = 7.8) are: Cd < 3.0, Pb < 120, Zn < 400 mg · $kg^{-1}$ DW).

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

1. *Rhizophagus clarum*, isolated from the rhizosphere of *Zea mays*, grown on soil contaminated by the repeated addition of sludge containing low amounts of metals (ref. EEZ 37).
2. *Claroideoglomus claroideum*, isolated from the rhizosphere of *Lavandula stoechas* grown on soil naturally rich in heavy metals (ref. EEZ 54).

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-month-old oat pot cultures.

Three treatments were compared: 1 – control, non-inoculated plants (NI), 2 – plants inoculated with *R. clarum* EEZ 37 (Rc), 3 – plants inoculated with *C. claroideum* EEZ 54 (Cc).

### Determination of root colonization

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

### Acid phosphatase activity

Acid phosphatase activity (APA, EC 3.1.3.2) was determined in roots and soil according to the method

of Schneider *et al.* (2000). Fresh root tissue was homogenized with 0.1 M sodium acetate buffer (pH 5.0). After centrifugation, the supernatant was assayed for 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 was 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.

## Easily extracted and total extracted glomalin-related soil proteins

Extraction was done according to Wright and Upadhyaya (1996). The soil (2 g) was mixed with 8 ml of 20 mM sodium citrate at pH 7.0. The samples were autoclaved for 30 min (121°C) and immediately centrifuged at 5,000x g for 15 min. The supernatant contained the easily extracted glomalin-related soil proteins (EE--GRSP). To total extract glomalin-related soil proteins (TE-GRSP) 2 g of soil was autoclaved in 8 ml of 50 mM sodium citrate at pH 8.0 for 60 min. Immediately after autoclaving, centrifugation at 5,000x g for 15 min was done. The supernatant was stored at 4°C until needed for analysis. Glomalin quantification (EE-GRSP and TE-GRSP) was done using the Bradford (1976) assay based on measuring absorbance at 595 nm against protein dye reagent and bovine serum albumin as a standard.

## 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 3 : 1 (v/v) $HNO_3$ : $HClO_4$ solution. The samples were dried by heating on a heating block at 200°C. The residue was dissolved in 25 ml of 1 N HCl (Doumett *et al.* 2008). Metal concentrations were determined on the inductively – coupled Plasma Mass Spectrometer (CCD Simultaneous ICP OES, Varian, Austria).

## Antioxidant capacity assays

### Total antioxidant capacity

Free radical-scavenging activity in fruit tissues was determined from the bleaching of the purple methanol solution (2,2-diphenyl-1-pircylhydrazyl, 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:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100,$$

where: $A_{blank}$ – the absorbance of the control reaction (containing all reagents except the test compound), $A_{sample}$ – the absorbance of the test compound, i.e. *P. peruviana* fruit extracts.

### Ferric ion reducing power (FRAP assay)

The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ (tripyridyltriazine) solution (10 mM TPTZ in 40 mM HCl), and $FeCl_3$–$6H_2O$ (20 mM) at a ratio of 10 : 1 : 1 (Benzie and Strain 1996). To perform the assay, 900 µl of FRAP reagent, 90 µl of distilled water and 30 µl of the fruit extract were mixed and incubated at 37°C for 15 min. The absorbance was measured at 595 nm using FRAP working solution as a blank.

### Determination of total phenolic compounds and flavonoids

Dry fruit samples (1 g) were ground and thoroughly extracted with 96% (v/v) methanol. Concentrations of phenolic compounds were determined spectrophotometrically using the Folin-Ciocalteu reagent and calculated as caffeic acid equivalents (Pfeffer *et al.* 1998). Flavonoids in plant tissues were measured spectrophotometrically according to Zhishen *et al.* (1999), using the standard curve of catechin.

### Antioxidant potential assay

Spectrophotometric quantification of water-soluble and lipid-soluble antioxidant capacity (WS-AOC, LS-AOC), expressed as equivalents of ascorbate and α-tocopherol, were performed through the formation of the phospho-molybdenum complex (Prieto *et al.* 1999). The assay was based on the reduction of Mo (VI) to Mo (V) by the sample analysis and the subsequent formation of a green phosphate/Mo (V) complex at an acidic pH. Dry fruit material (0.5 g) was ground with pestle and mortar to a fine powder and 3 ml $dH_2O$ was added and the suspension was homogenized, transferred to tubes and shaken for 1 h at room temperature in the dark. The suspension was filtered and the extraction repeated with 3 ml $dH_2O$. The pellet was washed again with 2 ml $dH_2O$. For lipid soluble antioxidant capacity (expressed as α-tocopherol), the procedure was the same except that the extraction was carried out with hexane as a solvent. The method has been optimized and characterized on linearity interval, repeatability and reproducibility and molar absorption coefficients for the quantitation of water-soluble and lipid-soluble antioxidant capacities expressed as equivalents of ascorbate and α-tocopherol (Prieto *et al.* 1999). Absorption coefficients were: (3.4±0.1) × 103 $M^{-1} \cdot \cdot cm^{-1}$ for ascorbic acid and (4.0±0.1) × 103 $M^{-1} \cdot cm^{-1}$ for α-tocopherol. The $H_2O_2$ content was determined

