Acta Physiologiae Plantarum (2021) 43:26
https://doi.org/10.1007/s11738-020-03191-0

ORIGINAL ARTICLE

Rosmarinic and caffeic acids contents in Basil (*Ocimum basilicum* L.) are altered by different levels of phosphorus and mycorrhiza inoculation under drought stress

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Received: 18 September 2019 / Revised: 12 December 2020 / Accepted: 15 December 2020

Abstract

Basil (*Ocimum basilicum* L.) is a high-value medicinal herb cultivated for economical applications, especially pharmaceutical purposes. There is less information on the effects of arbuscular mycorrhizal (AM) fungi, drought stress, and phosphorus (P) supply on important phenolic compounds like caffeic (CA) and rosmarinic (RA) acids. Therefore, an experiment was performed to examine whether treatments: AM (inoculated with *Glomus hoi* and non-inoculated (NM) plants; drought stress (60% field capacity (FC)) and well-irrigated (90% FC) and phosphorus supply (0.1, 0.2 and 0.3 g/kg of soil) could influence plant growth and contents of total phenols (TPC), RA, CA and phenylalanine ammonia-lyase (PAL) activity. The experiment was conducted as a factorial oriented by completely random design with three repetitions. Shoots of 8-week-old plants that were subjected to all mentioned treatments were harvested and analyzed. High-performance liquid chromatography (HPLC) was used to quantify RA and CA contents. Results indicated that both P supply and AM inoculation enhanced plant growth, tissue P content, RA and CA production and PAL activity. There was an increase of 1.84, 1.59, and 2.22 times for TPC, RA and CA contents respectively in AM plants compared to NM plants in P2 levels and in drought stress. There was a positive relationship between the phenol content and PAL activity for all the treatments. Consequently, mycorrhizal inoculation and application of an appropriate level of P could serve as an adaptive strategy to enhance productivity and plant synthesis of phenolic compounds under restricted irrigation with health-promoting activities.

Keywords *Ocimum basilicum* L. · Rosmarinic acid · Caffeic acid · P level · Mycorrhizae · Phenolic compounds

Abbreviations

AM	Arbuscular mycorrhizae
CA	Caffeic acid
DW	Dry weight
FC	Field capacity
GAE	Gallic acid equivalents
HPLC	High-performance liquid chromatography
NM	Non-mycorrhizal
OPA	*ortho*-Phosphoric acid
P	Phosphorus
PAL	Phenylalanine ammonia-lyase
RA	Rosmarinic acid
TPC	Total phenol content

Communicated by R. Baczek-Kwinta.

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Introduction

Water deficit is one of the most serious environmental constraints which threatens agriculture and decreases crop productivity. Drought stress affects the plant's growth via physiological mechanisms such as photosynthetic efficiency, cell and membrane integrity, and results to increasing plant susceptibility to disease. However, water deficit causes the accumulation of some secondary metabolites such as phenolic compounds (Sinclair et al. 2014).

Arbuscular mycorrhizae (AM) have been found to improve plant growth and stress resistance. Moreover, it can affect the secondary metabolites level in plants (Zubek et al. 2012; Ceccarelli et al. 2010). The presence

Published online: 19 January 2021

of mycorrhizal fungi in the soil enhances phosphate solubility via secreting phosphatase enzymes and therefore improves plant growth and nutrition (Roy-Bolduc and Hijri 2011).

Basil (*Ocimum basilicum* L., Lamiaceae), originated from the warm tropical climates of India, Africa, and southern Asia, is now cultivated worldwide under various ecological circumstances (Adham 2015). It is a popular herb which is utilized in culinary, cosmetic, industrial, and pharmaceutical applications. Basil can be consumed as a digestive stimulant with anticarcinogenic, antibacterial, and anticonvulsant properties (Swedan 2013).

Due to having the ability as free radicals scavenger and electron donor, these compounds are strong antioxidants and can protect the plants against pathogens and predators. In the cellular level, they can protect the cells from the adverse impacts of reactive oxygen species (ROS) created against different abiotic stresses (Riaz et al. 2019). Accordingly, the plants containing a high level of antioxidants are resistant to ROS-induced oxidative damages. These antioxidants can also act as defensive compounds (Meot-Duros and Magne 2009). The presence of phenolic compounds depends on several factors including the type of soil, plant species, genetic factors, growth stage, and geographical location (Scagel and Lee 2012).

Caffeic acid (CA) and rosmarinic acid (RA, an ester of CA), the main phenolic compounds responsible for the antioxidant activity in basil, are mainly produced in the leaves and roots (Toussaint et al. 2008; Kwee and Niemeyer 2011). CA is actively involved in plant physiology and the mechanism of stress tolerance (Riaz et al. 2019). In recent years, RA has been utilized as an anti-inflammatory, anti-proliferative, and chemoprotective agent (Srivastava et al. 2016). It has been proposed that in plants RA is synthesized via phenylpropanoid and the tyrosine-derived pathways. Phenylalanine ammonia-lyase (PAL) is a main enzyme in the phenylpropanoid path catalyzing the biotransformation of L-phenylalanine to trans-cinnamic acid and ammonia. Former studies confirmed the inclusion of PAL in RA biosynthesis (El-Naggar 2010; Kim et al. 2013). Increasing stress-induced of PAL activity may be regarded as the onset of the cells acclimation under drought conditions (Hazzoumi et al. 2015).

