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Response to Drought Stress of In Vitro and In Vivo Propagated *Physalis peruviana* L. Plants Inoculated with Arbuscular Mycorrhizal Fungi

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Abstract: *Physalis peruviana* L. is one of the most favorable tropical fruit due to its fast growth and nutritional properties. The current research outlined the response to drought stress of *golden berry* plants inoculated with arbuscular mycorrhizal fungi *Claroideoglomus claroideum* and propagated in vitro as well as from seeds. The implementation of mycorrhizal symbiosis was determined by root colonization, glomalin content, and alkaline and acid phosphatases in roots and soil. The plant protection was assured by enzyme and non-enzyme antioxidants. The adapted in vitro propagated plants demonstrated higher resistance to drought than plants developed from seeds indicated by increased growth parameters (shoot, root biomass, fruit number), plastid pigment content, antioxidant activity, and less enhance mentofoxidative markers levels in water-deficient conditions. The findings in the present research are relevant to obtain the optimal mycorrhizal association and type of propagation in an adverse environment for golden berry development and will lead to the establishment of a database and model of varied plant responses to stressful conditions such as drought.

Keywords: *Physalis peruviana* L.; arbuscular mycorrhizal fungi; antioxidant protection

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1. Introduction

Physalis peruviana L. (golden berry, cape gooseberry) belongs to the family *Solanaceae* and is a South American plant native to Colombia, Ecuador and Peru. Widely introduced in the 20th century, *P. peruviana* is cultivated or grows wild across the world intemperate and tropical regions [1]. *P. peruviana* is a valuable food supplement with therapeutic properties since the fruits contain biologically active substances such as phytosterols, tocopherols, vitamins, vital minerals, and various groups of phenols and flavonoids [2]. The fruits' orange color is due to the presence of β-carotene, the main active component of vitamin A. The bioactive content of *P. peruviana* provides its anti cancerous function, associated with preventing the accumulation of free radicals in tissues. In particular, their physalin content is a very important, biologically active component for the fruits' anti-inflammatory, antimicrobial, antitumor, immune-modulatory, and antiparasitic properties [3]. The fruits are also rich in iron, manganese, zinc, sodium, magnesium, potassium, calcium, phosphorus, sulfur, aluminum, boron, and copper [4]. Sexual reproduction, from the seeds, is the preferred approach for the natural propagation of *P. peruviana* in which plants are reproduced in their native environment or under controlled conditions. Successful seed emergence depends on environmental parameters such as humidity, temperature, light, and oxygen. Moreover, the climate conditions in the temperate latitudes do not allow the full realization of the vegetation cycle of this species when grown from seeds. Another approach for *P. peruviana* reproduction is the asexual in vitro method, which employs micropropagation and grafting.

Asexual in vitro propagation is an alternative option for the rapid production of healthy, genetically uniform, and pathogen-free plantlets [5,6]

Arbuscular mycorrhizal fungi found in nature form symbioses with about 95% of all earth plants [7]. There is a direct physical connection between the soil and plant roots thanks to mycorrhizal symbiosis. The mycorrhizae's role includes increasing plant tolerance to stressful environments, promoting water and nutrition intake, and reducing plant pathogen infections [7,8]. Environmental stress conditions, such as drought stress, increase the generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals, which may cause cell damage [9,10]. To mitigate and repair the damage, plants possess enzymatic (SOD, CAT, and peroxidases) and non-enzymatic (ascorbate, glutathione, carotenoids, and tocopherols) mechanisms to detoxify ROS. The soil-borne arbuscular mycorrhizal fungi (AMF) possesses the potential to improve plant growth and development by modulating key physiological and biochemical characteristics, reducing the adverse effects of abiotic stresses, such as drought [11]. No information is available on the effects of mycorrhizal inoculation of *P. peruviana* on the degree of antioxidant potential in adapted, in vitro and in vivo propagated plants as a consequence of drought-induced stress.

The present study aimed to determine the physiological parameters and to evaluate the potential of *P. peruviana* L. in vitro and seed-propagated plants to cope with drought-induced stress conditions following mycorrhizal symbiosis.

2. Materials and Methods

2.1. Shoot In Vitro Propagation, Root Initiation, and Ex Vitro Acclimatization

The *Physalis peruviana* L. seeds after efficiently surface sterilizing with 70% ethanol for 2 min and 15% bleach solution (commercial bleach) for 15 min, followed by three washes for 15 min with sterilized distilled water, were planted in pots containing an unsterilized soil: perlite mixture (2:1) for four weeks until reaching an approximate height of 10 cm [12]. The acquired seedlings were sterilized superficially for 30 min with 0.04% mercury chloride ($HgCl_2$), followed by three rinses in sterile water for 15 min. The seedlings were segmented into 4–16 pieces and cultured for three weeks in Murashige and Skoog's [13] medium which includes vitamins, sucrose (3.0%), agar-agar (7.0 g L^{-1}), and 1.0 mg L^{-1} $CaCl_2$. The pH of the MS media was adjusted to 5.8. The media was autoclaved (121 °C, 20 min, pressure = 1.1 kg cm^{-2}). *P. peruviana* plantlets were transferred to $\frac{1}{2}$ MS medium (2% sucrose and 0.5 mg L^{-1} IBA) for root initiation [14]. The in vitro cultures were acclimated in a growth room: 22 °C temperature, 70% humidity, and 16 h photoperiod (40 µmol m^{-2} s^{-1} full spectra cool white fluorescent light, (Philips 36 W)).

