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Foliar application of bio-fertilizers influenced the endogenous concentrations of phytohormones and amino acids in leaves and roots of olive trees (*Olea europaea* L. cv. Chemlali)

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The chemical composition of olive leaves and roots of Chemlali variety was evaluated regarding the contents of amino acids and endogenous phytohormones under foliar application of biofertilizers (F1: rich in nitrogen (N), phosphorus (P), potassium (K); F2: rich in calcium (Ca); F3: application of F1 and F2). The results of this study demonstrated significant effects of tested fertilizers on the concentrations of amino acids. Glutamic acid and asparagine had the highest concentrations in leaves and the lowest concentrations in roots under F1 and F3. Regarding the concentrations of endogenous phytohormones, high contents of indole-3-acetic acid and salicylic acid were found in leaves and roots under the application of F1 biofertilizer. Jasmonic acid (JA) was of higher concentration in root than leaves, while abscisic acid occurred in lower concentration. Moreover, the analysis of isoprenoid cytokinins by ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS) showed that these cytokinins were improved and became more abundant in roots under foliar treatments. Particularly, Zeatin-N7-glucoside and Dihydrozeatin-N9-Glucoside were enhanced under F1 and F3. The results of this study suggest that foliar application of biological fertilizers may improve physiological status of olive trees throughout the enhancement of some phytohormones and amino acids.

Key words: Foliar fertilization, olive trees, amino acids, indole-3-acetic acid, isoprenoid cytokinins.

INTRODUCTION

Olive tree (*Olea europaea* L.) is the most important tree in the countries of the Mediterranean basin. Deficiencies in mineral elements such as Nitrogen (N), Phosphorus (P), Potassium (K) and Calcium (Ca) are among the

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Table 1. Physico-chemical characteristics of the soil (0-40 cm) of the experimental orchard.

Depth (cm)	0 - 40
Clay (%)	20
Silt (%)	11
Sand (%)	69
pH	8.5
Electrical conductivity (mmhos/cm)	2.7
Organic carbon (%)	0.67
Organic matter (%)	1.15
Total nitrogen (%)	0.073
Available phosphorus (P_2O_5) (ppm)	5
Exchangeable potassium (K_2O) (ppm)	459

major factors which are negatively influencing olive tree growth. Foliar fertilization appeared among the most used methods against these deficiencies and aimed to help the plant during the development and ripening process. Various macronutrients and micronutrients not only play a nutritional role, but also have an additional independent function signaling their metabolism (Krapp and Castaings, 2012). In this case, the application of some known minerals is necessary to have a positive effect on the physiological status in different plants. For example, it has been shown that the main nitrogen source nitrate, could act as a signal molecule controlling gene expression and plant development (Krapp and Castaings, 2012). Recent studies showed how nitrogen and phytohormone signals are involved in the modification of plant physiology and morphology (Gu et al., 2018). Moreover, N is known to be a vital element incorporated in proteins, nucleic acids, chlorophyll and numerous plant metabolites including phytohormones. Also, P is a building block of many secondary metabolites of plants (Gan et al., 2015) and is involved in the growth and development of all crops (Zhu et al., 2018). K is also a necessary macronutrient for trees; it carries out vital functions in metabolic and stress adaptation (Armengaud et al., 2004). Moreover, Ca is necessary for membrane stability and, together with P, it plays a central role in maintaining osmotic homeostasis and cell signalling, coupled with stress tolerance and appropriate photosynthetic functions (Qu et al., 2012). It is involved also in plant growth and development, cytoplasmic streaming, cell division, and photosynthesis (Huang et al., 2017). It plays a crucial role in the heat stress, in the cold acclimation of plants, and in the pathogen attack in the reactive oxygen species (ROS) response (Sharma et al., 2017).

Some studies were reported on the effects of foliar fertilization on the amino acid content in different plant species (Gutiérrez-Gamboa et al., 2016; Hannam et al., 2015). However, nothing is known about the effects on the endogenous hormone levels. The plant hormones, including the classical auxins indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and cytokinins (CKs), are chemical messengers which play a vital role in the regulation of plant growth, development and responses to environmental conditions (O'Brien and Benková, 2013). The combined activities of several hormonal pathways, such as ABA and development-related hormones, such as CK and auxin resulted when a plant is exposed to abiotic stresses and are involved in plant development. Also, SA and JA are activated when a plant is exposed to pathogenic attack or various-stress conditions mainly low temperature, drought and salinity (Kiba et al., 2011). In fact, many studies showed good evidence for a relation between plant hormones and availability and/or deficiency of mineral elements, for example in maize (Gawronska et al., 2003; Zhao et al., 2016), *Betula pendula* and in *Acer pseudoplatanus* L. (Darrall and Wareing, 1981). However, there has been no detailed study that aimed to evaluate the response of the olive tree to an exogenous supply of mineral elements by foliar fertilization. Thus, the aim of this study was to assess the effects of foliar application of some fertilizers based on minerals (N, P, K and Ca) extracted from natural sources, on the content of amino acids and phytohormones in leaves and roots of olive trees (cv. Chemlali).