Phosphorous (P) is an essential element in the biosynthesis of secondary metabolites in plants. Therefore, its availability may alter the quantity and composition of phenolic compounds. There is a competition between the synthesizing phenolic compounds and the necessary proteins for growth. AM fungi can affect the composition and accumulation of phenolic compounds via increasing the phosphate ions uptake (Toussaint et al. 2007).

Since AM fungi can improve plant productivity and nutritional quality of crops, so the replacement of chemical fertilizers with them as environment-friendly technologies has received increasing attention in recent years.

There are some reports considering the effect of mycorrhiza and drought stress on the production of phenolic compounds in several plants. However, as far as the authors inform, no research about the production of phenolic compounds in basil in response to mycorrhizal inoculation has been carried out at different P levels under drought stress.

The present work aimed at determining the effects of three P levels and the inoculation of roots by AM on the accumulation of CA and RA in basil plants under drought stress. The study was conducted to clarify whether the effect of AM on the production of phenolic compounds in basil is associated with AM-mediated impacts on P uptake. Furthermore, the results of this study will elucidate the best level of P and drought stress on the quantity of RA and CA. The results of this research will shed light on future AM applications in basil cultivation to improve the medicinal components of this beneficial species.

Materials and methods

Plant material

Sweet basil (*O. basilicum* L.) seeds were prepared from Seed and Plant Improvement Institute, Karaj, Iran. They were surface-sterilized in a 10% sodium hypochlorite solution for 10 min followed by three times rinsing with distilled water. The pots were randomly arranged in a growth chamber at 25 ˚C and a photoperiod of 14/10 h (light/darkness). Seeds were germinated in the plastic pots (23 cm in diameter and 21.5 cm in height) filled with sterilized clay and sand (1:1 w/w). Isolated fungus (*Glomus hoi*) were prepared from the Touran Biotechnology Company. The mycorrhiza-bearing soil (*G. hoi*) (ratio of 100 g per 1 kg of soil) was added to some pots (12,000–15,000 spores per 1 kg of soil). Accordingly, the plants were inoculated with the AM fungi. For control groups, the plants were sown in un-inoculated soil (NM). Phosphorus was separately added to the soil at three different levels: 0.1, 0.2, and 0.3 g $CaHPO_4$/kg of soil (corresponding to P1, P2, and P3, respectively). The plants were irrigated as needed in the first few days, and they were subjected to drought stress (60% field capacity (FC) 10 days after emergence. So, three P levels, fungal inoculation, and non-fungal inoculation and two irrigation regimes [60% and 90% FC, namely drought stress and non-stress (NS) conditions, respectively] were evaluated in a factorial test in terms of completely random design with three replications. Shoots and roots of 8-week-old plants were separated and weighted by digital scale (0.001 g accuracy). Dry weights (DW) of samples were measured after their drying in oven at 40 °C for 3–4 days. This temperature was selected to prevent CA

and RA breakdown. In continue, P content of the tissues, PAL activity, soluble protein content, total phenolic content (TPC), RA and CA contents were measured and recorded for each sample. The RA and CA standards were purchased from Sigma (St. Louis, MO, USA).

Determination of P content in plants

P content of tissues was determined calorimetrically using a phosphovanado-molybdate method (Hanson 1950). Briefly, 50 mg of dried specimens were digested in a nitric–perchloric acid mixture (6:1) overnight at 180 °C. Then, diluting the products to 25 ml was performed in water, and 8 ml aliquot was created to 25 ml with 2 ml of dye reagent containing nitric acid, 5% ammonium molybdate, 0.25% ammonium vanadate, and water (1:1:1 v/v/v). The absorbance of samples was read at 390 nm after 30 min. P content in the digested samples was determined by a standard curve prepared to utilize known concentrations of KH_2PO_4 (0–10 µg/ml).

Extraction and estimation of TPC

TPC was assessed based on the process by Singleton and Rossi (1965). The assay mixture contained 2.5 ml of Folin–Ciocalteau reagent (Sigma Chemical Co.), 4 ml of distilled water, 1.25 ml of 2.1% Na_2CO_3 and 1 ml of the methanolic extract. Incubation of the mixture was performed in the darkness for 30 min and its absorbance was measured at 735 nm. A standard curve of Gallic acid solution (25, 50, 75, 100, 150, and 200 µg/ml) was prepared, and TPC of the extract was stated as mg Gallic acid equivalent/g dry weight extract sample (mg GAE/g DW).

Determination of RA level by spectrophotometric method

A stock solution of RA was prepared in a methanol/water mixture (20:80; v:v) and its dilutions (25–200 µg/ml) were prepared using the same solvent. They were kept in darkness in a refrigerator. 50 mg of dry leaves was mixed with 25 ml of methanol–water (70:30) mixture. After mixture sonication for 20 min, the extracts were filtered (Juliani et al. 2008). A rapid spectrophotometric method was used to determine the RA level in sample extracts (Ozturk et al. 2010). Briefly, 200 µl of the extract solution was introduced to 200 µl of 0.5 M zirconium (IV) oxide chloride and 4.6 ml of ethanol. After 5 min, the absorbance was defined at 362 nm against a reagent blank. The contents of RA in the extracts were determined based on the following equation attained from the standard RA curve ($R^2 = 0.992$). Absorbance = 0.0266 × µM acid − 0.0269.