After four weeks, the micro propagated plants following careful removal from the tubes and washing to remove the adhering agar-agar were acclimatized under ex vitro conditions. The plantlets were adapted in pots (d = 8 cm) with unsterilized soil: peat: sand (2:1:1/*v:v:v*), protected by a transparent polyethylene membrane to ensure high humidity (90%) and cultivated for 2 weeks in a growth chamber at 20 °C with a 14 h photoperiod (50 µmol m^{-2} s^{-1}, full spectra fluorescent light). Following two weeks of cultivation, the plants were adapted in a green house and one week later, planted in plastic containers with 4 kg unsterilized soil: sand (3:1/*v:v*). The soil type is leached cinnamon forest soil (according to FAO/UNESCO-ISRIC Soil Map- Chromic Luvisols) with pH (H_2O)—6.2, total mobile nitrogen (N-NO_3^- + N-NH_4^+)—0.008 g kg^{-1}, P_2O_5—0.03 g kg^{-1}, K_2O—0.120 g kg^{-1}, and 1.88% organic matter.

2.2. Seed-Propagated Plants

The *P. peruviana* seeds were sown in a laboratory into4 kg pots (two plants per pot) in unsterilized soil: sand (3:1 *v/v*) for germination at the end of January. At the end of April, the pots were adapted in a greenhouse (from April to July, with a photoperiod of approximately 15 h; t = from 15 °C to 30 °C night/day; humidity between 40% and 65%).

Four replicates per treatment were prepared. At the end of July, the plant samples were collected when the fruits to be used for analyses were visually ripened.

2.3. Mycorrhizal Inoculation

Mycorrhizal inoculation was carried out by laying the seeds over a thin layer of the AMF inoculum (2 g kg^{-1} soil substrate) [15]. The inoculums contained of colonized roots and soil (4-month-old oat pot cultures). The AMF strain (*Claroideoglomus claroideum*, ref. EEZ 54) was kindly provided by the Estación Experimental del Zaidín (Spain) mycorrhizal strain collection.

2.4. The Experimental Design

(1) Non-mycorrhizal seed-propagated plants, grown at 60% soil moisture (**NM-Ws**);
(2) AMF-inoculated and seed-propagated plants, grown at 60% soil moisture (**AM-Ws)**;
(3) Non-mycorrhizal in vitro propagated plants adapted in 60% soil moisture (**NM-Win**);
(4) AMF-inoculated in vitro propagated plants adapted in 60% soil moisture (**AM-Win**);
(5) Non-mycorrhizal seed-propagated plants, grown at 40% soil moisture (**NM-Ds**);
(6) AMF-inoculated and seed-propagated plants, grown at 40% soil moisture (**AM-Ds)**;
(7) Non-mycorrhizal in vitro propagated plants, adapted in 40% soil moisture (**NM-Din)**;
(8) AMF-inoculated in vitro propagated plants, adapted in 40% soil moisture (**AM-Din)**.

2.5. Determination of Mycorrhizal Colonization

The gridline intersect method was implemented to determine the mycorrhizal root colonization rate [16]. To prove the mycorrhizal association, the roots were first washed with 10% KOH and stained with Trypan blue (0.05% in lactic acid, *v/v*) [17]. Pots were irrigated at different levels based on 60% soil water holding capacity (control), and 40% soil water holding capacity (drought-stressed plants).

2.6. Soil and Root Acid (AcP) and Alkaline Phosphatase (AlP) Activity

The activity of the enzymes that catalyze the hydrolysis of phosphate esters in an acidic or alkaline pH (AcP and AlP) in roots and soil samples were tested according to the Tabatabai and Bremner assay [18] with the modifications by Schneider et al. [19]. Root tissues (1 g fresh weight) were ground in 0.1 M sodium acetate buffer (pH 5.0) for the AcP activity reaction or in alkaline medium containing 100 mM sodium phosphate buffer (pH = 8.0) for the AlP activity determination. Following centrifugation, the supernatant was incubated in the respective homogenizing buffers (pH 5.0 or pH = 8.0 sodium acetate buffer) with added 5 mM p-nitrophenyl phosphate. The reaction was stopped using 0.2 M NaOH, and the absorbance was measured at 405 nm (UV/VIS Spectrophotometer, Shimadzu UV 1601). Rhizosphere soil samples were collected around the roots of *P. peruviana* plants for determination of soil alkaline and acid phosphatase levels. The activity rate was determined colorimetrically as a result of p-nitrophenol released due to the phosphatase activity throughout the buffered 1 g of fresh soil in sodium p-nitrophenyl phosphate solution and toluene (37 °C, 60 min). The enzyme activity was calculated as the amount of µg p-nitrophenol produced by g soil (fresh weight, FW) per hour.

2.7. Easily Extractable and Total Extracted Glomalin-Related Soil Proteins

The rhizosphere soil was collected to analyze the concentration of soil easily extractable and total extracted glomalin-related soil proteins (EE-GRSP, TE-GRSP) using the Wright and Upadhyaya assay [20]. EE-GRSP was derived from 2 g soil samples mixed with 8 mL 20 mM sodium citrate (pH 7.0), autoclaved (30 min, 121 °C), and centrifuged at 5000× *g* (15 min). The supernatant was stored at 4 °C until further analysis. The extracting method for TE-GRSP included autoclaving of a 2 g soil sampled mixed with 8 mL 50 mM sodium citrate (pH 8.0) for 60 min and centrifugation at 5000× *g* (15 min). The protein content was determined using the Bradford assay [20] using bovine serum albumin as the standard. GRSP analyses were done by soil samples of five replicate pots and three soil subsamples.

2.8. Analyses of Plant Pigments

Plant leaf pigments [chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoids] were analyzed after extraction in 80% acetone. The pigments were determined spectrophotometrically (UV/VIS Spectrophotometer, Shimadzu UV1601) as described by Lichtenthaler [21].

2.9. Analyses of Stress Markers

The stress markers were analyzed using fresh plant samples. The extraction was done by homogenizing 300 mg of fresh leaves or fruit samples with 0.1% trichloroacetic acid (*w/v*).

Free proline was derivatized with acid ninhydrin and the absorbance was measured at 520 nm (UV/VIS Spectrophotometer, Shimadzu UV1601) according to Bates et al. [22].