MATERIALS AND METHODS

Field experiment and treatments

The present study was carried out on mature olive trees of "Chemlali" cultivar grown in a rainfed orchard located at Jemmel, Tunisia (North latitude 35° 63′, East longitude 10° 68′ and Altitude: 44 m). The mean annual rainfall is 313 mm and the mean annual temperature is 18.2°C. The experimental soil has a sandy texture. A composite soil sample (0 to 40 cm depth) taken from the soil of the experimental farm was analyzed (Table 1). The experimental trees were planted at 10 × 10 m apart and arranged in a randomized block design with three blocks (9 trees in each block) and four treatments that consisted of the annual application of F0, F1, F2

Table 2. Mineral compositions (w/v: g/l) of the four foliar fertilizers used in this study.

Foliar treatment	N	P_2O_5	K_2O	CaO
F0	-	-	-	-
F1	40	20	60	-
F2	-	-	-	160
F3	40	20	60	160

and F3 (obtained from Agronutrition (France, Carbonne)) during two successive growing seasons (2014 and 2015):

F0: Control, without foliar fertilization (water spray).
F1: A ready liquid formulation made up of amino acids and extracts of Molasses beet (sources of nitrogen (N) and potassium (K)) and the edge of fish (source of phosphorus (P)). The mineral composition of this fertilizer is shown in Table 2. Foliar fertilization consisted of four applications with 15 day interval, starting from petal fall, with a dose of 5 L/ha.
F2: This is a liquid formulation of calcium concentrate (Table 2). Five applications with 15 days interval were conducted, starting from fruit set, with a dose of 2 L/ha.
F3: The trees received both F1 and F2 foliar fertilizers.

Plant

Fresh olive leaves and roots of *O. europaea* were collected in the second week of March (spring 2016, after two years of foliar fertilization). Leaves were collected from three random locations in each plot and root samples were collected at 10 to 15 cm depth. Fresh olive leaves and roots were washed with distilled water to eliminate any traces of dust. The samples were immediately protected in isothermal bins with dry ice and transferred to the laboratory. In order to stabilize the byproduct and to avoid quality losses and undesirable degradation during storage and transportation, plant materials were homogenized by grinding in liquid nitrogen and kept at -80°C before analysis of phytohormones and amino acids.

Determination of amino acids

Plant material was homogenized using a MagNA Lyser instrument (Roche Life Science, USA). Amino acids were extracted in formic acid (0.125 v%, 1 µl/mg fresh weight). Heavy labeled L-Glutamine-2, 3, 3, 4, 4-d5 (0.1 nmol, Sigma Aldrich, Overijse, Belgium) was added as an internal standard to quantify recovery and ionization efficiency. Prior to analysis, samples were filtered using a Chromafil AO20/3 (0.2 µm, 3 mm) Polyamine syringe filter (MV, Düren, Germany). Samples were analyzed using an UPLC system linked to an Acquity triple quadrupole detector (Waters, Milford, MA) equipped with an electrospray interface. Samples (6 µl) were injected on an ACQUITY BEH Amide Column (1.7 µm × 2.1 mm × 100 mm, Acquity UPLC, Waters, Milford, MA) and eluted with a gradient of solvent A (0.1% FA in H_2O) and solvent B (0.1% FA in CAN) over 7.5 min at 0.5 ml/min. The effluent was introduced into the electrospray source (source temperature: 120°C, capillary voltage: 1.8 kV, component dependent cone voltages between 13 and 23 V). Quantification was done by multiple reactant monitoring (MRM) of the MH+ ion (dwell time: 0.016) and the proper product ion. This corresponded to 175>70 and 175>130 for arginine, 156>110 for histidine, 148>84 and 148>130 for glutamic acid, 147>84 and 147>130 for glutamine, 133>74 and 133>88 for asparagine, 133 > 70 for ornitine, 116>70 for proline, 106 > 70 and 106>88 for serine, 90>44 for alanine, and 152>89 and 152>135 for L-Glutamine-2,3,3,4,4-d5. All data were processed using Masslynx/Quanlynx software V4.1 (Waters, Milford, MA).

Measurement of phytohormones

Measurement of endogenous SA, JA, IAA and ABA levels

Extraction of SA, JA, IAA and ABA: Plant materials were extracted overnight in 80% methanol (10 µl/mg FW, -20°C, 16 h). 100 pmol of $C_6{}^{13}$-phenyl-IAA (Cambridge Isotope Laboratories Inc., Andover, Massachusetts, USA), 150 pmol of D6-ABA ([$^2H_6$](+)-*cis, trans* ABA (OlChemIm, Olomouc, Czech Republic)), 100 pmol of [2H4]-SA (OlChemIm) and 100 pmol of dehydro-Jasmonic acid (OlChemIm) were added as internal standards. After a purification step removing pigments on a C18 cartridge (Bond Elut C18 6 cc, 500 mg, Agilent, CA, USA) in 80% methanol, the extract was diluted and acidified with formic acid 6% so that hormones bind to a C18 cartridge. Diethyl ether was used to elute the hormones. The residual water was removed and the ether phase was evaporated under a stream of N_2 gas (Turbovac L V Evaporator, Hopkinton, MA, USA). After methylation with diazomethane (Schlenk and Gellerman, 1960), the samples were dried under N_2 gas and dissolved in 100% hexane (15µ l) for GC-MS/MS analysis of salicylic acid (SA) and jasmonic acid (JA). The remaining of the samples (after JA and SA analysis) were dried and redissolved in 50 µl of 10% for LC-MS/MS analysis of indole-3-acetic acid (IAA) and abscisic acid (ABA).