High-performance liquid chromatography (HPLC) analysis

Separation of RA and CA was achieved using an Agilent 1260 series HPLC with a ZORBAX Eclipse plus C_{18} column (Gemini-N × 100 mm × 4.6 mm × 3.5 µm particle diameter). The solvent used for separating individual polyphenols included 0.1% (v/v) *ortho*-phosphoric acid (OPA) in HPLC grade water (mobile phase A) and methanol (HPLC grade, Merck) + 0.1% OPA (v/v) in methanol (mobile phase B). By testing different gradient phases for isolating CA and RA, it was indicated that a gradient program similar to the one reported by Srivastava et al. (2014, 2016) was the best way for separating the compounds. Hence, the flow rate of 1.0 ml/min and the injection volume of 20 µl was used. A detection wavelength of 280 nm along with unknown samples was recognized by comparing with the standard calibration and retention time of CA and RA (Sigma) over a content range from 20 to 100 mg/l (Fig. 1a,b). The results were represented as mg RA/g DW and mg CA/g DW (Fig. 2a,b).

Protein extraction and PAL activity assay

Leaf samples (300 mg fresh mass) were extracted in 6.5 ml of buffer (50 M Tris–HCl pH 8.8, 15 mM 2-β-mercaptoethanol). At 5000×*g*, centrifuging the homogenate was performed for 30 min at 4 °C. The Bradford (1976) assay, and bovine serum albumin as a standard method were used to collect the supernatants and determine the protein content. The Bradford reagent was made

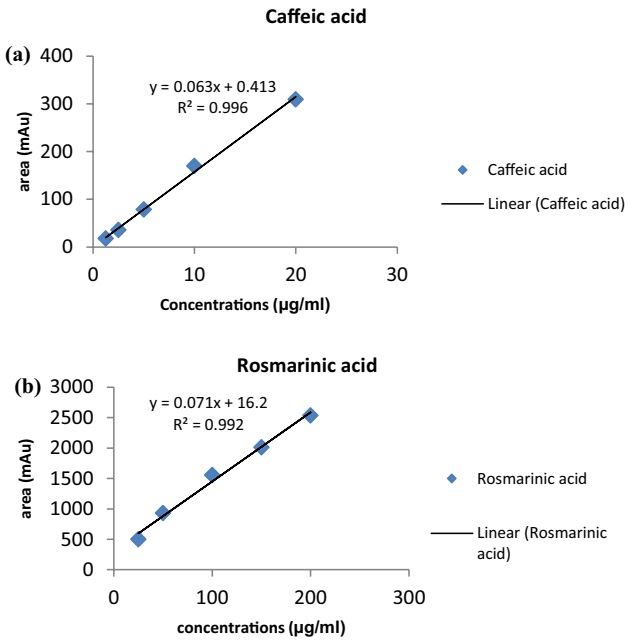

Fig. 1 Calibration curve for caffeic acid (**a**) and rosmarinic acid (**b**)