Lipid peroxidation was assayed by the malondialdehyde (MDA) amount, a product of unsaturated fatty acid peroxidation, as a thiobarbituric acid-reagent product according to Kramer et al. [23] using the extinction coefficient 155 mM^{-1} cm^{-1}.

The hydrogen peroxide concentration was measured colorimetrically (UV/VIS Spectrophotometer, Shimadzu UV1601) by KI reaction according to Alexieva et al. [24].

The compounds with free thiol groups were determined by incubation of 40µL supernatant in 150µL Ellman's reagent (10 min at 20 °C) [25]. The absorbance was measured at 412 nm (UV/VIS Spectrophotometer, Shimadzu UV 1601).

2.10. Analyses of Antioxidant Capacity

Enzyme activity analyses [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX)] were performed on the fresh leaf and fruit extracts. The leaf samples were homogenized at 4 °C. The enzyme extract was prepared according to Hristozkova et al. [26] following with enzymes specific reactions: total SOD activity (EC 1.15.1.1) [27], CAT activity (EC 1.11.1.6) [28], APX activity (EC 1.11.1.1) [29] and GPX activity (EC 1.11.1.7) [30] and determined spectrophotometrically (UV/VIS Spectrophotometer, Shimadzu UV-1601). Soluble protein content was identified using the Bradford assay and bovine serum albumin as the standard [31].

For the antioxidant analysis, the samples were prepared by homogenizing 0.3 g dry samples (leaves and fruits) in 96% (*v/v*) methanol. The content of total phenols was measured using the Folin–Ciocalteu reagent method and caffeic acid as the standard [32]. Total flavonoid concentration (leaf and fruit samples) was quantified by Zhishen et al.'s method with a catechin as the standard [33].

The free radical-scavenging activity of the lea fand fruit extracts was analyzed by the DPPH method [34] using the antioxidant's ability to bleach the purple methanol solution containing the free radical DPPH• (1,1-diphenyl-2-picrylhydrazyl) that can readily undergo reduction by an antioxidant. The following equation was used to calculate the percent inhibition of the DPPH_ radical (I%): $I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$, where, A_{blank} is the absorbance of the control reaction (including all reagents except the plant extract)at 517 nm, and A_{sample} is the absorbance of the extract.

The ferric reducing antioxidant power (FRAP method) was carried out according to the procedure of Benzie and Strain [35]. The FRAP assay is based on the rapid reduction of ferrous-tripyridyltriazine (Fe^{III}-TPTZ), to a ferric-tripyridyltriazine (Fe^{II}-TPTZ) a blue product at low pH by antioxidants present in the samples.

The antioxidant capacity was determined by spectrophotometric assay of lipid-soluble (LS-AOM) and water-soluble (WS-AOM) metabolite profiles [36]. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample and formation of a green phosphate/Mo (V) complex at a low pH. The method of Prieto et al. [36] features a linearity interval, repeatability, and reproducibility and the molar absorption coefficients for the quantitative assessment of WS- and LS-AOM as analogs of ascorbate and α-tocopherol. The coefficients of absorbance were $(3.4 \pm 0.1) \times 10^3$ M^{-1} cm^{-1} for ascorbate and $(4.0 \pm 0.1) \times 10^3$ M^{-1} cm^{-1} for α-tocopherol.

2.11. Reagents

All solvents were analytical. Ascorbate, guaiacol, hydrogen peroxide, ascorbate, and ammonium molybdate were obtained from Merck (Germany). Nitroblue tetrazolium, riboflavin, and methionine were purchased from Sigma (USA). The other chemicals were of all analytical grades.

2.12. Statistical Analysis

The data are expressed as mean ±standard error, where n varied between 3 and 10, depending on the type of analysis. Letters in common indicate no significant differences. Comparison of means was performed by the Fisher least significant difference (LSD) test at $p \leq 0.05$ following ANOVA. A statistical software package (StatGraphics Plus 5.1) was used.

3. Results and Discussion

Physalis peruviana has great potential, since it grows in different soil types, has low fertilizer requirements, and has a high tolerance to unfavorable environmental factors [2]. The present research revealed the effect of three factors influencing *P. Peruviana* growth: in vitro propagation, drought stress, and arbuscular mycorrhizal association.

3.1. Drought Influence on the Growth of P. peruviana

AMF unstressed experimental variants (AM-Ws; AM-Win) led to an increase in the biomass of the aboveground parts of the plants, the biomass accumulation of the roots and fruits, as well as fruit number per plant (Table 1).

Table 1. Growth parameters of *P. peruviana* non-mycorrhizal (**NM**), AMF-inoculated **(AM**), well-watered (**W**), drought-stressed (**D**), from seed-propagated (**s**), and in vitro propagated (**in**) plants.

Variants	Shoots	Roots	Fruits	Fruit Number
	g.plant^{-1} DW	**g.plant^{-1} DW**	**g.plant^{-1} DW**	**plant^{-1}**
NM-Ws	11.01 ± 0.55 c *	4.14 ± 0.21 c	0.46 ± 0.02 c	5.25 ± 0.26 d
AM-Ws	12.91 ± 0.65 e	5.78 ± 0.29 e	0.58 ± 0.03 f	7.75 ± 0.39 e
NM-Win	12.31 ± 0.61 d	4.93 ± 0.25 d	0.49 ± 0.02 d	5.30 ± 0.27 d
AM-Win	14.25 ± 0.71 f	6.29 ± 0.60 f	0.65 ± 0.03 g	9.50 ± 0.47 f
NM-Ds	8.93 ± 0.45 a	3.27 ± 0.16 a	0.33 ± 0.02 a	2.00 ± 0.10 a
AM-Ds	9.24 ± 0.46 b	3.96 ± 0.20 b	0.48 ± 0.02 d	3.25 ± 0.16 b
NM-Din	9.45 ± 0.47 b	3.69 ± 0.22 b	0.39 ± 0.02 b	3.03 ± 0.15 b
AM-Din	10.95 ± 0.55 c	4.31 ± 0.18 c	0.58 ± 0.03 e	3.77 ± 0.19 c
LSD	0.97	0.51	0.021	0.48

Values are the mean ± SE, $n = 9$; * common letters indicate no significant differences as assessed by Fisher LSD test ($p \leq 0.05$) following ANOVA.