according to Patterson *et al.* (1984). For the determination of superoxide dismutase, catalase, ascorbate peroxidase (APX) and guaiacol peroxidase (GPO) activities, fresh fruit material (0.50 g fresh weight «FW») was homogenized in 0.1 mM K-phosphate buffer (pH 7.8), containing 2.0 mM $Na_2$-EDTA (ethylenediaminetetraacetic acid, 1 mM PMSF (phenylmethylsulfonyl fluoride), 2% polyvinylpyrrolidone K-40 (w/v) and 10% glycerol. The homogenate was centrifuged at 12,000x g for 30 min and the supernatant was used as a crude enzyme extract. All steps in the preparation of the enzyme extract were carried out at 0–4°C.

Total CAT (EC 1.11.1.6) activity was measured according to the method of Beers and Sizer (1952), with minor modifications. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 0.1 M EDTA, 15 mM $H_2O_2$ and 0.1 ml enzyme extract. The reaction was started by the addition of $H_2O_2$. The decrease of $H_2O_2$ was monitored at 240 nm and quantified by its molar extinction coefficient (36 $M^{-1} \cdot cm^{-1}$) and the results expressed as units – mol $H_2O_2$ destroyed per min per mg protein.

Total APX (EC 1.11.1.1) activity was assayed according to Nakano and Asada (1987). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 M EDTA, 0.5 mM ascorbate, 0.1 mM $H_2O_2$ and 0.2 ml enzyme extract. The reaction was started by the addition of $H_2O_2$ and ascorbate oxidation measured at 290 nm for 1 min. Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 $mM^{-1} \cdot cm^{-1}$) and the results expressed as mol $H_2O_2$ destroyed per min per mg protein.

Total GPO (EC 1.11.1.7) activity was determined as described by Urbanek *et al.* (1991) in a reaction mixture (2.0 ml) containing 100 mM phosphate buffer (pH 7.0), 0.1 µM EDTA, 5.0 mM guaiacol, 15.0 mM $H_2O_2$ and 100 µl enzyme extract. The addition of the enzyme extract started the reaction and an increase in absorbance was recorded at 470 nm for 1 min. Enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient (26.6 $mM^{-1} \cdot cm^{-1}$). The results were expressed as µmol $H_2O_2$ $mg^{-1}$ protein $min^{-1}$.

Total SOD (EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), as described by Giannopolitis and Ries (1977). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 M EDTA, 13 mM methionine, 33 µM NBT, 3.3 µM riboflavin and enzyme extract (50, 100, 150 and 200 µM). Riboflavin was added last and tubes were shaken and illuminated. The reaction was allowed to proceed for 15 min, after which the lights were switched off and the tubes covered with a black cloth. The absorbance of the reaction mixture was read at 560 nm. One unit of SOD activity (U) was defined as the amount (mg) of protein required to cause 50% inhibition of the NBT photoreduction rate. Soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

### Gas chromatography (GC) analysis of total lipids and fatty acid methyl esters (FAME) in fruits

The dried biomass of *P. peruviana* fruits was extracted three times with chloroform – methanol (2 : 1, v/v) for 30 min under reflux. The extracts were evaporated *in vacuo* and the residues were re-extracted with chloroform. Total lipids were determined gravimetrically and calculated as a percentage of dry biomass. Fatty acids from the lipids were converted to FAME by heating in methanol containing 6% anhydrous HCl at 60°C for 1.5 h. The FAME were extracted with hexane and purified by thin-layer chromatography on silica gel. Using gas-chromatography the purified FAME were analysed on a Perkin-Elmer instrument as previously described (Iliev and Petkov 2006). Fatty acids were identified using reference substances.

### Statistical analysis

Data were expressed as means ± standard error, where n varied between 3 and 10, depending on the type of analysis. Comparison of means was performed by the Fisher's least significant difference (LSD) test at $p \leq 0.05$ following ANOVA. Statistical software package StatGraphics Plus, version 5.1 for Windows, USA was used.

## Results and Discussion

*Physalis peruviana* has great potential, since it grows in different soil types, has low fertilizer requirements, and high tolerance to unfavorable environmental factors (Puente *et al.* 2011). This study revealed that *P. peruviana* plants, grown in metal-contaminated soil developed successful mycorrhizal association, flowered and formed fruits without visual phytotoxicity symptoms despite the elevated concentrations of Cd and Pb.