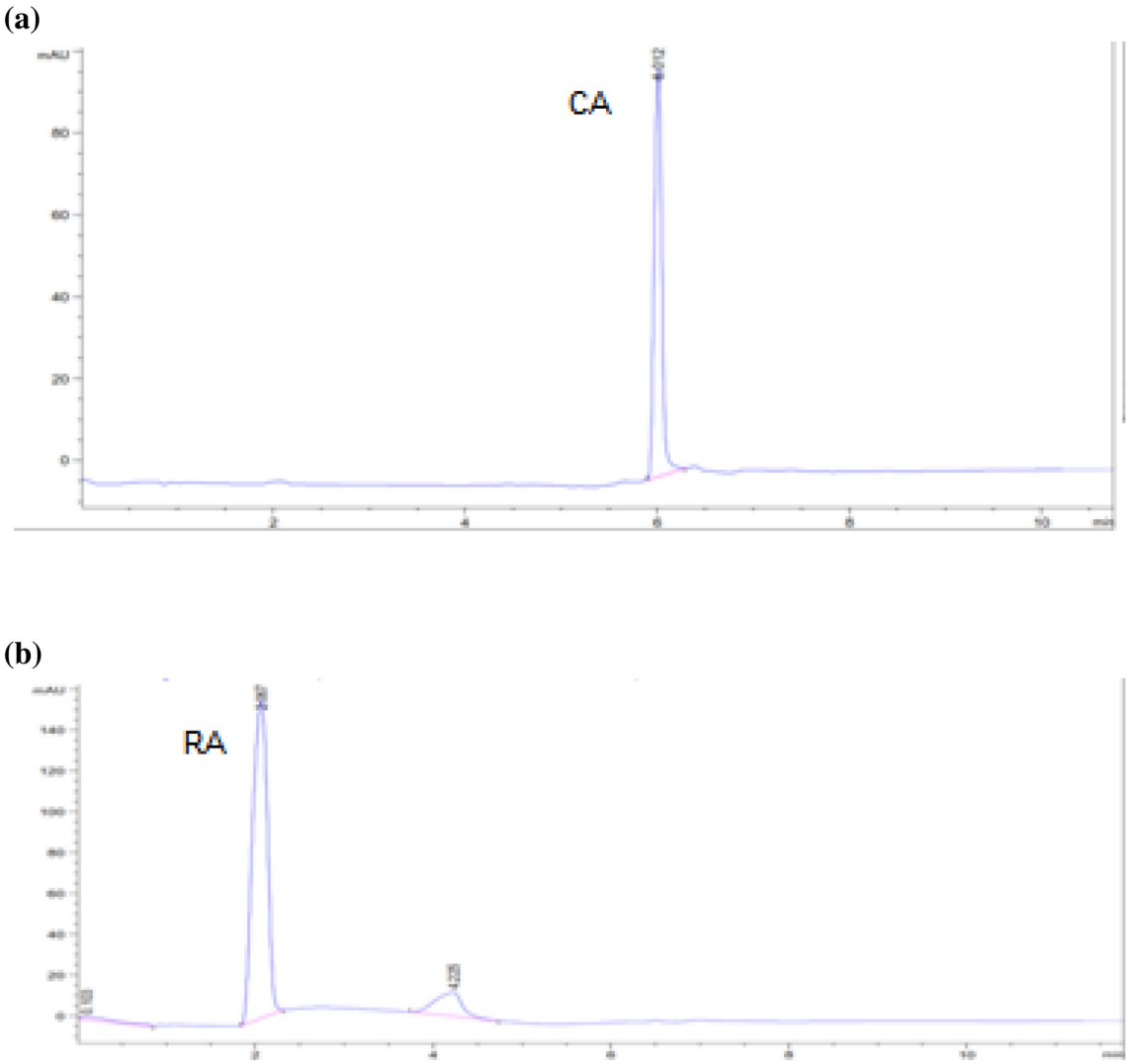

Fig. 2 HPLC chromatograms with UV and mass spectra of caffeic acid (**a**) (Retention time 6.012 min) and rosmarinic acid (Retention time 2.067 min.) (**b**) in standards sample

as follows: the solution was diluted with distilled water to one liter by adding 100 ml 85% phosphoric acid to a mixture of 100 mg coomassie brilliant blue dissolved in 50 ml 95% (v/v) ethanol. To perform the assay, 200 µl of dye reagent and 50 µl of each sample were mixed for 60 s. After 5 min, the samples' absorbance was measured at 595 nm at room temperature.

According to the method of Wang et al. (2006), PAL activity was determined in terms of the rate of cinnamic acid production. The enzyme extract (0.1 ml) was incubated with 1 ml 50 M Tris–HCl, 0.5 ml of L-phenylalanine (10 M), and 0.4 ml of distilled water at 37 °C for 60 min. Afterwards, the products were extracted with 15 ml ethyl acetate, by addition of 0.5 ml of 6 M HCl, after evaporation for removing the extraction solvent. The solid residues were suspended in 3 ml of 0.05 M NaOH while measuring the absorbance at 290 nm. The enzyme activity was expressed in µmoles cinnamic acid/mg protein/min.

Statistical analysis

The test was performed with three replications for each treatment. SPSS 16.0 software was used for all statistical analyses. The data were analyzed for normality and homogeneity of variance (Levene's test). All the data were expressed as mean values. Univariate analysis of variance (ANOVA) was utilized to define the treatment effects, moreover, to compare the differences among treatments. Duncan's multiple range test was used at 5% significance level.

Results and discussion

Here, the content of therapeutic compounds of aerial parts, as an important parameter in the medicinal value of *O. basilicum was* measured.

Dry weight (DW)

According to the results, biomass DW decreased under drought stress, regardless of AM inoculation. In NM plants, the effect of drought stress on DW was more severe than AM plants. In AM plants, DW significantly ($P \leq 0.05$) increased in both stressed and non-stressed plants and for all P levels (Fig. 3). The results of the study revealed that moisture regimes and P application affected DW significantly ($P \leq 0.05$). The highest (0.63 g) and the lowest (0.15 g) amount of DW was observed in AM plants (non-stressed, P3) and NM plants (stressed, P1) respectively. Higher levels of P enhanced the DW in both AM and NM plants, however, there was no significant difference between P2 and P3 levels in both irrigation regimes. DW in AM plants was similar to the well-irrigated NM plants for all P applications.

In this study, the influence of drought stress on DW was more evident in NM than in AM plants. According to Baslam and Goicoechea (2012), the biomass of drought-exposed AM lettuce was similar to NM plants grown under well-irrigated conditions. Similarly, dry weights of AM water-stressed *Rosmarinus officinalis* were enhanced as compared with the non-AM stressed plants (Sanchez-Blanco et al. 2004). Low-soil water supply decreases the turgor pressure leading to a reduction in cell division and elongation and therefore decreasing biomass production under water stress conditions (Shao et al. 2008). Therefore, water-stressed plants have smaller leaf areas and height, and all growth processes dependent on pressure turgor especially photosynthetic activity and carbon assimilation are decreased in drought-exposed plants. Drought stress also decreased P diffusion rates in soil and affected the plant metabolic process (Baslam and Goicoechea 2012). Under drought stress conditions, mycorrhizal hyphae could impact the host water balance in drying soils and contribute to drought tolerance of crops through increasing the root water absorption or improving the root contact with soil particles. Mycorrhizal inoculation improves rhizosphere leading to more efficient nutrient uptake (especially P), increased photosynthesis ability, enhanced disease resistance and reduced the negative effects of drought stress on plant growth (Sinclair et al. 2014).

