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Relation between leaf and stem biochemical constituents and rooting ability of olive cuttings

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Abstract
The relationships between rooting potential with endogenous phenolic compounds, nitrogen and soluble carbohydrates of cuttings for five olive cultivars (‘Rowghani’, ‘Dusti’, ‘Tokhmekabki’, ‘Konservalia’ and ‘Amigdalolia’) were investigated. Extracts of the leaves and the base of the cuttings were used for analysis of biochemical compounds. Results showed that there were significant differences in rooting potential among olive cultivars. There was no correlation between leaves and stems nitrogen contents and rooting ability of the cuttings. Moreover, leaf and stem soluble sugars, leaf total phenolics, stem caffeic acid, narengin and chlorogenic acid contents of the leaf and stem, did not affect the rooting potential of the cuttings. However, leaf catechin, stem total phenolics and vanillic acid had positive effects on rooting potential of the cuttings. There was a negative correlation between rooting percentage of the cuttings and leaf gallic acid and narengine contents.

Keywords*:* biochemical components, leafy cuttings, olive, rooting potential.

Introduction

Olive (*Olea europaea* L.) is mainly propagated by leafy-stem cuttings. There is a variation in the rooting potential among olive cultivars and they have been classified into three groups namely: easy, moderate and hard-to-root cultivars (Denaxa *et al*., 2012). A balance among endogenous stimulatory and inhibitory factors, as well as, nutritional factors is required to promote rooting of olive cuttings (Wiesman and Lavee, 1995). Nitrogen levels appeared to be an important predictor for rooting potential of stem cuttings. In general, but not always, high N supply to the stock plants or high N content of cutting tissues decreases propagation success through cuttings (Porfírio *et al*., 2016; Dag *et al*., 2012; Hambrick *et al*., 1991; Druege *et al*., 2000). Nitrogen content strongly influences carbon allocation and partitioning in plants. Reduced propagation rate when high N levels are applied is due to reduction in starch reserves in the cutting tissue (Rowe *et al*., 1999). Carbohydrates are considered as optimal markers because they are the direct products of photosynthesis and constitute an important source of energy (Aslmoshtaghi and Shahsavar, 2010; Denaxa *et al*., 2012). Although many studies have shown a positive correlation between rooting potential and carbohydrate content of cuttings, others have failed to establish such

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correlation (Denaxa *et al.*, 2012). It has been reported that seasonal changes in rooting ability of olive cuttings is related to the seasonal changes of carbohydrates in reproductive and vegetative shoots (Del-Rio *et al.*, 1991). Cuttings of easy-to-root cultivars have been characterized with higher carbohydrate content than the cuttings of difficult-to-root cultivars (Denaxa *et al.*, 2012; Yoo and Kim, 1996). Supply and redistribution of carbohydrates in cuttings may sometimes limit their rooting potential (Hartman *et al.*, 2001).

Variations in rooting of cuttings have been associated with the changes in the levels of endogenous growth regulators and other metabolites. Such regulatory processes are controlled through qualitative and quantitative changes in enzymes, such as peroxidases, IAA-oxidase and polyphenol oxidase (Trobec *et al.*, 2005).

Phenolic compounds play important roles in formation of adventitious roots (Aslmoshtaghi and Shahsavar, 2010). However, the mechanism by which phenolics act is still largely unknown. Phenolic compounds are secondary metabolites derived from pentose phosphate, shikimate and phenylpropanoid pathways in plants. They can be classified into non-soluble compounds such as tannins, lignins, cell-wal bound hydroxycinammic acid, and soluble compounds such as phenoic acids, flavonoids, phenylpropanoids and quinines. Phenolic compounds are the most widely occurring groups of phytochemicals in plants. They have considerable physiological and morphological importances and play a crucial role in plants growth and reproduction (Balasundram *et al.*, 2006; Silva *et al.*, 2006). Some phenolic compounds can affect adventitious root formation through preventing auxin degradation via IAA-oxidase system, forming auxin – phenol complex and inhibiting auxin decarboxylation (Haissig, 1986; Wilson and van Staden, 1990).