### The effect of arbuscular mycorrhizal fungi strains on dry weight and fruit number of *Physalis peruviana* plants

An analysis of dry-weight biomass demonstrated an increment in the root, shoot and fruit dry weight values of *Claroideoglomus claroideum* (Cc) treatments compared with the other studied variants (Table 1). Root DW rates following *Rhizophagus clarum* (Rc) inoculation were statistically similar to non-inoculated plants (NI), but dry

**Table 1.** Dry weight and fruit number of *Physalis peruviana* plants inoculated with *Rhizophagus clarum* and *Claroideoglomus claroideum*

| Treatments | Roots [g · plant$^{-1}$ DW] | Shoots [g · plant$^{-1}$ DW] | Fruits [g · plant$^{-1}$ DW] | Fruit number per plant |
|---|---|---|---|---|
| NI | 3.583 a | 9.02 a | 0.579 a | 6 a |
| Rc | 3.708 a | 10.125 b | 0.562 a | 7 a |
| Cc | 4.734 b | 10.801 c | 0.615 b | 10 b |

Letters in common within a column indicate no significant differences assessed by Fisher LSD test ($p \leq 0.05$) after performing ANOVA (n = 6). DW – dry weight, NI – non-inoculated plants, Rc – *Rhizophagus clarum*, Cc – *Claroideoglomus claroideum*

shoot biomass was higher in those treatments. The increased fruit number in Cc plants was followed by higher DW than control and Rc plants (Table 1). The positive effect of AMF strains on plant biomass under the studied unfavorable conditions was markedly expressed in the Cc plants, while the fungi affected only shoot DW in Rc variants (Table 1). Previous studies have found that the AMF strains derived from the soil with a naturally high level of metals are evolutionarily more adapted to higher metal concentrations (Cornejo *et al.* 2013). As pointed out in the "Material and Methods" section, the strain *C. claroideum* (ref. EEZ 54) was isolated from the rhizosphere of *L. stoechas* growing on soil naturally rich in heavy metals (Rio Tinto, Spain). The positive effect of AMF inoculation on *P. peruviana* growth has been observed in a limited number of investigations (Radhika and Rodrigues 2010; Miranda *et al.* 2011). In both studies only the stimulatory role of mycorrhizal inoculation on plant development was described.

## Concentrations of Pb and Cd in soil and plant parts after harvest of *Physalis peruviana*

The order of partitioning of Pb and Cd was: soil > root > shoots > fruits for all treatments (Table 2). In the fruits, the highest content of Pb and Cd was observed in NI. In the shoots (stems and leaves), we found the highest content of both Pb and Cd in NI. In both mycorrhizal treatments, the level of HM in the belowground parts increased compared to the NI. Rc preferably accumulated Pb, whereas Cc accumulated Cd. The HM accessibility to plants and thus their toxicity depends on the interplay of complicated rhizospheric activities involving not only transfer processes between soil and plants but also microbial activities. Mycorrhizal fungi appear to play a central modulating role (Schützendübel and Polle 2002). In our previous research with *Calendula officinalis* (Hristozkova *et al.* 2016), we found an opposite trend – an increased content of Cd and Pb in the shoots compared to roots. The successful mycorrhizal association functioned as a barrier decreasing HM uptake and translocation to the aboveground parts. Different mechanisms are responsible for the Cd and Pb reduction in the shoots. One possible explanation is a biomass dilution effect due to the positive growth response to AM symbiosis (Li *et al.* 2016). The ability to bind HMs in the rhizosphere by releasing glomalin may also account for the reduction of HM. Moreover, plants inoculated with AMF under HM stress may result in the expression of specific genes, which are responsible for the production of proteins (including metallothioneins) that increase the resistance of plants to stress (Ferrol *et al.* 2016).

## Acid phosphatase activity (soil and roots), percentage of mycorrhization, easily extracted glomalin-related soil proteins (EE-GRSP), total extracted glomalin-related soil proteins (TE- GRSP) and total protein concentration

The mycorrhizal colonization somewhat helped distinguish between the two inoculated roots (Fig. 1). The

**Table 2.** Concentrations of Pb and Cd (mg · kg$^{-1}$) in soil and plant parts after harvest of *Physalis peruviana* plants inoculated with *Rhizophagus clarum* and *Claroideoglomus claroideum*

| Treatments | Soil | | Roots | | Shoots | | Fruits | |
|---|---|---|---|---|---|---|---|---|
| | Pb [mg · kg$^{-1}$ DW] | Cd [mg · kg$^{-1}$ DW] | Pb [mg · kg$^{-1}$ DW] | Cd [mg · kg$^{-1}$ DW] | Pb [mg · kg$^{-1}$ DW] | Cd [mg · kg$^{-1}$ DW] | Pb [mg · kg$^{-1}$ DW] | Cd [mg · kg$^{-1}$ DW] |
| NI | 142.94 a | 1.94 b | 5.56 a | 0.38 b | 5.08 c | 0.35 c | 0.74 b | 0.18 b |
| Rc | 199.75 c | 1.86 a | 10.33 b | 0.34 a | 4.1 b | 0.24 b | 0.46 a | 0.04 a |
| Cc | 171.72 b | 3.89 c | 5.93 a | 0.82 c | 3.72 a | 0.12 a | 0.49 a | 0.05 a |