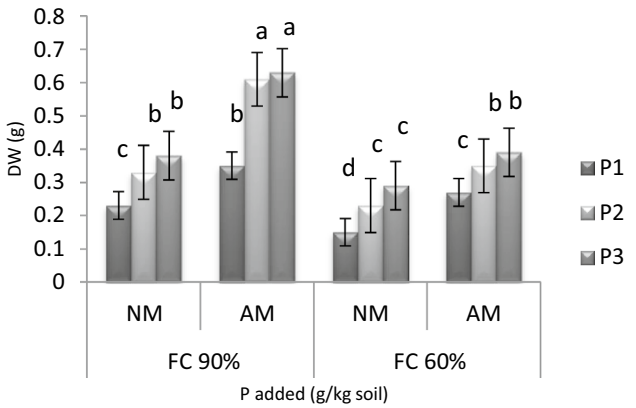

Fig. 3 Dry weight (DW) of *O. basilicum* plants grown under three different levels of P amendments. The plants were non-mycorrhizal (NM) or colonized by *Glomus hoi* (AM). Various letters reveal the significant differences based on Duncan's test ($P \leq 0.05$). *P* phosphorus

Tissue P content

In both irrigation regimes, tissue P contents in NM basil plants were significantly increased at P3 level. Totally, AM plants had higher tissue P content than NM plants (Table 1). For all AM plants, P content was similar to that of NM plants grown at both P2 and P3 levels (Fig. 4). The highest amount of phosphorus (6.17 mg/g FW) belonged to non-stressed AM plants (P3) while the lowest was observed in stressed NM plants (3.12 mg/g FW) (P1). In non-stress conditions, tissue P contents of AM and NM plants revealed significant difference only at P3 level ($P \leq 0.05$), while in drought stress, tissue P contents of AM and NM plants were significantly different at P2 and P3 level ($P \leq 0.05$).

For all treatments, increasing P supply enhanced tissue P content. Moreover, AM plants had higher tissue P content than NM plants for both irrigation regimes. Tissue P content in AM plants was lower under drought stress as compared to the well-irrigated condition which was similar to the reported results in mycorrhizal cotton plants (Mai et al. 2018).

P availability changes the nutrient accumulation patterns in plants and consequently affects their ability to utilize nutrients for growth and metabolic processes. Plant nutrient status can significantly influence the production of secondary metabolites. Furthermore, plants can change their morphology and metabolism to adapt to P-limited conditions. Such adaptive alterations contribute to the increment of the P availability in the rhizosphere and improve P uptake through extra radical hyphae to maintain metabolic efficiency (Scagel and Lee 2012). These beneficial effects for plant growth might were carried out due to improving P nutrition via the AM fungus (Toussaint et al. 2007).

Table 1 Growth parameter and contents of phytochemicals in mycorrhizal (AM) and non-mycorrhizal (NM) *O. basilicum* plants grown at different levels of P and drought stress

FC (%)	Mycorrhiza inoculation	P level	DW (g)	P content (mg/g FW)	TPC (mg GAE/g DW)	PAL activity (μ/mg protein)	RA content (mg/g DW)	CA content (mg/g DW)
90	AM	1	0.35^{b}	4.11^{b}	9.91^{cd}	38.11^{cd}	0.61^{e}	0.32^{d}
	AM	2	0.61^{a}	5.32^{ab}	11.82^{c}	41.20^{c}	0.92^{d}	0.59^{c}
	AM	3	0.63^{a}	6.17^{a}	15.25^{c}	47.34^{c}	0.96^{d}	0.67^{c}
	NM	1	0.23^{c}	4.54^{b}	5.17^{d}	18.99^{e}	0.52^{e}	0.12^{f}
	NM	2	0.33^{b}	5.15^{ab}	9.53^{cd}	25.26^{ed}	0.65^{ed}	0.17^{ef}
	NM	3	0.38^{b}	5.81^{a}	11.76^{c}	27.98^{d}	0.82^{d}	0.25^{e}
60	AM	1	0.27^{c}	3.33^{c}	19.31^{c}	61.34^{b}	2.12^{b}	0.92^{b}
	AM	2	0.35^{b}	4.61^{b}	31.66^{a}	78.27^{a}	3.05^{a}	1.51^{a}
	AM	3	0.39^{b}	4.72^{b}	38.97^{a}	89.13^{a}	2.97^{a}	1.45^{a}
	NM	1	0.15^{d}	3.12^{c}	12.24^{c}	35.31^{cd}	1.03^{d}	0.41^{d}
	NM	2	0.23^{c}	4.15^{b}	17.18^{c}	43.71^{c}	1.91^{c}	0.68^{c}
	NM	3	0.29^{c}	4.25^{b}	25.23^{b}	59.24^{b}	2.12^{b}	0.83^{b}

P1, P2 and P3 are phosphorus amendments as 0.1, 0.2 and 0.3 g/kg soil, respectively