AMF inoculation of both in vivo and in vitro cultivated *P. peruviana* plants subjected to drought stress (experimental variants AM-Ds; AM-Din), reduced the harmful effect of water deficits in the soil, which was established by the measured increase in the values of the morphological parameters.

The data clearly underline that AMF symbiosis stimulated plant development, notably in drought-stress conditions. The plants' growth indices were more markedly increased in micro-propagated and adapted plants compared to seed-propagated plants, irrespective AMF colonization. Progressive biomass accumulation of the in vitro propagated plants could be due to the stimulating effect of growth regulators present in the nutrient medium during the first stages of their cultivation. In a small number of studies, the stimulating effect of AMF inoculation on *P. peruviana* growth has been noted [37,38]. The beneficial influence of mycorrhizal symbiosis on plant development was discussed in both studies.

3.2. Mycorrhizal Symbiosis Parameters in P. peruviana Roots and Rhizosphere in a Drought-Stressed Environment

A relatively high percentage of colonization was observed in plants propagated in vitro, especially those subjected to drought (Figure 1). Mycorrhizal inoculation performed better in the roots of micro-propagated inoculated plants compared to seed-propagated plants under optimal and drought conditions. Slightly higher values were observed in the inoculated plants under drought conditions. The presence of local strains in the soil affected the low rate of colonization in non-inoculated plants (Figure 1).

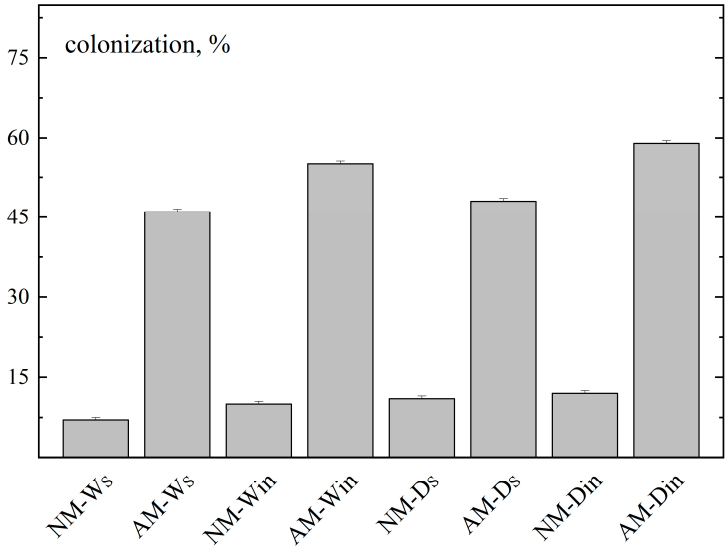

Figure 1. Mycorrhizal colonization (%) of *P. peruviana* roots: non-mycorrhizal (**NM**), inoculated with AMF (**AM**), well-watered (**W**), drought-stressed (**D**), seed-propagated (**s**), and in vitro propagated (**in**) plants. Values are mean ±SE, $n = 9$; common letters indicate no significant differences as assessed by Fisher LSD test ($p \leq 0.05$) following ANOVA.

The drought stress did not noticeably affect the rate of AMF colonization of plants [39]. The profile of mycorrhizal colonization was rounded out with alkaline and acid phosphatase activity (roots and soil), which were in correspondence with AMF root colonization (Figure 2).

Noticeably, lower values were reported for non-inoculated plants propagated in both ways, compare to higher values in mycorrhizal plants subjected to drought. Inoculation led to an increase in these values, without a significant difference between adapted micro-propagated plants (AM-Din) and those from seeds (AM-Ds). Drought stress stimulated root and soil AcP activity, while AlP activity in the same plants slightly decreased.

While alkaline phosphatases are produced by bacteria, fungi, and earthworms and function catalytically above pH 7 [40], acid phosphatases are enzymes of plant origin [40]. As a result, their activity is lower in plant roots than in soil, even though soil amendment is dependent on the presence of mycorrhizal fungi in the rhizosphere. On the other hand, in mycorrhizal plants grown at optimum soil moisture conditions, the increase in both glomalin-related soil proteins (EE-GRSP and TE-GRSP) content is higher than in drought-stressed conditions, assuming that lack of water suppresses their production and content in the rhizosphere. Inoculation with mycorrhizal fungi significantly increased the concentrations of EE-GRSP and TE-GRSP, regardless of the cultivation regimes (well-watered soil and drought-stressed) [41], since in dried plants it was insignificant. Extra radical hyphae development and soil aggregation of mycorrhizal plants were enhanced by drought acclimation, suggesting that they improved drought resistance by facilitating water uptake from the soil. The varied levels of the glomalin proteins that mycorrhiza produce have a number of important implications. A community may contain strains like *C. claroideum* that secrete large amounts of protein. Such fungi strains may be highly helpful

in agro-ecosystem applications because of their fundamental capacities to promote nutrient cycling, boost water infiltration, and aggregate soil [42].