Analysis of SA and JA: Gas chromatography-mass spectrometry analysis was performed using a Waters Micromass Quattro micro GC (Waters; MA,USA): a triple quadrupole with an integrated Agilent 6890N gas chromatography oven, and using an electron impact (EI) ion source, positive ion mode, 70 eV, Collision Energy 10 eV, inter-channel delay 10 ms and inter-scan delay 10 ms. The gas chromatography column used was a 15 m × 0.25 mm Agilent J&W DB-5ms, film thickness: 0.25 um (Agilent Technologies; CA, USA), injection volume: 10 µl, Carrier gas: helium, flow rate: 1 ml min^{-1}. The oven started isothermally at 50°C for 2 min and increased linearly to 300°C at a rate of 25°C min^{-1}. 300°C was held for 3 min. The diagnostic ions used for quantification of SA and JA in MRM mode are 152> 20 m/z for MeSA, 156>124 m/z for D4-MeSA, 224>151 m/z for MeJA and 226>153 m/z for DHMeJA (dwell time: 0.010 s).

Analysis of IAA and ABA: IAA and ABA were analyzed by UPLC-MS/MS after methylation (Acquily TQD, TQD, Waters, Manchester, UK), 6 µl injection by partial loop, ACQUITY BECH18, 1.7 um column (WATERS), column temperature: 30°C, flow of 400. The solvent gradient was as follows: 0 to 2 min: 95/5 of 10% methanol in NH_4OAc 1 mM/methanol; 2 to 4 min: 10/90 of 10% methanol in NH_4OAc 1 mM/methanol; 4 to 6 min: isocratic 10/90 of 10% methanol in NH_4OAc 1 mM/methanol. MS conditions: Polarity MS ES(+), capillary: 2 kV, cone: 20 V, collision energy: 20 eV, source temperature: 120°C, desolvation temperature: 450°C, Cone gas

flow: 50 L/h, desolvation gas flow: 750 L/h, collision gas flow: 0.19 ml/min. The diagnostic ions used for quantification for IAA and ABA were: 190>130 m/z for Me-IAA, 196>136 m/z for Me-C^{13}-IAA, 279>173 m/z for Me-ABA and 285>179 m/z for d_6-Me-ABA (dwell time: 0.02 s). Methanol and water used for MS were UPLC grade form Biosolve (Valkenswaard, the Netherlands). Data were expressed in pmol per gram of fresh weight (pmol. g^{-1} FW).

Measurement of endogenous CK levels

Cytokinin extraction: Plant material was extracted overnight in 80% methanol (10 µl/mg FW, -20°C, 16 h). [$^2H_3$]Dihydrozeatin (d-DHZ), [$^2H_3$]Dihydrozeatin Riboside (d-DHZR), [$^2H_6$]N^6-*Iso*pentenyladenine (d-*i*P), [$^2H_6$]N^6-*Iso*pentenyladenosine (d-*i*PA), [$^2H_5$]Zeatin-*7*-Glucoside (d-Z*7*G), [$^2H_5$]Zeatin-*9*-Glucoside (d-Z*9*G), [$^2H_6$]N^6-Isopentenyladenine-*7*-Glucoside (d-*i*P*7*G) and $^2H_6$]N^6-*Iso*pentenyladenine-*9*-Glucoside (d-*i*P*9*G) (10 pmol each, OlChemIm) were added as internal standards. After centrifugation (20000 g, 15 min, 4°C, 5810R, rotor FA-45-30-11 Eppendorf, Hamburg, Germany), the supernatant was passed over a C18 cartridge (500 mg, Varian) to retain pigments and was consecutively filtered (Chromafil Xtra PA-20/25, 0.2 µm, ϕ 25 mm, MN, Duren, Germany). Samples were dried in a SpeedVac (Christ RNC2-25 vacuum concentrator, with KNF N860.3FT.40.18 pump, Freiburg, Germany), and redissolved in 50 µl of 10% methanol for analysis.

Cytokinin analysis: Isoprenoid cytokinins were analyzed by UPLC-MS/MS (WATERS): 6 µl injection by partial loop, ACQUITY BEH-C18, 1.7 µm column (WATERS), column temperature: 30°C, flow 400, solvent gradient: 0 to 0.5 min: 95/5 of 10% methanol in NH_4OAc 1 mM/methanol; 0.5 to 3 min: 75/25 of 10% methanol in NH_4OAc 1 mM/methanol; 3 to 5 min: isocratic 75/25 of 10% methanol in NH_4OAc 1 mM/Methanol; 5 to 6 min: 5/95 of 10% methanol in NH_4OAc 1 mM/methanol; 6 to 6.5 min: isocratic 5/95 of 10% methanol in NH_4OAc 1 mM/methanol. MS conditions: Polarity MS ES(+), capillary: 2 kV, cone: 20V, collision energy: 20 eV, source temperature: 120°C, desolvation temperature: 400°C, Cone gas flow: 20 L/h, desolvation gas flow: 800 L/h, collision gas flow: 0.22 ml/min). The diagnostic ions used for quantification for isoprenoid cytokinins were: 222>136 m/z for DHZ, 220>136 m/z for Z, 353>222 m/z for DHZR, 352>220 m/z for ZR, 204>136 m/z for *i*P, 336>204 m/z for *i*PA, 384>222 m/z for DHZ*7*G and DHZ*9*G, 382>220 m/z for Z*7*G and Z*9*G, 366>204 m/z for *i*P*7*G and *i*P*9*G, 225>136 m/z for d-DHZ, 356>225 m/z for d-DHZR, 210>136 m/z d-*i*P, 342>210 m/z for d-*i*PA, 389>227 m/z for d-Z*7*G and d-Z*9*G, 372>210 m/z for d-*i*P*7*G and d-*i*P*9*G. Methanol and water used for MS were UPLC grade from Biosolve. Data were expressed in pmol per gram of fresh weight (pmol.g^{-1}FW).