There was no significant difference in means within a column followed by the same lower case letter at $P \leq 0.05$. *FC* field capacity, *P* phosphorus, *DW* dry weight, *TPC* total phenol content, *RA* rosmarinic acid, *CA* caffeic acid, *PAL* phenylalanine ammonia-lyase

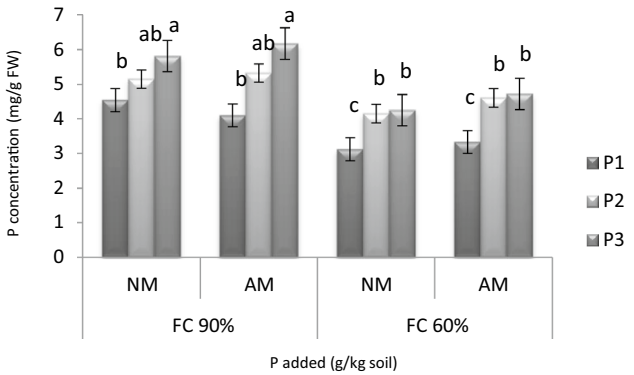

Fig. 4 Total plant phosphorus (P) content (mg/g FW) of *O. basilicum* plants grown under three different levels of P amendments. The plants were non-mycorrhizal (NM) or colonized by *Glomus hoi* (AM). Various letters reveal the significant differences based on Duncan's test ($P \leq 0.05$). *P* phosphorus

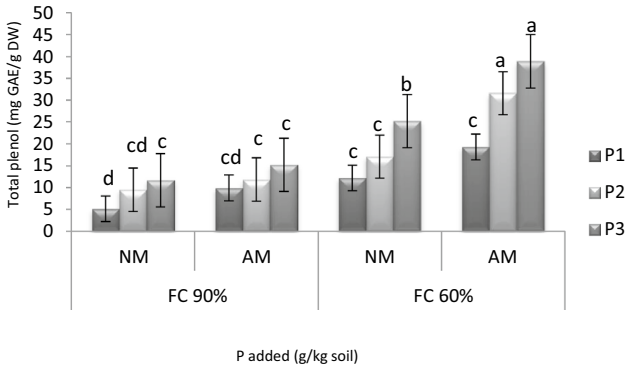

Fig. 5 Total phenol content (mg GAE/g DW) in *O. basilicum* grown under three different P amendments. The plants were non-mycorrhizal (NM) or colonized by *Glomus hoi* (AM). Various letters reveal the significant differences based on Duncan's test ($P \leq 0.05$). *P* phosphorus

Total phenol content (TPC)

TPC under three different P levels in both AM and NM plants are indicated in Fig. 5. Total phenol content varied from 5.1 to 38.9 mg GAE/g DW among treatments. Plants under drought stress showed higher TPC than NM groups (control). Furthermore, increasing P supply and mycorrhizal inoculation enhanced the TPC of basil plants. TPC in AM plants was found to be about 1.67 times more than that of measured in NM plants. The highest TPC (38.9 mg GAE/g DW) was observed in AM plants at P3 level under drought stress, while the least TPC (5.1 mg GAE/g DW) belonged to NM plants at P1 and NS condition. In non-stressed condition, NM plants significantly differed in terms of TPC for all P treatments ($P \leq 0.05$). Similar results were obtained for drought stress. Under drought stress, the TPC of AM plants increased 1.84 times more than NM plants when P application was P2 levels.

The results showed that drought stress and P levels had significant effects on TPC, and it was increased in AM plants at the highest P level. In the present study, AM plants revealed higher total phenols under water-deficit conditions. Similarly, high phenolic content due to drought stress was previously reported in *Solanum scabrum* spp. (Okello et al. 2017).

Plants synthesize a large number of phenolic compounds against abiotic and biotic factors. The phenolic compounds are the main non-enzymatic plants' antioxidants balancing

the destructive impacts of free radicals formed as the result of oxidative stress. Furthermore, these compounds can prevent oxidative damage of DNA, lipid peroxidation of the cell membrane, and protein and chelate metal ions. They also can improve the function of antioxidant enzymes (Gnanasekaran and Kalavathy 2017).

Drought stress-induced free radical production and altered the biosynthesis of specific metabolites. Under environmental stresses, the metabolic processes will be shifted towards the biosynthesis of highly reduced secondary metabolites through the xanthophyll cycle. The xanthophyll cycle protects the photosynthetic apparatus against drought-induced oxidative damage (Gnanasekaran and Kalavathy 2017).

Significant effects of mycorrhiza or P level on the production of phenolic compounds could be attributed to their impacts on plant biomass (Scagel and Lee 2012). Evidences indicated that AM symbiosis influences the activation of defense mechanisms, structural modifications, and phytohormone dynamics possibly through improved P and N supply (Ceccarelli et al. 2010). Chitin (a component of fungal cell walls) can induce the production of secondary metabolites in basil (Scagel and Lee 2012). Vogt (2010) showed biosynthesis of phenylalanine and tyrosine (precursors for all phenolic compounds, e.g., CA and RA) was highly dependent on the N metabolism. Zhu and Yao (2004) found a negative correlation between phenolic compounds in mycorrhizal tomatoes with disease severity.