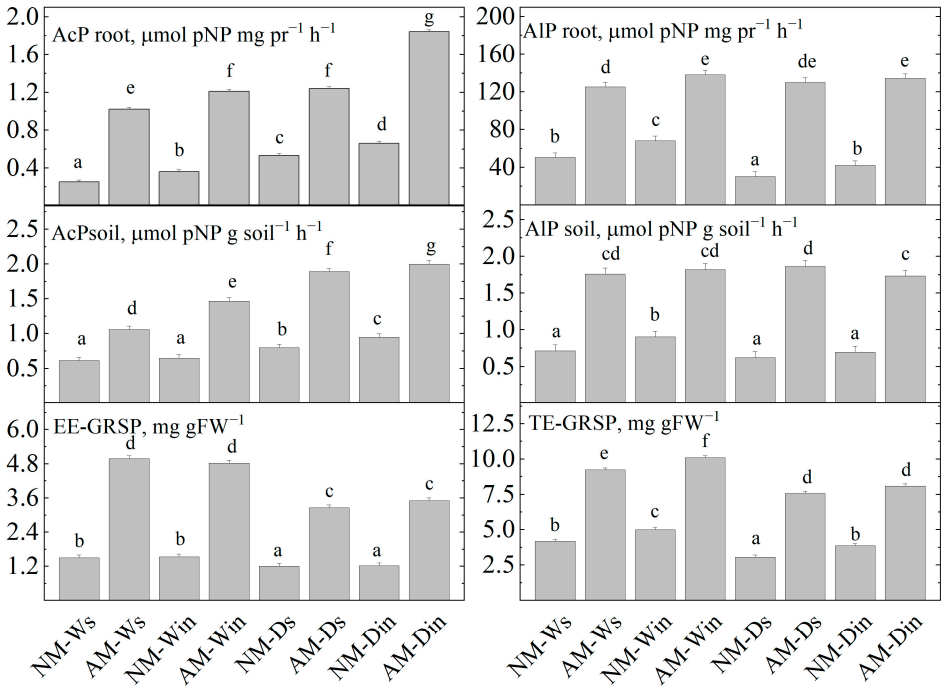

Figure 2. Activity of glomalin-related soil proteins, acid and alkaline phosphatases (AcP, AlP) in the roots and rhizosphere of *P. peruviana*: non-mycorrhizal (**NM**), AMF-inoculated **(AM**), well-watered (**W**), drought-stressed (**D**), seed-propagated (**s**), and in vitro propagated (**in**) plants. Values are means ± SE, $n = 5$; common letters within a graph show no significant differences as determined by Fisher LSD test ($p \leq 0.05$) following ANOVA.

3.3. Photosynthetic Pigments (Chla, Chlb, and Carotenoids) in P. peruviana Leaves Developed in a Drought-Stressed Environment

The important photosynthetic pigments Chla, Chlb, and carotenoids had relatively high values in well-watered inoculated plants propagated from seeds and in vitro (Table 2). In mycorrhizal plants propagated with both methods and grown in drought conditions, the plastid pigments values did not significantly decrease in comparison with non-inoculated plants. In the research of Figueiredo et al. [43], it was stated that the salinity of irrigation water reduced the quantum efficiency of photosynthesis in *P. peruviana* plants. The mycorrhizal symbiosis noticeably reduced the unfavorable results of water shortfall on the level of plant pigments, irrespective of propagation method. As a response to unfavorable environmental factors, plants attempt to adapt their photosynthetic system in order to use light more efficiently, but the mechanism varies among species, especially differing betweenC3 and C4 types [44]. According to Li et al.'s [44] analysis, the amount of the AMF-associated benefit in plant drought resistance, which is greater for the C3 species than the C4 species, may vary depending on the photosynthetic type.

Carotenoids play a role in preventing many types of abiotic stress from damaging the photosynthetic machinery [45]. The Chl a/b ratio in AM-Din was nearly twice higher compared to AM-Win. The same trend was observed for AM-Ws and AM-Ds. Under water stress, Chl-b is degrading into Chl-a, leading to a higher Chl-a/b ratio [46].

Table 2. Photosynthetic pigments Chl a, Chl b, and carotenoids in *P. peruviana* leaves: non-mycorrhizal (**NM**), AMF-inoculated (**AM**), well-watered (**W**), drought-stressed (**D**), from seed-propagated (**s**), and in vitro propagated (**in**) plants.

Variants	Chl a	Chl b	Chla + b	Chl a/b	Carotenoids
Leaves	**mg g FW^{-1}**	**mg g FW^{-1}**	**mg g FW^{-1}**		**mg g FW^{-1}**
NM-Ws	0.723 ± 0.04 c *	0.201 ± 0.010 c	0.924 ± 0.050 b	3.597 ± 0.186 d	0.150 ± 0.007 d
AM-Ws	1.269 ± 0.06 f	0.515 ± 0.031 e	1.784 ± 0.093 d	2.464 ± 0.126 a	0.312 ± 0.020 f
NM-Win	0.856 ± 0.04 d	0.268 ± 0.010 d	1.124 ± 0.061 c	3.194 ± 0.160 bc	0.167 ± 0.066 e
AM-Win	1.399 ± 0.07 g	0.590 ± 0.037 f	1.989 ± 0.100 e	2.371 ± 0.125 a	0.356 ± 0.028 g
NM-Ds	0.315 ± 0.02 a	0.106 ± 0.005 a	0.421 ± 0.025 a	2.971 ± 0.150 b	0.084 ± 0.004 a
AM-Ds	0.756 ± 0.04 c	0.183 ± 0.009 b	0.939 ± 0.051 b	4.131 ± 0.217 e	0.103 ± 0.005 b
NM-Din	0.387 ± 0.02 b	0.117 ± 0.006 a	0.504 ± 0.030 a	3.307 ± 0.171 c	0.092 ± 0.005 a
AM-Din	0.950 ± 0.05 e	0.199 ± 0.010 c	1.149 ± 0.064 c	4.773 ± 0.243 f	0.112 ± 0.006 c
LSD	0.072	0.029	0.109	0.300	0.041

Values are mean ± SE, n = 9; * common letters indicate no significant differences as assessed by Fisher LSD test ($p \leq 0.05$) following ANOVA.

3.4. Levels of Stress Markers Identifying P. peruviana Plants' Tolerance to Drought

The level of stress characteristics indicating the plants' sensitivity or resistance to drought stress. Proline, H_2O_2, and -SH group content was measured in *P. peruviana* leaves and fruits (Figure 3). Water deficiency during plant cultivation increased the generation of hydrogen peroxide, a reactive oxygen species, causing significant oxidative stress leading to lipid peroxidation in cell membranes as measured by the levels of malondialdehyde. Exposure of plants to drought increased the levels of the stress markers H_2O_2, MDA, and proline and decreased -SH group levels, both in the leaves and in the fruits of adapted micro-propagated *P. peruviana* plants and those from seeds.