Statistical analysis

All statistical values were calculated using SPSS for Windows v. 18.0 and expressed as mean ± standard deviation (SD) of three independent measurements. The post hoc Duncan's test was applied. The statistical significance level was fixed at P≤ 0.05.

RESULTS

Changes in amino acid concentrations in leaves and roots following foliar fertilization treatments

Under F1, this total increase is merely due to the clear increase of asparagine and glutamic acid, whereas under F3, the increase is due also to a minor increase of ornithine and serine in combination with a major increase of asparagine and glutamic acid. These results indicated that after N, P and K supply, leaves accumulated more amino acids than in control leaves or in those treated with F2.

Although, it was observed that the concentrations of amino acids were approximately 6 times higher in roots than in leaves, lower amino acid concentrations were observed in roots after fertilization as compared to those in control conditions (Table 3). Especially, glutamic acid, glutamine and to a lesser amount proline, became significantly lower when fertilizers were applied.

Variation of ABA, IAA, SA and JA concentrations in leaves and roots after foliar fertilization

In this experiment, ABA, IAA, JA and SA concentrations in leaves and roots were determined under the different treatments (Figure 1a to d). In leaves, a significant decrease of the ABA concentration was observed after foliar fertilization. The SA level increased significantly from 3900 pmol/g in F0 to 4700 in F1 and 7500 pmol/g in F3. Compared to F0, a significant increase in IAA concentrations were also observed in F1 (by about 73.33%) and in F2 (by 60%). These results were accompanied by an increase of the JA concentration in F3 and a clear decrease in F1 and F2 as compared to F0.

In roots, foliar fertilization did not significantly affect the endogenous ABA concentration. In contrast, the IAA root concentration increased only under F1 as compared to control (by about 59.47%). The concentration ranges of root IAA were comparable to leaf ones. IAA level was remarkably enhanced under F1 and F2 fertilization. Although the SA concentration in roots was 10 times lower than those observed in leaves, a slight increase of SA could be observed after F1 fertilization (560 pmol/g) as compared to F0 (350 pmol/g). Regarding JA level, being 10 times higher in roots than in leaves, JA concentration in roots decreased significantly under F1, F2 and F3.

Cytokinin concentrations in leaves and roots were differently affected under foliar fertilization treatments

The chemical structure of all the isoprenoid Cks analyzed (DHZR: Dihydrozeatin-N*9*-Riboside, DHZ: Dihydrozeatin, *trans*-ZR: *trans*-zeatin-N*9*-Riboside, *cis*-ZR: *cis*-zeatin-N*9*-Riboside, Z-N*7*-G: Zeatin-N*7*-glucoside, Z-N*9*-G: Zeatin-N*9*-glucoside, *i*P: *iso*pentenyladenine, *i*PA: *iso*pentenyladenosine, *i*P-N*7*-G: N6-*iso*pentenyladenine-N*7*-Glucoside, *i*P-N*9*-G: N6-isopentenyladenine-N*9*-Glucoside, *trans*-Z: *trans*-zeatin, *cis*-Z: *cis*-zeatin, DHZ-N*7*-G: Dihydrozeatin-N*7*-Glucoside, DHZ-N*9*-G:

Table 3. Effects of the foliar treatments on amino acid concentration (10^3 pmol/g FW) in leaves and roots of olive trees.

Amino acids (10^3pmol/g FW)	Leaves			
	F0	F1	F2	F3
Arginine	2.07±0.03^{b}	2.01±0.05^{b}	2.55±0.61^{ab}	3.06±0.52^{a}
Histidine	3.32±0.04^{c}	4.10±0.15^{b}	3.70±0.59^{bc}	6.14±0.02^{a}
Glutamic acid	28.79±10.73^{c}	71.79±7.17^{a}	50.95±0.22^{b}	108.99±38.79^{a}
Glutamine	5.17±0.67^{c}	9.86±1.60^{b}	9.96±1.30^{b}	15.69±0.13^{a}
Asparagine	103.44±15.01^{b}	199.10±25.33^{a}	89.58±6.65^{b}	167.36±45.57^{a}
Ornithine	10.32±0.59^{b}	8.43±1.70^{b}	7.65±3.86^{b}	44.78±2.81^{a}
Proline	6.15±0.25^{c}	11.53±0.82^{a}	8.41±1.24^{b}	5.99±0.12^{c}
Serine	19.43±0.21^{c}	16.01±1.72^{d}	41.97±0.51^{a}	32.45±6.85^{b}
Alanine	10.63±0.54^{a}	10.23±0.76^{a}	8.97±1.23^{a}	9.08±1.27^{a}
		Roots		
Arginine	69.55±1.50^{a}	65.90±5.50^{a}	70.78±4.12^{a}	61.22±27.48^{a}
Histidine	19.47±0.06^{a}	15.00±1.74^{a}	22.86±2.37^{a}	21.38±16.66^{a}
Glutamic acid	479.74±20.29^{a}	301.10±37.99^{c}	357.71±3.08^{b}	240.02±95.06^{c}
Glutamine	695.92±53.43^{a}	234.95±13.98^{d}	327.72±0.51^{b}	303.23±1.63E-05^{c}
Asparagine	180.90±2.89^{a}	82.78±16.53^{c}	128.74±2.69^{b}	81.93±37.18^{c}
Ornithine	92.89±4.45^{a}	28.01±7.40^{c}	75.08±0.31^{ab}	85.12±6.81^{a}
Proline	5.07±0.35^{a}	2.44±0.44^{c}	3.66±0.02^{b}	4.49±1.07^{ab}
Serine	64.83±2.02^{a}	37.37±7.36^{c}	56.07±4.35^{b}	53.58±2.50^{b}
Alanine	20.86±0.94^{a}	9.95±0.69^{c}	17.01±1.25^{b}	15.11±0.76^{b}