Quantification of RA and CA in basil leaves

RA and CA contents of NM and AM basil plants grown at different P levels and drought stress are demonstrated in Table 1. The RA content of leaves varied from 0.52 to 3.05 mg/g DW, while CA varied between 0.12 and 1.51 mg/g DW (Fig. 6a,b). Even though the amounts of RA and CA in AM plants exceeded that of NM plants, their fluctuation followed a similar pattern.

Increasing P levels significantly enhanced the RA and CA contents in both AM and NM plants. The highest contents of RA (3.05 mg/g DW) and CA (1.51 mg/g DW) were observed in AM plants at P2 level under drought stress; whereas the lowest contents of RA (0.52 mg/g DW) and CA (0.12 mg/g DW) were observed in NM plants at P1 level and NS condition. For all irrigation regimes, the amounts of RA and CA in both AM and NM plants were significantly enhanced by elevation of P level. These differences were not significant between AM (at P2) and NM plants (at P3) ($P \leq 0.05$). Under drought stress and in P2 level, the contents of RA (1.59-fold) and CA (2.20-fold), were increased in AM plants when compared with NM plants.

In this study, the values of RA content were in line with those reported for *O. basilicum*. Many authors have reported a large variability in RA content in the leaves of different

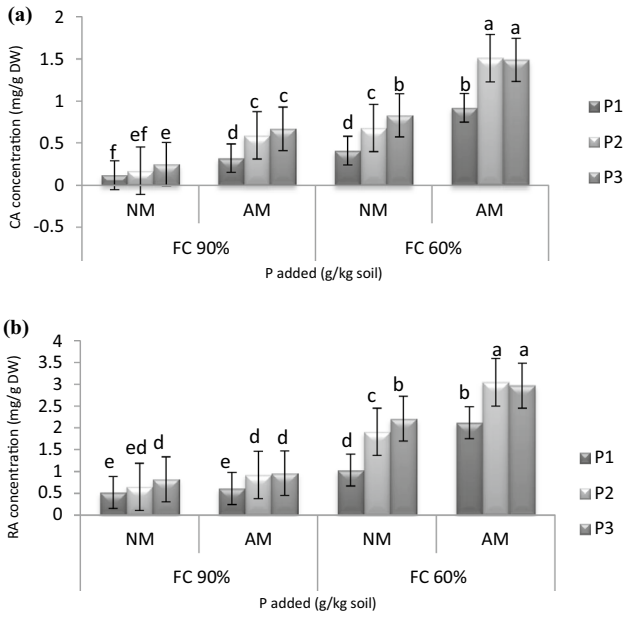

Fig. 6 **a** caffeic acid (CA) content (mg/g DW) in *O. basilicum* plants grown under three different levels of P amendments. **b** rosmarinic acid (RA) content (mg/g DW) in *O. basilicum* plants grown under three different P amendments. The plants were non-mycorrhizal (NM) or colonised by *Glomus hoi.* Various letters reveal the significant differences based on Duncan's test ($P \leq 0.05$). *P* phosphorus

species of *Ocimum*, which ranged from less than 0.1 mg/g DW to nearly 100 mg/g DW. In basil plants, RA biosynthesis strongly depends on the environmental conditions during the growth phase. The discrepancies in its reported levels could be due to quantification methods, growing conditions, genotypic differences, age, and the drying process (Flanigan and Niemeyer 2014). Most authors found several phenolic compounds in basil tissues (Kwee and Niemeyer 2011; Aburigal et al. 2017); however, RA was the most abundant phenolic compounds in many cases and is probably the major compound responsible for the basil antioxidant activity (Koroch et al. 2010; Kiferle et al. 2011; Flanigan and Niemeyer 2014).

Toussaint et al. (2007) showed that mycorrhiza enhanced the RA and CA production in basil shoots. Under the same P status, AM plants contained higher contents of CA and RA in comparison with NM plants. In our study, the effects of P supplementation on increasing TPC were independent of mycorrhiza inoculation. Such a result is consistent with the results of Pedone-Bonfim et al. (2012) in cebil (*Anadenanthera colubrine* Vell.), who showed that P supplementation to NM plants significantly increases RA and CA production. However, higher contents of phenolic compounds in the shoots of AM plants were not completely related to P availability.

In the present study, CA levels ranged from 0.12 mg/g DW to 1.51 mg/g DW, similar to the values previously

reported for other basil cultivars (Flanigan and Niemeyer 2014). Several studies have proved the potential of AM for increasing CA production in basil (Toussaint et al. 2007; Lee and Scagel 2012). The CA is actively involved in mechanisms of stress tolerance. Under drought stress, it is also responsible for the absorption of high energy radiations in mesophyll cells. Caffeic acid is also consumed for lignin biosynthesis in plants and induces the production of other phenolic compounds (especially, RA) which neutralize the effect of toxic ions in the cytoplasm (Riaz et al. 2019).