Letters in common within a column indicate no significant differences assessed by Fisher LSD test ($p \leq 0.05$) after performing ANOVA (n = 6). DW – dry weight, NI – non-inoculated plants, Rc – *Rhizophagus clarum*, Cc – *Claroideoglomus claroideum*

observed increase in Cc plants correspondended with the highest values of root and soil APA, root protein accumulation and glomalin production (EE-GRSP and TE-GRSP). As far as the root APA and protein content were concerned, inoculation with strain Rc had the lowest values, followed by NI plants and Cc (Fig. 1) despite the relatively high mycorrhizal colonization. The positive correlation between AM fungal root colonization and GRSP concentrations confirm the link between GRSP and AMF biological activity. There is evidence that different mycorrhizal species are capable of producing different amounts of GRSP (Wright and Upadhyaya 1996). The various amounts of glomalin protein which are produced by mycorrhizas has several significant implications. There may be strains in a community such as Cc which have high protein secretion rates. Based on its primary functions to aggregate soil, increase water infiltration and improve nutrient cycling, such fungal strains may be very useful in agro ecosystem applications (Rillig *et al.* 2002). Related to this, van Aarle *et al.* (2002) emphasized the importance of studying not only the amount of AM fungal mycelium but also the proportion of active mycelium. Plant roots, fungi and other microorganisms in the soil separately possess APA (Abd-Ala 1994), which is why in some cases there is no correlation with soil and root phosphatase activities. APA is associated with phosphorus acquisition in the rhizosphere, and the growth and development of the fungus within the host tissue (Prasad *et al.* 2012). It is managed by an unspecified mechanism of the plants.

## Concentration of $H_2O_2$ and activity of catalase (CCAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and guaiacol peroxidase (GPO) in fruits of *Physalis peruviana* plants

Simbiotic association with both mycorrhizal strains reduces the content of $H_2O_2$, in *P. peruviana* fruits, which is indicative of the alleviation of the stress. The capacity of CAT, SOD, APX and GPO enzymes significantly