Values are means± SD (n=3). Different letters indicate significantly different values at p≤0.05 according to Duncan test. Amino Acids not listed were below detection limit.

Dihydrozeatin-N*9*-Glucoside) are as shown in Figure 2. The effects of foliar biofertilization on the concentrations of these Cks are shown in Table 4. The results showed also that *i*P-N*9*-G was the most abundant cytokinin metabolite in both leaves and roots (Table 4). In leaves, the foliar treatments decreased considerably the level of *i*P-N*9*-G (by about 14.54% in F1, 10.90% in F2 and 9% in T3 comparing to control) and the level of *trans*-ZR (by about 42.10% in F1, 32.63% in F2 and 57.9% in F3 as compared to control). In roots, *i*P-N*9*-G decreased also under biofertilization with the three treatments. However, DHZR, *trans*-ZR, *i*PA, Z-N7-G, DHZ-N*9*-G and *i*P-N7-G were enhanced under foliar fertilization, especially with F1 and F3 spraying (Table 4).

DISCUSSION

Foliar biofertilization influenced amino acid compositions in olive leaves and roots

In this study, absorption of nutrients by foliar sprays resulted in an increase of amino acid levels in leaves, especially under F1 and F3 applications. This may be due to the action of the fertilizer based on nitrogen, phosphorus and potassium that served as nitrogen and phosphorus donors for the biosynthesis of all essentially amino acids, nucleic acids, and other nitrogen-containing compounds. Nitrogen contributes to the synthesis of the first amino acids glutamate and glutamine and thereafter, the synthesis of other amino acids (Hirel and Lea, 2011). Asparagine, aspartate, glutamine and glutamate are carriers to translocate inorganic nitrogen from source organs to sink organs (Peoples and Gifford, 1990). This may explain the increase of glutamic acid and asparagine in leaves and not in roots. Gutiérrez-Gamboa et al. (2016) studied the effect of different foliar nitrogen applications by organic source treatments on the amino acid composition in vineyard. They reported that these treatments improved the amino acid content and increased glutathione concentration.

The findings showed an increase in the content of proline under F1 (N, P and K-based fertilizer) and F2 (Ca^{2+}-based fertilizer). These results agree with those obtained by Gutiérrez-Gamboa et al. (2016) who observed an increase in proline after foliar urea application to grapevines. This is a positive feature for olive trees because it has been reported that the elevated proline concentration may act either as a compatible solute to protect plants from dehydration or contribute to stabilizing subcellular structures and scavenging free radicals, thus conferring tolerance of plants to abiotic stress (Ashraf and Foolad, 2007). Moreover, Zhao et al. (2009) found that nitric oxide (NO), where nitrogen is one