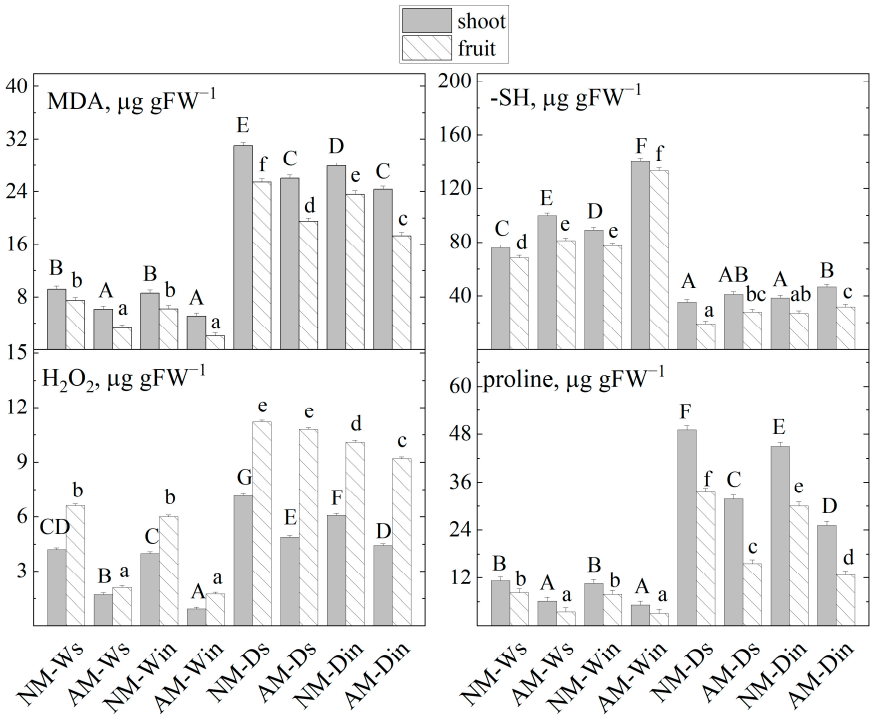

Figure 3. The levels of stress markers identifying the plants' tolerance to drought—MDA, -SH groups, H_2O_2, and proline in *P. peruviana* (leaves and fruits): non-mycorrhizal (**NM**), AMF-inoculated (**AM**), well-watered (**W**), drought-stressed (**D**), seed-propagated (**s**), and in vitro propagated (**in**) plants. Values are mean ± SE, n = 3; common letters within a graph show no significant differences as determined by Fisher LSD test ($p \leq 0.05$) following ANOVA. The statistical analyses of leaves (uppercase) and fruit (lowercase) were performed separately.

Inoculation of plants with *Claroideoglomus claroideum* reduced the level of oxidative stress indicated by a slight decrease in the content of H_2O_2, MDA, and proline, but a significant increase in the amount of -SH groups, both in the leaves and fruits of micro propagated and seed-propagated *P. peruviana* plants. Through the growth of extraradical AMF fungal mycelia, the AMF symbiosis facilitated plants in efficiently absorbing water and mineral nutrients from the soil. The greater water status in AMF-inoculated *P. Peruviana* plants decreased the harmful impact of the drought stress leading to the lower accumulation of proline and hydrogen peroxide [47,48]. There is evidence in the literature of both an increase [49] and a decrease [50,51] in proline and hydrogen peroxide content in inoculated plants compared to non-inoculated ones. Therefore, the level of stress markers depends on the type of AMF and plant species used. The results agrees with previous findings in trifoliate orange and *Erythrina variegate* plants under drought stress [50,51].

It was determined that the negative effects of drought were less pronounced in leaves and fruit of adapted, in vitro propagated plants regarding the levels of the examined stress markers by comparing the changes in their levels in leaves and fruit of plants grown from seeds. Similar conclusions were made in earlier studies using *Coleus forskohlii* (Willd.) [52], which pointed out a similar model in different plants to deal with stressful conditions of the environment such as drought.

The –SH groups, MDA, and proline levels were higher in the aboveground parts, but the H_2O_2 content was significantly higher in the fruits in all investigated treatments. Hydrogen peroxide is involved in the oxidation processes needed to start and promote the ripening of the fruit. During the ripening of peppers (*Capsicum annuum* L.) and tomatoes (*Solanum lycopersic* L.), which also belong to the family of Solanaceae, the activity of CAT is down-regulated [53]. This inhibition of CAT activity may reduce the H_2O_2-removing capacity and, accordingly, increase the ripening-associated nitro-oxidative burst in peroxisomes [54].

Drought increased the concentration of the osmotic regulator proline to different degrees depending on the AMF strain. Thus, the proline content of leaves may serve as a good parameter to measure water stresses in both inoculated and non-inoculated plants [39].