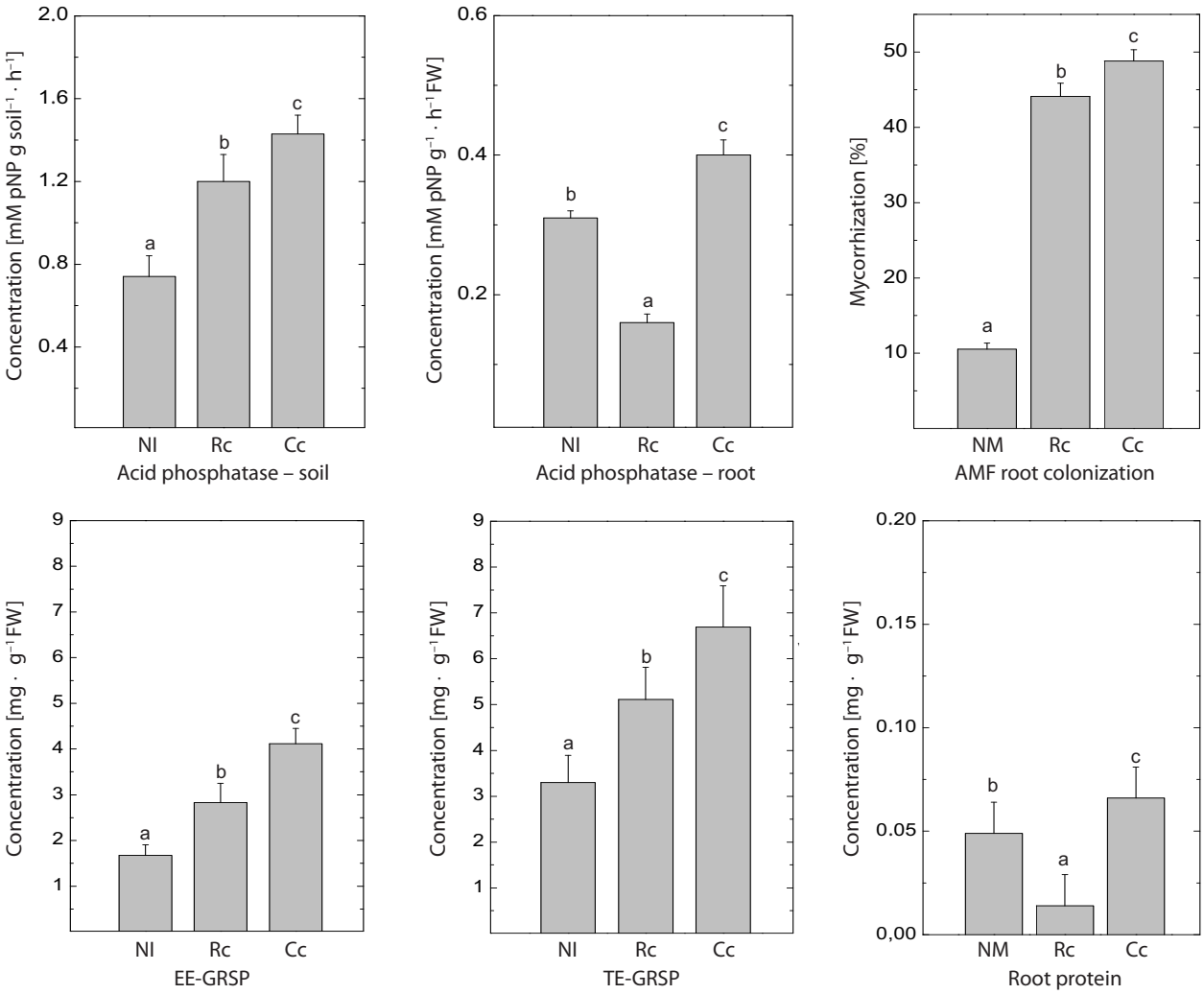


**Fig. 1.** Acid phosphatase activity (soil and roots), percentage of mycorrhization, easily extracted glomalin-related soil proteins (EE--GRSP), total extracted glomalin related soil proteins (TE-GRSP) and total protein concentration in *Physalis peruviana* plants inoculated with *Rhizophagus clarum* (Rc) and *Claroideoglomus claroideum* (Cc). Values are means ±SE, letters in common within a graph indicate no significant differences assessed by Fisher LSD test ($p \leq 0.05$) after performing ANOVA (n = 3). pNP – paranitrophenol

increased in the plants inoculated with strain Cc, followed by the control plants (Fig. 2). A decrease was recorded in Rc plants' enzyme functions compared to control and Cc variants, except for GPO activity. The assessment of antioxidant enzymatic activities (SOD, CAT, APX, GPO) and non-enzymatic low molecular metabolite concentrations (total phenols, flavonoids, carotenoids, lipid soluble and water soluble AOC) revealed a counteraction against harmful active oxygen species generated by HM oxidative stress. We found that both mycorrhizal strains reduced the content of $H_2O_2$, in *P. peruviana* fruits, which is indicative of the alleviation of the stress. When the plants were inoculated with strain Cc a significant increase in the activity of antioxidant enzymes (CAT, SOD, APX and GPO) occurred, followed by the control plants (Fig. 2). Enhanced activity of antioxidants mediates quick scavenging of ROS and hence protects cells from possible oxidative damage (Noctor and Foyer 1998; Mittler 2002). The study of Hashem *et al.* (2016) demonstrated that antioxidant enzymes increased in Cd-stressed tomato plants and their activity was further enhanced by inoculation with AMF. On the other hand, fruit ripening is an aerobic process generating ROS. These molecules initiate and improve degenerative processes associated with fruit maturation, which is considered a protracted form of senescence (Qin *et al.* 2009). The increased antioxidant system activity in *Prunus* and *Citrus* spp. greatly contributes to protecting fruit against oxidative damage during ripening and to delaying the senescence processes in harvested fruits (Racchi 2013).

## Total phenolic and flavonoid concentrations, water-soluble, lipid-soluble antioxidant capacities (AOC) (expressed as equivalents of ascorbate and α-tocopherol), free radical-scavenging activity (DPPH) and ferric reducing power (FRAP) of *Physalis peruviana* fruits

The concentration of total phenols and flavonoids in the Rc variant decreased while the level of flavonoids in NI