Several diphenols, o-diphenols, coumarins and polyphenols compounds can inhibit oxidation of IAA (Trobec *et al.*, 2005). On the other hand, phenolic compounds such as monophenols and m-diphenols can inhibit the rooting process by stimulating IAA oxidation or promoting IAA decarboxylation, while some other phenolics have no regulatory effect on IAA content in plant tissues.

The objective of this study was to investigate the relationship between rooting potential of cuttings and different endogenous phenolic compounds, nitrogen contents and soluble carbohydrate in leaf and stem of five olive cultivars.

Materials and Methods

Plant materials, cutting preparation and propagation

This experiment was conducted in Agricultural and Natural Resources Research and Education Center of Fars province in June 2013 and June 2014. In spring (June) 15 cm long cuttings with 2-4 leaves and 0.8 cm diameter were taken from 25 years old mother plants of five olive cultivars namely: 'Rowghani' (easy-to-root), 'Dusti' and 'Tokhmekabki' (moderate-to-root), 'Konservalia' and 'Amigdalolia' (difficult-to-root). Rooting ability of cuttings were measured in two years (2013 and 2014), whereas the analysis of biochemical compounds were performed only in the second year (Jun, 2014). At the beginning of the experiment (only in 2014), the leaves and the basal 3 cm section of the cuttings were sampled for determination of their biochemical compounds. The basal ends of the cuttings were dipped in 4000 mg/l Indol-3-butyric acid (IBA) solution for 5 seconds. Thereafter, cuttings were planted in containers and placed on greenhouse benches that filled with a mixture of perlite and sand (1:1). The benches were bottom heated (22 ˚C) and the samples kept under an automatic mist and fogging unit (80% to 90% relative humidity). Each experimental unit consisted of 75 cuttings. At the end of the rooting period (3 months), the percentage of rooted cuttings were recorded. Cuttings with one or more clearly visible root initials (1-2 mm or longer) and/or roots were classified as rooted

cuttings and were used for calculation of rooting percentage.

Extraction and analysis of phenolic compounds

Extraction, separation and quantification of phenolics were performed according to the method described by Misan *et al*. (2011) with some modifications.

Plant extracts were prepared by grinding 200 mg frozen samples with a solution of methanol/acetic acid (85:15) mixture for 24 h at 4 °C and were subsequently extracted in an ultrasonic bath at room temperature for 15 min. The resulting suspension was then centrifuged at 10000 rpm for 20 min at 0 °C. To remove compounds such as chlorophylls and lipids, the supernatant was extracted with 1 ml n-hexane and was centrifuged at 10000 rpm for 10 min. After removing the supernatant, the resulting solution was used for the analysis of total phenolic contents and their components.

Total phenolics were determined spectrophotometerically using Folin-Ciocalteu reagent and results were expressed as gallic acid equivalents. Quantification of total phenolics was performed using a microplate reader (Bio Tek ELx808) at 750 nm and gallic acid calibration curve (y= 119.02x – 22.348, R2= 0.9947).

Phenolic components were quantified by a HPLC (high performance liquid chromatograph) (Agilent 1200 series) equipped with a UV-Vis multi-wavelength detector at 280 and 330 nm. Data were evaluated using a Chemstation Software (Agilent Technologies) data processing system. The separation of components was achieved by an Agilent, XDB-C18, 5 µm, 4.6×150 mm column, at a flow-rate of 1 ml min^{-1}. Solvent gradient was performed by varying the proportions of solvent A (methanol) to solvent B (2% acetic acid in water) for separation of galic acid, vanillic acid, catechin and naringin at 280 nm and chlorogenic acid and caffeic acid at 330 nm. The total running time and post-running time were 30 and 10 min, respectively. The column temperature was 30 ºC. The volumes of injected samples and standards were 20 µl which was done automatically using autosampler.