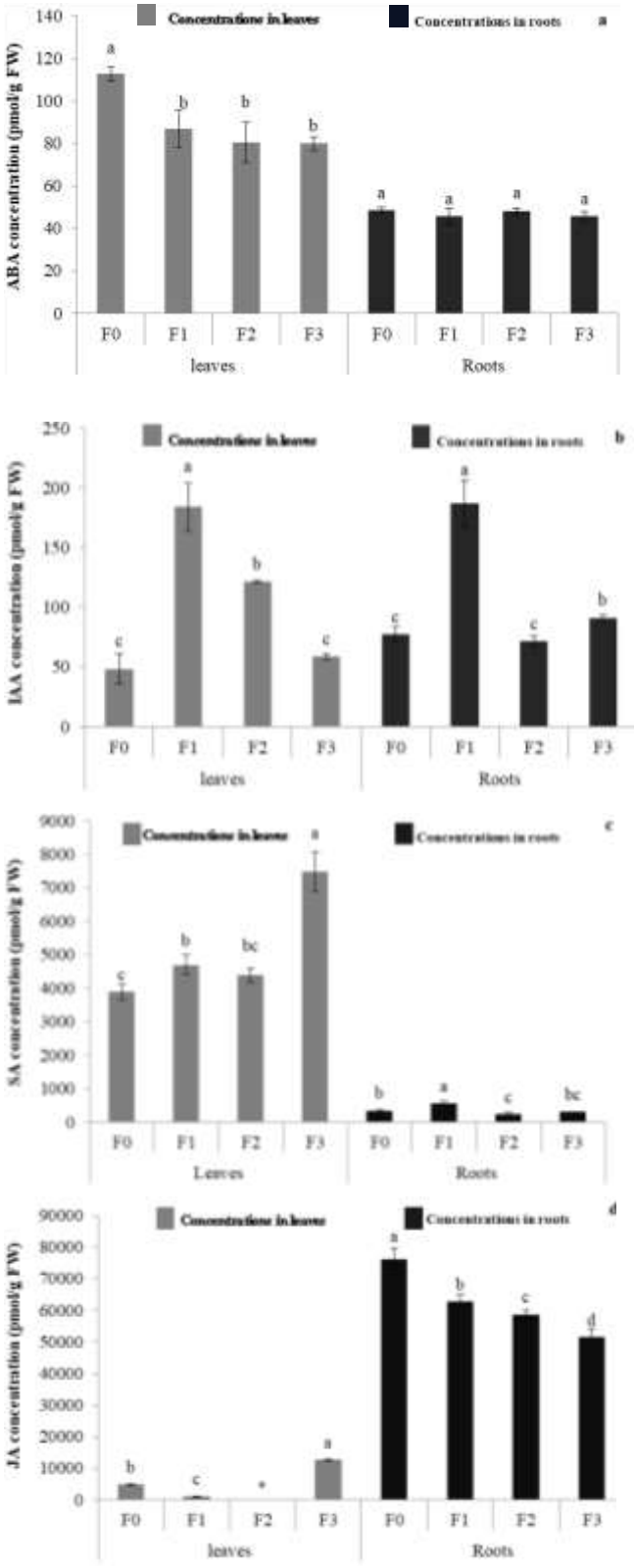

Figure 1. Effect of foliar treatments on the abscisic acid ABA) (a), indole-3-acetic acid (IAA) (b), salicylic acid (SA) (c) and jasmonic acid (JA) (d) concentrations (pmol/g FW) in leaves and roots of olive trees. Values are expressed in means ± SD (n=3). Different letters indicate significantly different values at p≤0.05 according to Duncan test. *Below detection limit.