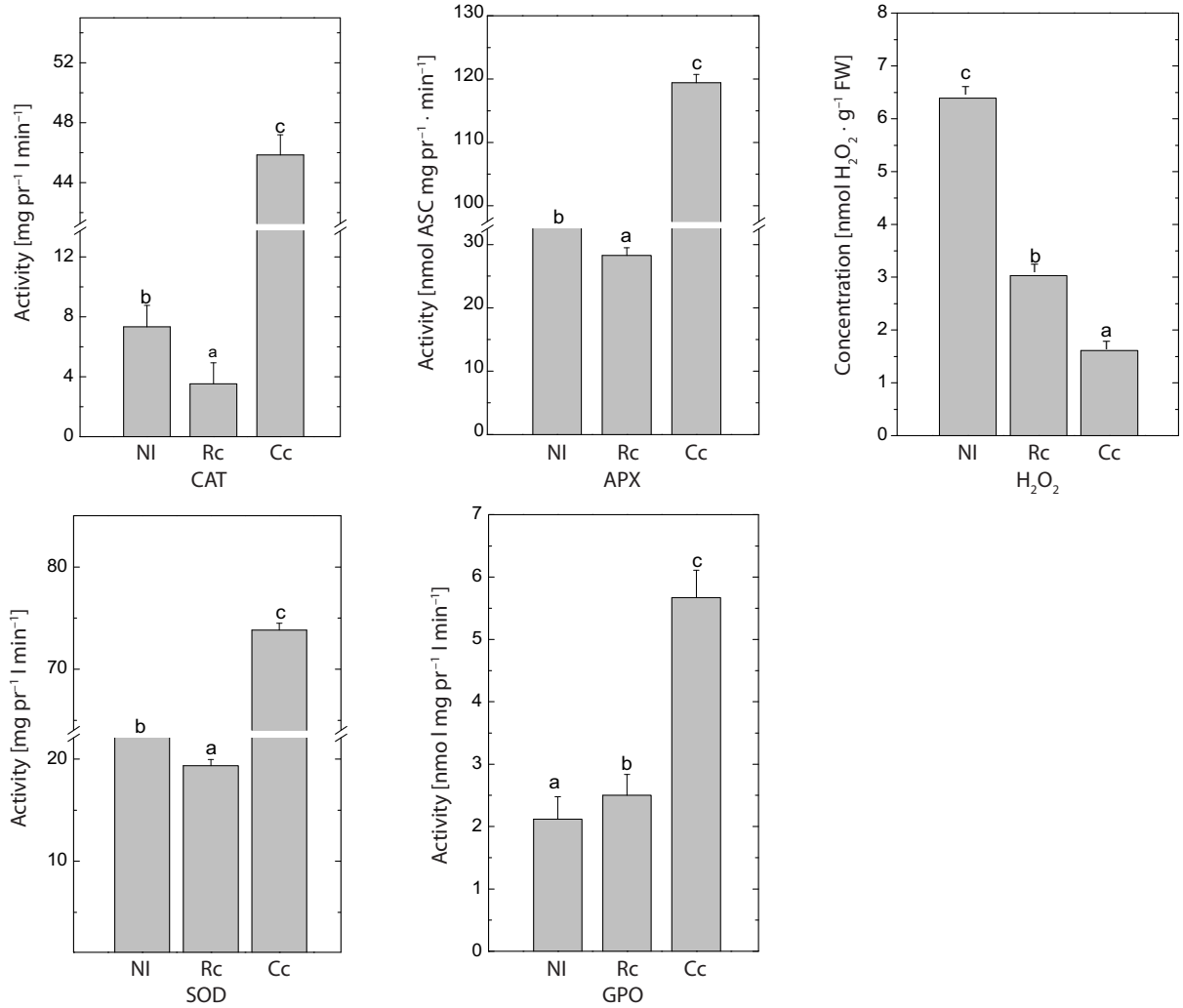


**Fig. 2.** Concentration of $H_2O_2$ and activity of catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and guaiacol peroxidase (GPO) in fruits of *Physalis peruviana* plants, inoculated with *Rhizophagus clarum* (Rc) and *Claroideoglomus claroideum* (Cc). Letters in common within a graph indicate no significant differences assessed by Fisher LSD test ($p \leq 0.05$) after performing ANOVA (n = 3). ASC – ascorbic acid

plants was the highest. A slight increase was observed in Cc total phenol concentration compared with flavonoids (Fig. 3). To further investigate the oxidative response of *P. peruviana* during its interaction with AMF in HM soil, we studied the concentration of water-soluble and lipid-soluble AOC (expressed as equivalents of ascorbate and α-tocopherol, respectively) (Fig. 3). We observed a reduction of their concentration in NI plants, coinciding with the results for total carotenoids (Table 3). FRAP activity and the content of water- and lipid-soluble metabolites with antioxidant activity in *P. peruviana* fruits was higher in both tested strains while DPPH activity did not change (Fig. 3). The antioxidant performance of phenolic compounds is due to their high tendency to chelate metals. The concentration of flavonoids and total phenols in Rc variations decreased while the level of flavonoids was the highest in NI. An opposite trend was observed in Cc variants. According to Nell *et al.* (2009), a published report has shown that AMF does not have positive effects on the composition of phenolic ingredients. On the other hand, the antioxidant capacity (FRAP) is associated with the total phenolic content, which probably is a consistent predictor of the cellular antioxidant activity of fruits (Fu *et al.* 2011). According to Racchi (2013), the decline in the antioxidant system paralleled decreased antioxidant activity (FRAP values) and consequently was correlated with enhanced oxidative stress associated with ripening in orange fruits. The similar tendency of increased FRAP activity and total phenol concentration, accompanying a significant increment of antioxidant enzyme levels (CAT, SOD, APX and GPO) in Cc plants, underlined the contribution of the *C. claroideum* strain in fruit metabolism activated to fight against oxidative stress induced by heavy metal contamination. The concentration of water soluble and lipid soluble AOC (expressed as equivalents of ascorbate and α-tocopherol, respectively) described the oxidative response of *P. peruviana* during its interaction with AMF in HM soil (Fig. 3). The observed concentration reduction in NI plants, coincided with the rates for total carotenoids. The obtained results underline the observation that ascorbate and α-tocopherol participate in the