3.5. Antioxidant Enzyme Activityin P. peruviana Plants Developed in a Drought-Stressed Environment

The analysis of antioxidant enzyme activity (SOD, CAT, APX, GPX) and concentrations of non-enzymatic low molecular metabolites (total phenols, flavonoids, carotenoids, lipid-soluble and water-soluble AOC) revealed a counteraction against harmful active oxygen species generated by oxidative stress. Enhanced activity of antioxidants mediates quick scavenging of ROS and hence protects cells from possible oxidative damage [55,56]. The effect of AMF inoculation on the antioxidant activity in fruits and leaves of adapted, in vitro propagated plants was compared with seed-propagated plants in water-deficient conditions (Figure 4). In the fruits, higher activity of total SOD (class of metalloenzymes that catalyze the dismutation of two molecules of $O_2\bullet$ into molecular oxygen and H_2O_2) was recorded compared to the aboveground parts in all studied treatments (Figure 4). The low activity of CAT corresponded with the high levels of H_2O_2 because CAT catalyzes the reaction in which hydrogen peroxide breaks down into water and oxygen. Higher activity of APX and GPX enzymes was noted in the fruits of drought-stressed and mycorrhizal *P. peruviana* plants versus the leaves. SOD, APX, and GPX activities were in correspondence with the observed stress markers and showed increased levels in drought-stressed and AMF-inoculated plants. Superoxide radicals are produced in organelles, where an electron transport chain is present (mitochondria and chloroplasts). $O_2\bullet-$ activation can also occur in microsomes, glyoxisomes, peroxisomes, apoplasts, and the cytosol. As phospholipid membranes are impermeable to O_{2-}, the presence of SOD at the sites of its formation is critical. CAT activity was higher in well-watered plants.

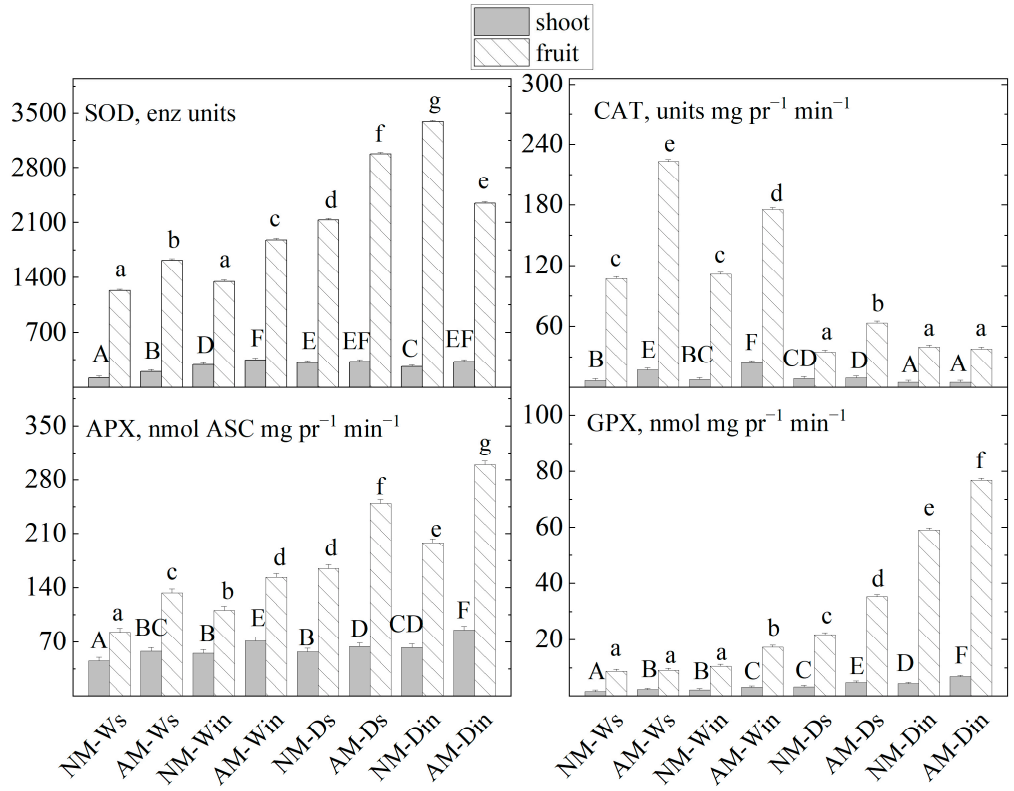

Figure 4. Activities of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) in *P. peruviana* (leaves and fruits): non-mycorrhizal (**NM**), AMF-inoculated (**AM**), well-watered (**W**), drought-stressed (**D**), seed-propagated (**s**), and in vitro propagated (**in**) plants. Values are average ±SE, $n = 3$; common letters indicate no significant differences as determined by Fisher LSD test ($p \leq 0.05$) after performing ANOVA analysis. The statistical analyses of leaves (uppercase) and fruit (lowercase) were performed separately.

Drought caused changes in the activity of antioxidant enzymes in the leaves of in vitro propagated *P. peruviana*, which depend on the stage of plant development. APX in the peroxisomes converts H_2O_2 to H_2O and O_2, and water stress signaling has a positive effect on the activity of the antioxidant enzymes which was higher in inoculated variants compared to controls. Higher H_2O_2 content in variants submitted to water stress is likely to lead to feedback inhibition of CAT. The drought of non-inoculated and inoculated in vivo and in vitro propagated plants caused an increase in SOD, CAT, APX, and GPX activity (Figure 4). Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) showed increased activity in *Pisum sativum* L. affected by drought compared with control well-watered plants [57]. Abedi and Pakniyat [58] found that severe water stress preferentially enhanced the activities of superoxide dismutase (SOD) and guaiacol peroxidase (POD) whereas it decreased catalase (CAT) activity in oilseed rape (*Brassica napus* L.) plants.

3.6. Antioxidant Potential of P. peruviana Plants Developed in a Drought-Stressed Environment

The balance between the rates and capacities of antioxidant production and turnover associated with antioxidant demand controls the amount of intracellular metabolites with antioxidant ability during drought stress [59]. The non-enzymatic plant antioxidants have been classified by Conklin [60] into two major types: (1) antioxidant-like scavengers, which react with H_2O_2, O_2^-, OH, and lipid hydroperoxidases and (2) pigments such as carotenoids. To further investigate the oxidative response of *P. peruviana* during its interaction with AMF in water deficiency, we studied the total antioxidant activity in leaf and fruit extracts by detecting the radical scavenging activity (DPPH method), ferric reducing antiox-

idant potential (FRAP method), and levels of non-enzymatic defenses (Figure 5). Higher levels of total flavonoids, phenols, and WS-AOM were observed, especially in the fruits but not in treatments subjected to drought. LS-AOM was higher in the leaves compared to WS-AOM content, while in the fruit samples the opposite tendency was observed.