Nitrogen content

Samples were placed in paper bags and dried at 65 °C for 48 h in an oven. The dried samples were ground and passed from a 40 mesh screen, and stored for future analysis. To determine the total nitrogen, 0.3 g dried samples were used in a Kjeldahl instrument (KjelFlex K-360, Buchi Co., Switzerland) based on micro-Kjeldahl method (Bremner *et al*., 1996).

Soluble sugars

Samples were dried in a air-forced oven at 65 °C for 48 h and then ground into fine powder. Dried leaf and stem samples (100 mg) were used for measuring soluble sugars. Samples were extracted in hot 80% ethanol and assayed using phenol-sulfuric acid method using glucose as standard (Masuko *et al*., 2005).

Statistical analysis

The experiments were conducted based on a completely randomized design with three replications and 25 cuttings in each replication. One-way ANOVA was used for analysis of data by Excel and SAS programs. Means were compared using LSD test at $\alpha = 0.01$ and 0.05.

Results

The means of rooting percentage for the five olive cultivars are shown in Figure 1. Significant differences were found among rooting percentages of the cuttings for the studied cultivars. Highest rooting percentage (31.3%) was observed in the easy-to-root cultivar ‘Rowghani’, while lowest rooting percentage, was observed in difficult-to-root cultivars ‘Konsrervalia’ (4.7) and ‘Amigdalolia’ (6.5%). Although, moderate-to-root cultivars (‘Dusti’ and ‘Tokhmekabki’) were ranked between easy-to-root and difficult-to-root cultivars, rooting percentage in ‘Tokhmekabki’ was

significantly higher than rooting percentage in 'Dusti' (Fig. 1).

Although, no significant differences ($P \leq 0.05$) was found in stem nitrogen content of five olive cultivars (Fig. 2). There were considerable differences in leaf nitrogen content among the studied cultivars. The highest leaf nitrogen content (24.15 mg g^{-1} DW) was detected in 'Tokhmekabki' and the lowest leaf nitrogen content (19.15 mg g^{-1} DW) was found in 'Amigdalolia' cultivar. There was no significant relationship between the leaf nitrogen content and the rooting capacity among the cuttings of the five studied cultivars. Accordingly, no significant difference ($P \leq 0.05$) was found for leaf nitrogen content between 'Tokhmekabki' and 'Konsrervalia' cultivars with moderate and low rooting ability, respectively (Figs. 1 and 2).

The lowest content (54 mg g^{-1} DW) for leaf soluble sugars was detected in 'Konservalia'; while highest content of leaf soluble sugars (69.2 mg g^{-1} DW) was found in 'Rowghani' cultivar. There was no statistical difference among other cultivars for leaf soluble sugars (Fig. 3).

A significant difference was found in the leaves of the cuttings with respect to their total phenol contents but these differences have no relation with rooting percentage and thus rooting potential of cuttings. Highest total phenol concentration in the stems (10.06 mg g^{-1} DW) was detected in 'Tokhmekabki' cuttings, while 'Konservalia' had the lowest phenol content (9.79 mg g^{-1} DW) among the studied cultivars (Fig. 4).

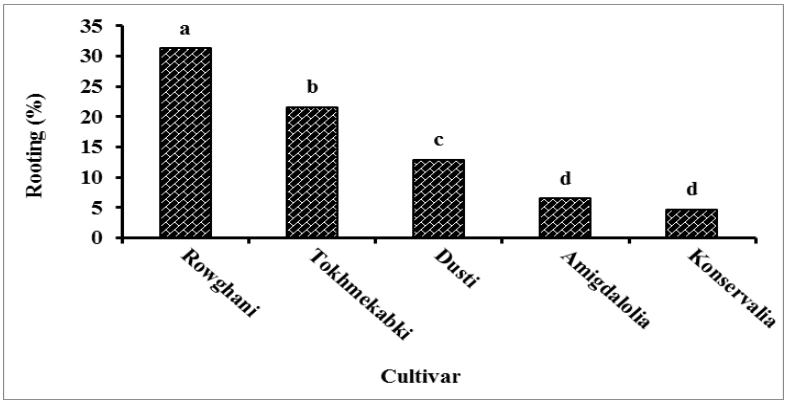

Fig. 1. Rooting percentage of cuttings for five olive cultivars. Columns followed by the same letters do not differ significantly at $P \leq 0.05$ using LSD test.