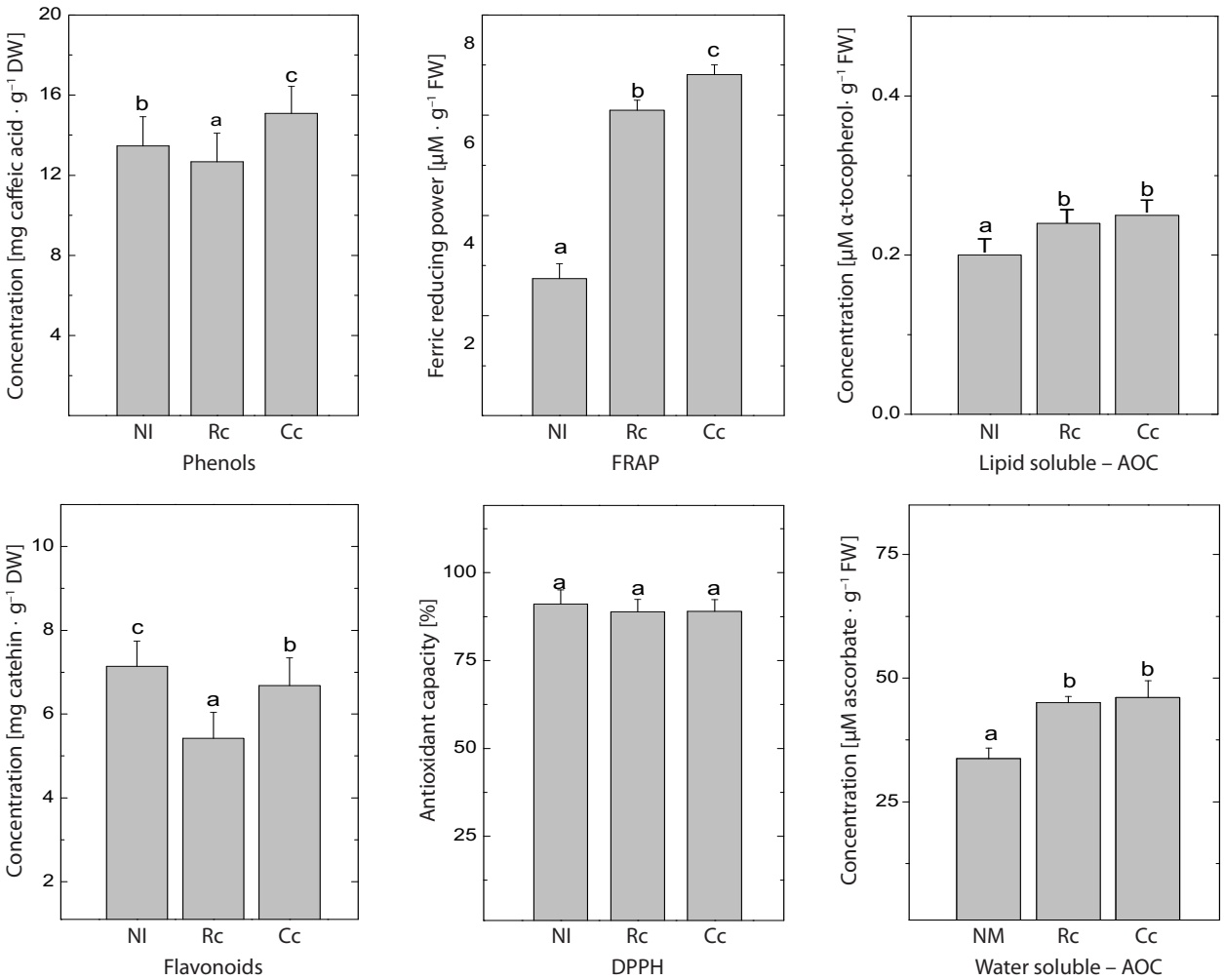


**Fig. 3.** Total phenolic, flavonoid concentration, water-soluble, lipid-soluble antioxidant capacities (AOC) (expressed as equivalents of ascorbate and α-tocopherol), free radical-scavenging activity (DPPH) and ferric reducing power (FRAP) of *Physalis peruviana* fruits inoculated with strains: *Rhizophagus clarum* (Rc) and *Claroideoglomus claroideum* (Cc). Letters in common within a graph indicate no significant differences assessed by Fisher LSD test ($p \leq 0.05$) after performing ANOVA (n =3)

maintenance of the redox balance of the cell and the free radical scavenging cycle (Racchi 2013).

The antioxidant capacity of *P. peruviana* has been reported in previous studies (Ramadan and Mörsel 2007; Vasco *et al.* 2008; Puente *et al.* 2011; Valdenegro *et al.* 2012). Ramadan and Mörsel (2007) researched the antioxidant activity of *P. peruviana* juice according to reaction time with DPPH. They found that the antioxidant capacity of this fruit was related to its fat-soluble bioactive contents such as tocopherols, sterols, and carotenoids. Vasco *et al.* (2008) analyzed the antioxidant capacities of different types of fruits and determined that *P. peruviana* has antioxidant activity at a low level compared to other fruits. According to Valdenegro *et al.* (2012), the total content of antioxidants in the fruits of *P. peruviana* depends on the species and cultivar and can be affected by many factors such as environmental conditions of growing, harvest time, ripening stage, storage and processing conditions.