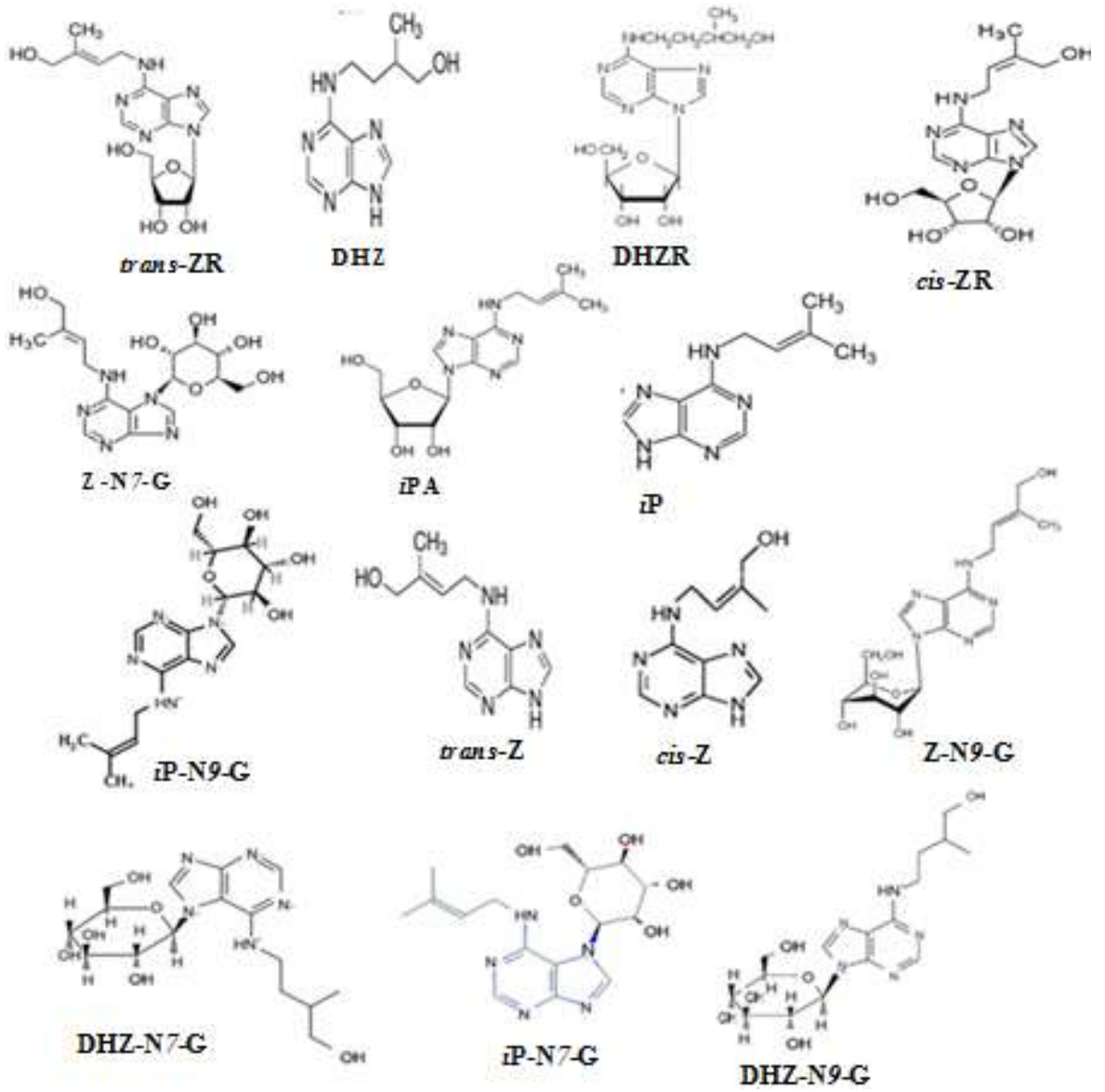

Figure 2. Chemical structures of the tested isoprenoid cytokinins. DHZR: Dihydrozeatin-N9-riboside, DHZ: Dihydrozeatin, *trans*-ZR: *trans*-zeatin-N9-riboside, *cis*-ZR: *cis*-zeatin-N9-iboside, Z-N7-G: Zeatin-N7-glucoside, Z-N9-G: Zeatin-N9-glucoside, *i*P: *iso*pentenyladenine, *i*PA: *iso*pentenyladenosine, *i*P-N7-G: N^6-*iso*pentenyladenine-N7-glucoside, *i*P-N9-G: N^6-*iso*pentenyladenine-N9-glucoside, *trans*-Z: *trans*-zeatin, *cis*-Z: *cis*-zeatin, DHZ-N7-G: Dihydrozeatin-N7-glucoside, DHZ-N9-G: Dihydrozeatin-N9-glucoside.

of component, is a highly active and an important signaling molecule involved in diverse pathophysiological processes and may be associated with the accumulation of proline by transcriptionally promoting proline synthesis and suppressing proline degradation, thus contributing to enhanced abiotic stress tolerance in plant. The results discussed here support the hypothesis that exogenous application of nutrients, particularly nitrogen, may play an important role in the way of proline signaling.

In addition to N, K is known to have a positive effect on amino acid content. According to Tovar-Méndez et al. (2003), in pea seedling, pyruvate dehydrogenase kinase activity is stimulated by NH_4^+ and K^+. Furthermore, the calcium cation Ca^{2+} acts as a regulator. It activates pyruvate dehydrogenase phosphatase by other divalent cations, which in turn activates the pyruvate dehydrogenase component of the pyruvate dehydrogenase complex (Tovar-Méndez et al., 2003). Therefore, this increases the rate of reaction of several cycle steps, and thus increases the flow of compounds through this metabolic pathway such as amino acids.

F1, F2 and F3 foliar applications increase clearly the individual amino acids in leaves. This beneficial effect in plant leads to improvement in the primary metabolism

Table 4. Isoprenoid cytokinin concentrations (pmol/g FW) in olive leaves and roots under different foliar treatments.

Variable	Leaves				Roots			
	F0	F1	F2	F3	F0	F1	F2	F3
DHZR	b.d	b.d.	b.d.	b.d.	4.0±0.4^{b}	5.2±0.8^{a}	b.d.	b.d.
trans-ZR	95±7^{a}	55±4^{b}	64±3^{b}	40±9^{b}	12.2±0.3^{d}	18.2±0.3^{a}	13.5±0.2^{c}	15.4±0.3^{b}
cis-ZR	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
DHZ	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
trans-Z	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
cis-Z	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
iPA	b.d.	b.d.	b.d.	b.d.	14.8±0.2^{b}	14.8±0.6^{b}	18±2^{a}	16.01±0.09^{ab}
*i*P	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
DHZ-N7-G	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Z-N7-G	b.d.	b.d.	b.d.	b.d.	67±2^{b}	81±9^{a}	62±4^{b}	89±1.8^{a}
DHZ-N9-G	0.8±0.8^{a}	b.d.	b.d.	b.d.	111.±16^{b}	170±20^{a}	110±10^{b}	160±6^{a}
Z-N9-G	b.d.	b.d.	b.d.	b.d.	31±8^{a}	b.d.	b.d.	37±9^{a}
*i*P-N7-G	b.d.	b.d.	b.d.	b.d.	8.4±1.1^{c}	16.4±0.2^{a}	13.9±1.2^{b}	8.9±0.7^{c}
*i*P-N9-G	550±21^{a}	470±6^{b}	490±22^{b}	500±13^{b}	149±6^{a}	130±12^{b}	130±4^{b}	119±12^{b}

DHZR: Dihydrozeatin-N9-riboside, DHZ: dihydrozeatin, *trans*-ZR: *trans*-zeatin-N9-riboside, *cis*-ZR: *cis*-zeatin-N9-riboside, Z-N7G: Zeatin-N7-glucoside, Z-N9G: Zeatin-N9-glucoside, *i*P: *iso*pentenyladenine, *i*PA: Isopentenyladenosine, *i*P-N7-G: N^6-Isopentenyladenine-N7-glucoside, *i*P-N9-G: N^6-*iso*pentenyladenine-N9-glucoside, *trans*-Z: *trans*-zeatin, *cis*-Z: *cis*-zeatin, DHZ-N7-G: Dihydrozeatin-N7-glucoside, DHZ-N9-G: Dihydrozeatin-N9-glucoside. b.d. = below detection limit. Values are means± SD (n=3). Different letters indicate significantly different values at p≤0.05 according to Duncan test.

(protein synthesis) and polyamine biosynthesis playing an important role at flowering, fruit set and the plant development (Gény et al., 2007).