The AM-induced alterations in the contents of RA and CA in *O. basilicum* can be explained by different reasons. These changes can be related to the mycorrhizal-derived cytological alterations. At the cellular level, mycorrhiza can induce increasing gene expression, activating key-enzymes, enhancing the content of precursors of these compounds, and increasing some organelles which can activate tricarboxylic acid and biosynthetic plastidial pathways. These pathways are responsible for the production of by-products which are consumed in the synthesis of phenolic compounds such as tyrosine and phenylalanine (precursors for all phenolic compounds, e.g., CA and RA) (Santos et al. 2017; Lohse et al. 2005). Plant response to AM colonization and signaling mechanisms between the host plant and fungi may modify the contents of these compounds and alter secondary metabolite balance. In general, the production of phenolic compounds is regarded as a defensive reaction to fungal infection. Induction in the synthesis of phenolic acids in mycorrhizal plants can be the result of better phosphorus and/or nitrogen nutrition due to AM colonization (Zubek et al. 2012).

Various metabolites can be produced through optimization of P uptake (Pedone-Bonfim et al. 2012). Increased CA contents in NM plants were reported by the enhancement of P level (Toussaint et al. 2007; Scagel and Lee 2012). In our study, P levels significantly influenced RA and CA production in NM plants, which is in agreement with previous findings.

PAL activity

PAL catalyzes the initial step of the phenylpropanoid pathway that finally leads to the formation of phenolic compounds (especially RA and CA). The effects of P application, drought stress, and mycorrhiza inoculation on PAL activity are shown in Table1. In this experiment, increasing P level led to elevated PAL activity in basil leaves (Fig. 7). Under drought stress, PAL activity was significantly increased in AM plants at P3 level (89.1 u/mg protein) ($P \leq 0.05$). The lowest PAL activity (18.9 u/mg protein) was measured in NM plants (non-stressed, P1). Drought stress-exposed AM plants, which had the maximum PAL activity (at P3), accumulated the highest amount of TPC, RA, and CA. There

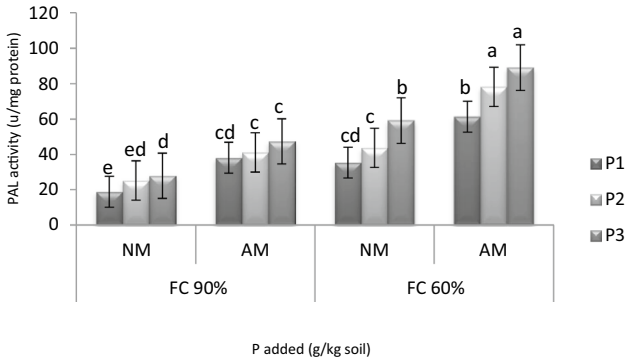

Fig. 7 PAL activity (u/mg protein) in *O. basilicum* plants grown under three different levels of P amendments. The plants were non-mycorrhizal (NM) or colonized by *Glomus hoi* (AM). Various letters reveal the significant differences based on Duncan's test ($P \leq 0.05$). *P* phosphorus

is consistency between this result and the results of Blilou et al. (2000) indicating the increased levels of transcripts encoding PAL in mycorrhizal plants (*Oryza sativa*). Swedan (2013) showed that the gene expression trend of PAL was associated with phenolic contents and the plants producing the highest phenolic compounds had the highest PAL gene expression. Studying the effect of chitosan and chitin oligomer (from fungal cell walls) on soybean leaves, Khan et al. (2003) showed that total phenolic content and PAL activity of soybean leaves increase following these treatments. A positive correlation between total phenolic content and PAL activity was also reported. The higher levels of phenolic compounds and PAL activity in AM-inoculated of *O. basilicum* under drought stress are in line with the previous reports about increased production of phytochemicals due to mycorrhiza symbiosis (Toussaint et al. 2008; Hura et al. 2007).

Conclusions

Basil is considered as functional plant food for its high content of secondary metabolites and antioxidant features preventing oxidative stress-induced diseases. Mycorrhizal inoculation represents a useful biotechnological application that affects the biosynthesis of secondary metabolites with health-promoting activities. The results showed inoculation of plants with AM fungus and appropriate content of P and suitable level of drought stress can be a simple and useful method to optimize the quality of basil composition and achieve higher contents of phenolic compounds for pharmaceutical purposes and phytotherapeutic market. Accumulation of phenolic compounds in Basil plants can be increase by a direct effect of the mycorrhiza and improved P nutrition. Both drought stress and P levels enhanced secondary

metabolites production. In this experiment application of 0.2 g P per kg of soil in AM plants under drought stress was the best approach to reach to high contents of RA and CA, but further research is necessary to determine whether either biotic (fungus or bacteria) and abiotic elicitors can effectively improve the biosynthesis of these compounds. The capability of various AM species at regulating genes related to secondary metabolites should be further assessed by metabolomics and transcriptomic analyses. These studies should explore the differential expression of genes included in biosynthesizing health-promoting plant compounds in AM plants. In this study we report for the first time, a large increase of TPC, CA, and RA level in sweet basil plants inoculated with *G. hoi* under drought stress and adequate P supply (0.2 g/kg soil).

Author contribution statement MZ: collected the data, performed all experiments, contributed data, measured and recorded data, performed the statistical analysis, wrote the paper; AG: conceived and designed the experiments and analysis, performed the statistical analysis, wrote the paper, performed text mining analysis, discussed the results and contributed to the final manuscript; ML: collaborated in the design of the experiment, wrote the paper.

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