## Fatty acid profile, total lipids (%) and total carotenoids

According to the results represented in Table 3, the fatty acid profile gave the proportion of the essential fatty acids in a whole berry (including full berry oil, seed oil, and pulp oil). In all of the tested variants, linoleic acid was the dominating fatty acid followed by palmitic and oleic acid. The highest content of linoleic acid was detected in the NI plants (74%) while the percentage of palmitic acid remained constant in all of the tested plants. The highest oleic acid content was in Rc. An analysis of fatty acids also revealed that the *P. peruviana* fruits from Rc had the greatest stearic and linolenic acid content. Three minor fatty acids, namely palmitoleic (C16 : 1), stearic (C18 : 0), and α-linolenic (C18 : 3) were also detected in *P. peruviana* fruits. The carefully chosen mycorrhizal treatment changed the composition and concentration of fatty acids of *P. peruviana* fruits under heavy metal stress, resulting in increased unsaturated fatty acid proportions (Table 3). The values obtained for total lipids (between 7 and 9%) demonstrated that fruits of *P. peruviana* are relatively rich in lipophilic substances (Table 3). The highest value for NI plants followed the above mentioned tendency for linoleic acid. The fatty acid profile gave the proportion of the essential fatty acids in a whole berry (including full berry oil, seed oil and pulp oil). The detected components in all three samples were typical for *P. peruviana* fruits as described by Ramadan and Mörsel (2003). In all of the tested variants, linoleic acid was the dominating fatty acid followed by palmitic and oleic acids. The highest content of linoleic acid was detected in the NI plants (74%) while the percentage of palmitic acid remained constant in all of the tested plants. The oleic acid content was highest in Rc. The analysis of fatty acids also revealed that the *P. peruviana* fruits from Rc had the greatest content of stearic and linolenic acids. Three minor fatty acids, namely palmitoleic (C16 : 1), stearic (C18 : 0), and α-linolenic (C18 : 3) were also detected in *P. peruviana* fruits. The carefully selected mycorrhizal treatment changed the composition and concentration of fatty acids of *P. peruviana* fruits under heavy metal stress, resulting in an increase in the proportion of unsaturated fatty acids. We hypothesized that the increased levels of total lipids and linolenic acid in control plants and Cc variants might be related to an enhanced rate of lipid biosynthesis while the lowest total lipids and a higher percentage of some fatty acids in Rc were a result of heavy metal-induced lipid peroxidation or suppression of the biosynthesis. Also, the supposed lipid peroxidation could explain lower antioxidant enzyme activities (CAT, APX, and SOD). Also, remarkable changes in the membrane lipids were reported to be an adaptive response to different environmental stresses (including HM), to restore excellent physical membrane properties (Upchurch 2012). In general, under heavy metal stress, the saturated FA and those with shorter chains decreased, while there was increase in the unsaturated FA content (Prasad 2004).

**Table 3.** Fatty acid profile (as a percentage of total fatty acid methyl ester, FAME), total lipids (%) and total carotenoids (mg · · $g^{-1}$ DW) in the fruits of *Physalis peruviana* plants inoculated with *Rhizophagus clarum* and *Claroideoglomus claroideum*

| Fatty acid | Treatments [%] | | |
|---|---|---|---|
| | NI | Rc | Cc |
| saturated | | | |
| C16 : 0 (Palmitic) | 13.1 a | 13.7 b | 13.7 b |
| C18 : 0 (Stearic) | 0.2 a | 0.6 c | 0.5 b |
| ω–3 Unsaturated | | | |
| C18 : 3 (α-Linolenic) | 0.2 a | 1.9 c | 0.3 b |
| ω–6 Unsaturated | | | |
| C18 : 2 (Linoleic) | 74.5 c | 71.1 a | 72.6 b |
| ω–7 Unsaturated | | | |
| 16 : 1 (Palmitoleic) | 1.1 b | 0.7 a | 1.3 b |
| ω–9 Unsaturated | | | |
| C18 : 1 (Oleic) | 10.8 a | 12.0 c | 11.7 b |
| Lipids (%) | 9.5 c | 7.8 a | 8.7 b |
| Total carotenoids [mg · $g^{-1}$ DW] | 0.63 a | 0.70 b | 0.67 a |

Letters in common within a row indicate no significant differences assessed by Fisher LSD test ($p \leq 0.05$) after performing ANOVA (n = 6), DW – dry weight, NI – non-inoculated plants, Rc – *Rhizophagus clarum*, Cc – *Claroideoglomus claroideum*

## Conclusions

Golden berry grown under conditions of heavy metal pollution (Cd, Pb) performed as a tolerant plant species. This was based on the absence of visible toxicity symptoms and plant biomass reduction. More research is necessary to reveal a plant's ability to grow under field conditions with a higher concentration of heavy metals and targeted mycorrhizal treatment. The fruit number, root and shoot dry weights increased in plants associated with *C. claroideum* as a consequence of successfully developed symbiosis with golden berry roots and corresponded with higher acid phosphatase activity and glomalin production. The type of antioxidant defense was AMF strain dependent. Antioxidant activity and $H_2O_2$ neutralization were more enzymatic than the non-enzymatic process in the fruits of plants inoculated with *C. claroideum* compared to those forming an association with *R. clarus*. The developed mycorrhizal association changed the composition and concentration of fatty acids of golden berry fruits in response to heavy metal stress, appearing as an increased proportion of unsaturated fatty acids.

Our outcomes underline the fact that the AMF strains depend on the genotype, have different abilities to develop an effective mycorrhizal association with the host plant and have an influence on the plant metabolism. Plants grown in symbiosis with an appropriate AMF are often more competitive and better able to tolerate different types of stress than normally grown plants, potentially enhancing plant tolerance. The findings in this study are important in order to obtain the optimum benefits of mycorrhizal association under unfavorable conditions for golden berry bioactive compound accumulation in edible parts. The *P. peruviana* fruits could be considered as an important source of biologically active components with high antioxidant activity to meet the requirements of consumers interested in the potential role of functional foods. This study would encourage future research on other AMF strains in association with *P. peruviana* to achieve general benefits of bioactive compounds in its fruits.

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