Foliar fertilization modified the phytohormone profile in olive trees

The observed increase of IAA level in both leaves and roots may be due to the action of some minerals known to have a positive effect on the IAA synthesis, such as N that is one of the chemical components of IAA. Furthermore, IAA seems to be translocated from leaves to root in response to foliar nitrogen-based fertilization. The supply of nitrogen will therefore allow the plant to multiply cells and thus stimulates vegetative growth by the formation of IAA which promotes the proliferation of buds and slows down the formation of root tips (Tu et al., 2017). Furthermore, potassium status may also affect IAA accumulation in the plant. According to Tu et al. (2017), K fertilization increased the contents of IAA and ZR, but also reduced ABA content consistently in vegetable soybean seeds. The results were not in agreement with those of Armengaud et al. (2004), who observed a reduction in the expression of genes controlling IAA biosynthesis when resupplying potassium of potassium-deprived *Arabidopsis* plants. On the other hand, it was suggested that under low-nitrate conditions, soybean and *Arabidopsis* plants accumulated higher levels of IAA in the root when compared with plants grown under high-nitrate conditions (Rubio et al., 2009). Studies on the effects of nutrient supply on JA and SA signaling are very limited. A significant reduction of JA was observed in leaves and roots of olive trees fertilized with F1, which is in agreement with the results of Armengaud et al. (2004) who reported that K^+ starvation allows an increase in the levels of JA by increasing transcription of proteins such as lipoxygenase while after a K^+ resupply, JA decreased rapidly. This indicates that foliar treatments rich in K mitigated the transcriptional response of JA biosynthesis genes (Armengaud et al., 2004). On the other hand, no JA-responsive genes were identified in response to nitrogen and phosphorus starved plants (Hammond et al., 2003; Wu et al., 2003). JA plays a role in plant defense responses against pathogen attack (Kunkel and Brooks, 2002). One might hypothesize that the observed increase of JA in F3 may contribute to the protection of olive trees against insect herbivores and fungi attack.

Regarding SA, the most remarkable thing is that there is an increase in the level of this phenolic compound in leaves and roots after foliar supply with N, P and K. Del Río et al. (2003) observed that N supply caused the rise of leaf phenolic compound concentrations in olive trees. Besides, in some cases SA and JA act by antagonism, where the SA blocks JA biosynthesis or negatively control the expression of genes regulated by JA. In other cases, the signals act synergistically to activate defense responses (Calatayud et al., 2013). The production of SA increases locally and systemically after infection by various pathogens. Similarly, JA, as a signal molecule in defense, increases also. Both SA and JA led to the

production of plant defensive compounds to activate the plant defensive pathways (War et al., 2015; Ibrahim et al., 2018). In the other side, SA and Ca play crucial roles in plant development and mediate plant response to biotic and abiotic stress (Guo et al., 2018). Thus, the increase of SA especially after F1 and F3 fertilization may promote the natural defense mechanism of olive trees against abiotic stress.

ABA acts as an internal signal enabling plants to survive under adverse environmental conditions (Keskin et al., 2010). In agreement with the results of the present study, Kiba et al. (2011) observed that ABA content in *Arabidopsis* roots did not show a significant difference between seedlings grown under high nitrate or low nitrate conditions. In leaves, the reduction in the ABA content is in agreement with the results reported by Gawronska et al. (2003) for maize (*Zea mays* L.) showing that the ABA concentration in tissue is negatively affected by nitrogen availability. The study of Gawronska et al. (2003) also demonstrated that ABA content in leaves and xylem sap was negatively correlated with nitrogen availability. ABA and IAA are known to be possible effectors for cytokinins (Gawronska et al., 2003). In fact, the decrease of the ABA content in leaves may be related to the cytokinin increase in the roots after foliar bio-fertilization, because of the antagonistic relationship between ABA and CKs. In the literature, it was shown that under water stress, plants reduced the level of CKs and increased the content of ABA (Pospíšilová, 2003). This meant that the ratio of cytokinins to ABA in the leaves and the roots substantially changes by the availability of nitrogen sources.

CKs level increased slightly under F1 and F3 in roots (Table 4). It has been reported that for maize and *Arabidopsis*, a nitrate supply caused an accumulation of CKs in roots followed by a flow of these cytokinins to the aerial parts (Takei et al., 2004). Darrall and Wareing (1981) have found in *B. pendula* that a supply of ammonium, nitrate, or ammonium nitrate showed the highest level of cytokinin-like activity, implying that CKs are a nitrogen status signal (Kiba et al., 2011). Also, Gu et al. (2018) noted that CKs interacted with N coordinating various metabolic processes and developmental programs in plants. The increase in CK levels, in turn, enhances growth and developmental processes in many plants (Nishiyama et al., 2011; Kang et al., 2012). Therefore, it is suggested that the increase of CKs under foliar bio-fertilization could be a positive feature regarding its implication on olive tree growth.

Conclusion

This study revealed that nutrient availability by foliar fertilization is an important factor determining the amino acid synthesis and the profile of phytohormone in both leaves and roots of olive tree. It was also shown that foliar bio-fertilization induced increase significantly on the production of endogenous phytohormones and amino acids in Chemlali cv. This study shows that the use of foliar bio-fertilizers (N, P, K and Ca-based) separately or in combination enhances the adaptation against biotic factors (microbes or herbivores) by increasing the SA and JA and improving the olive tree performances by increasing the amino acid, auxin and cytokinins. Thus, it is suggested that foliar bio-fertilizers have an important role as secondary signals. It can initiate a cascade of signaling events which lead to the modification and translocation synthesis of metabolites in trees. As an enticing area of future study, application of similar treatments is viewed in the olive tree under stressful conditions such as drought stress to determine if the foliar bio-fertilizers tested give usually the same effects.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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