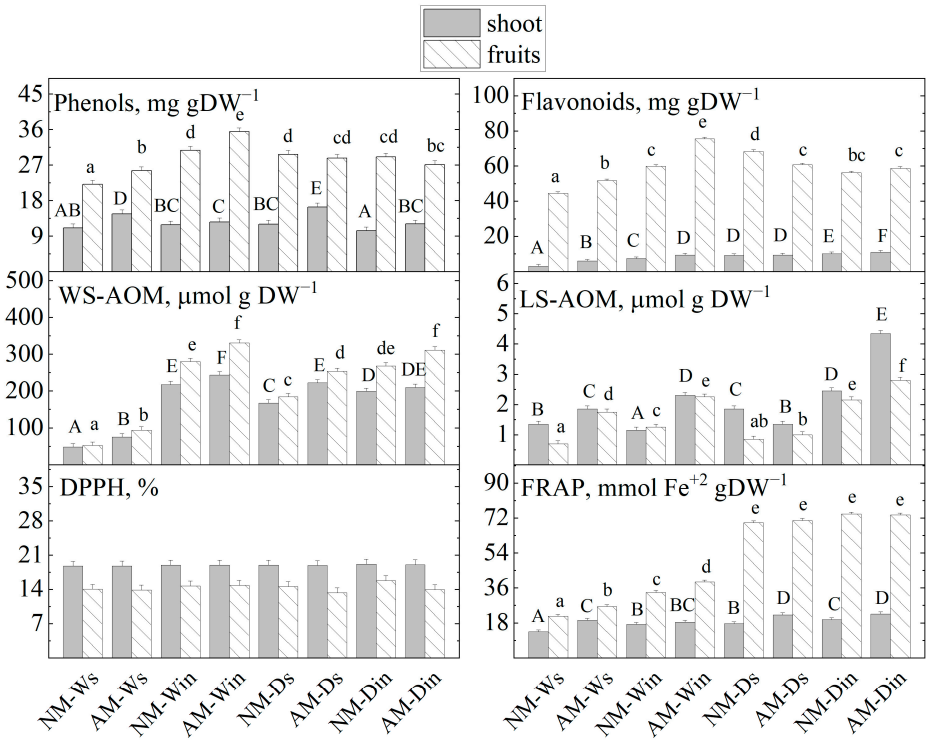

Figure 5. Metabolites with antioxidant power and total antioxidant potential in *P. peruviana* (leaves and fruit): non-mycorrhizal (**NM**), AMF-inoculated (**AM**), well-watered (**W**), drought-stressed (**D**), seed-propagated (**s**) and in vitro propagated (**in**) plants. Values are mean ±SE, n = 3; different letters indicate significant differences as assessed by Fisher LSD test ($p \leq 0.05$) after performing ANOVA multi-factor analysis. The statistical analyses of leaves (uppercase) and fruit (lowercase) were performed separately.

In drought-stressed plants, the levels of WS-AOM increased as a consequence of mycorrhizal colonization. The antioxidant capacity detected by the DPPH method did not show any statistical differences between the investigated treatments. The differences in antioxidant activity expressed by the ferric reducing potential showed an adequate reaction to drought stress and increased levels were measured both in the plants propagated from seeds and in micro propagated and adapted plants (Figure 5).

The extent to which the activity of antioxidant enzymes and the amount of metabolites with antioxidant activity increased with the drought stress was extremely variable according to the type of AMF strain, type of plant species, and even between two varieties of the same species. The level of reaction depended on the type, development, and metabolic state of the plant species, as well as the stress duration and intensity. Our results are in accordance with numerous studies which showed increased content of anthocyanins, flavonoids, and total phenolic compounds in *Triticum aestivum* plants colonized by *Glomus mosseae* [61], in maize colonized by *Glomus versiforme* [62]. Enhanced concentrations of carotenoids in *Zinnia elegans* [63] and *Citrus aurantifolia* [64] under drought stress have also been reported.

It can be assumed that AMF stimulated an additional increase in enzymatic antioxidant activities and the levels of antioxidant capacity measured with FRAP with a smaller increase in the levels of oxidative markers such as MDA, H_2O_2, and proline; therefore, the micro propagated plants are more resistant to drought than those grown from seeds. According to the results, it was obvious that the morphological parameters (shoot and root biomass, and

number the fruits), as well as plastid pigments content were enhanced in micro propagated plants with all studied treatments (AMF inoculation and drought stress) compared with plants grown from seeds. Concerning mycorrhizal parameters, the mycorrhizal status was slightly better in the roots of micro propagated plants inoculated with *Claroideoglomus claroideum* than in vivo propagation by seeds. Inoculation led to an increase in the values of acid phosphatase in roots and soil in the plants subjected to drought and propagated with either method.

4. Conclusions

P. peruviana plants grown under drought-stressed conditions performed as a tolerant species, based on the absence of visible symptoms and significant plant biomass reduction. Our outcomes underline that mycorrhizal golden berry plants are more competitive and better able to adapt to drought stress than non-mycorrhizal plants, potentially enhancing plant tolerance. On the other side, the adapted micro-propagated *P. peruvian* plants were more stable to drought than seed-grown plants. Presumably, the increased drought stress tolerance of adapted micro-propagated *P. peruviana* plants compared to seed-propagated plants could be due to the formed epigenetic variations through in vitro culture under stressful environmental conditions. Epigenetic mechanisms could be responsible for the flexibility of some plants in coping with changing environmental conditions. Most probably, this is the reason why micro-propagated *P. peruviana* plants are better adapted to drought.

The findings in the present research are relevant to obtain the optimal mycorrhizal association and type of propagation under unfavorable conditions for golden berry development and tested these conditions with diverse plant types the will lead to a databases and model to study the response of varied plants to stressful environmental conditions such as drought.

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