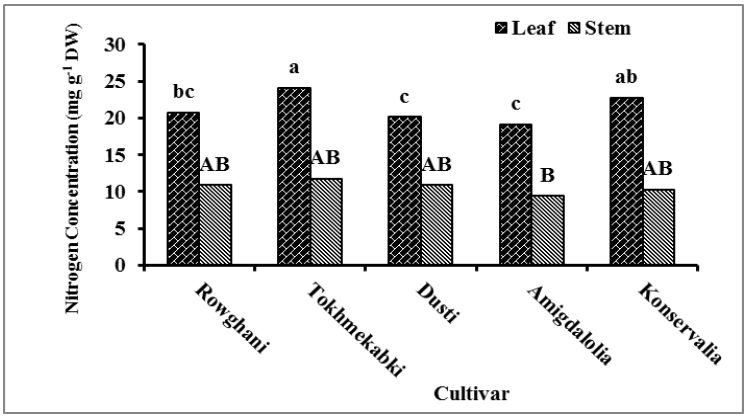

Fig. 2. Leaf and stem nitrogen contents of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at $P \leq 0.05$ using LSD test.

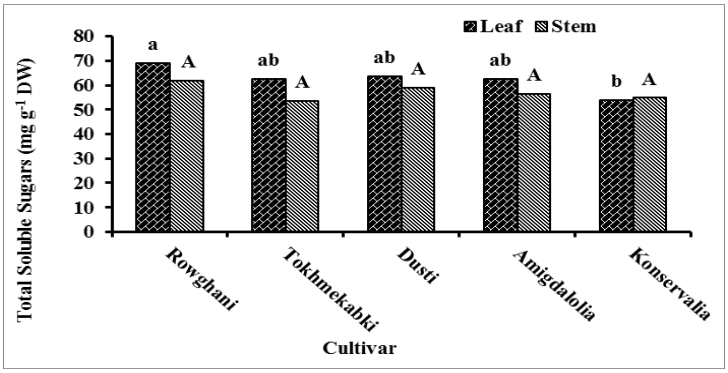

Fig. 3. Leaf and stem total soluble sugars contents of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at *P*≤0.05 using LSD test.

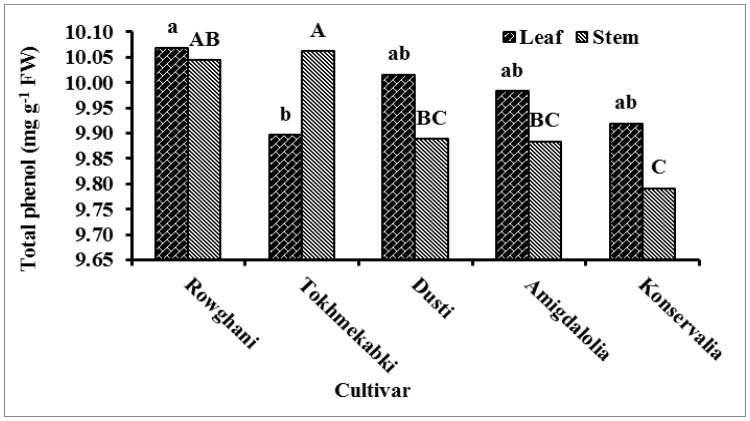

Fig. 4. Leaf and stem total phenol contents of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at *P*≤0.05 using LSD test.

Same amounts of caffeic acid, narengin and catechin were found in the stem cuttings of the cultivars. Highest and lowest leaf caffeic acid contents were found in 'Rowghani' and 'Amigdalolia' cuttings respectively, however, the leaf caffeic acid contents were not related to the rooting ability of the cuttings (Figs. 1 and 5). No differences were found in chlorogenic acid content in the leaf of the cuttings for the five studied cultivars (Fig. 6). Catechin was detected at relatively low concentrations (2.0 mg g^{-1} FW in 'Tokhmekabki and 1.46 mg g^{-1} FW in 'Rowghani') and lower concentrations in the other cultivars (Fig. 11). A significant negative relationship was found between the rooting potential and leaf narengine concentrations among cultivar's cuttings (Figs. 1 and 7). The same relationship was observed for galic acid contents in the leaves (Fig. 8). The highest concentration of vanillic acid was found in the leaves of 'Rowghani' cuttings, its concentrationwas slightly decreased in 'Tokhmekabki' and 'Dusti' and the lowest vanillic acid concentration was found in 'Amigdalolia' and 'Konservalia' cultivars (Fig. 9). Although C/N ratio in the leaf and stem of the cuttings was statistically different among cultivars but have no clear pattern and therefore seems that it had no relation with rooting potential of the cuttings (Fig. 10).

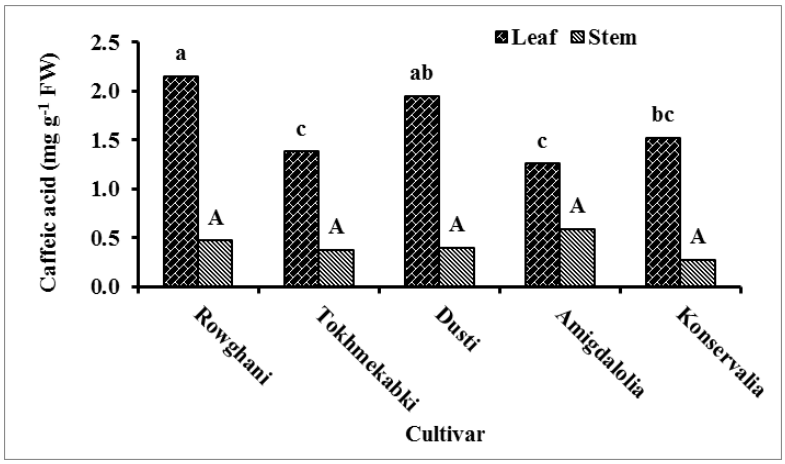

Fig. 5. Leaf and stem caffeic acid contents of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at $P \leq 0.05$ using LSD test.

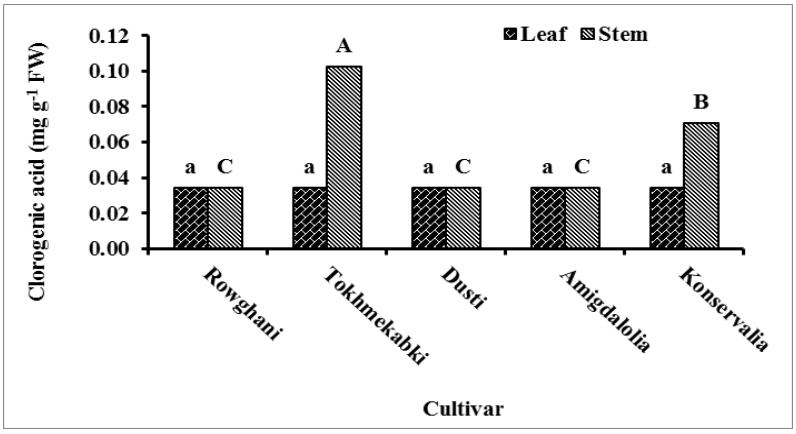

Fig. 6. Leaf and stem chlorogenic acid contents of five olive cultivars. Columns Followed by the same capital or small letters do not differ significantly at $P \leq 0.05$ using LSD test.

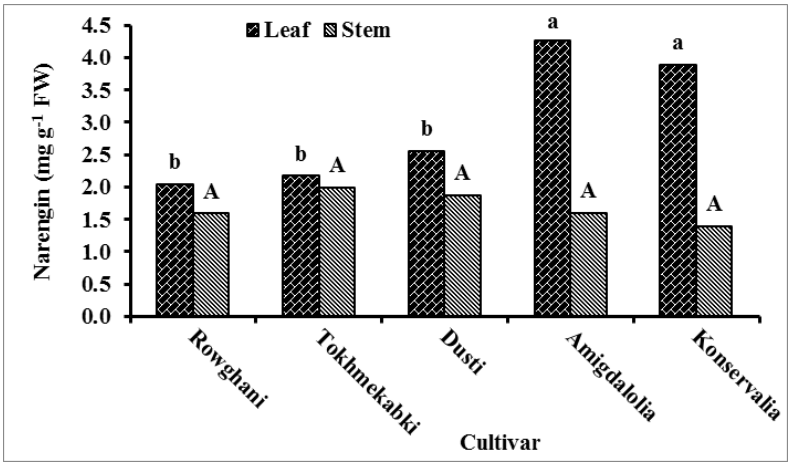

Fig. 7. Leaf and stem narengin contents of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at P≤0.05 using LSD test.

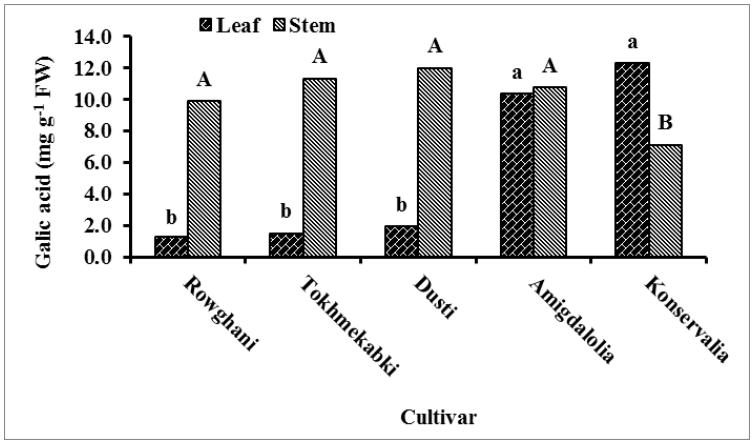

Fig. 8. Leaf and stem gallic acid contents of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at $P \leq 0.05$ using LSD test.

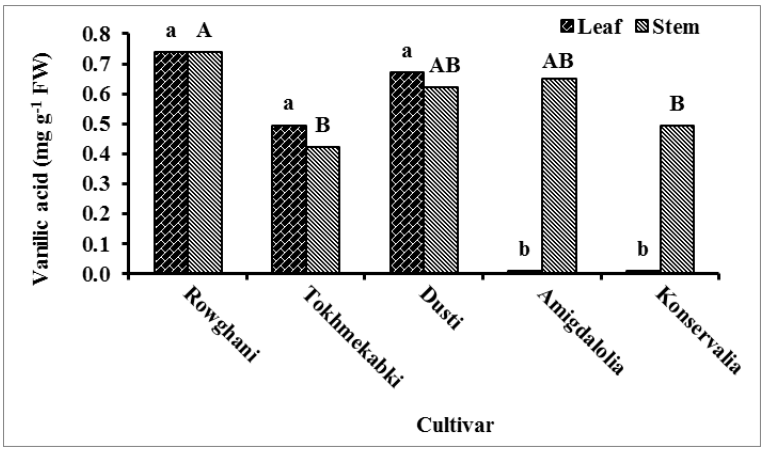

Fig. 9. Leaf and stem vanillic acid contents of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at $P \leq 0.05$ using LSD test.

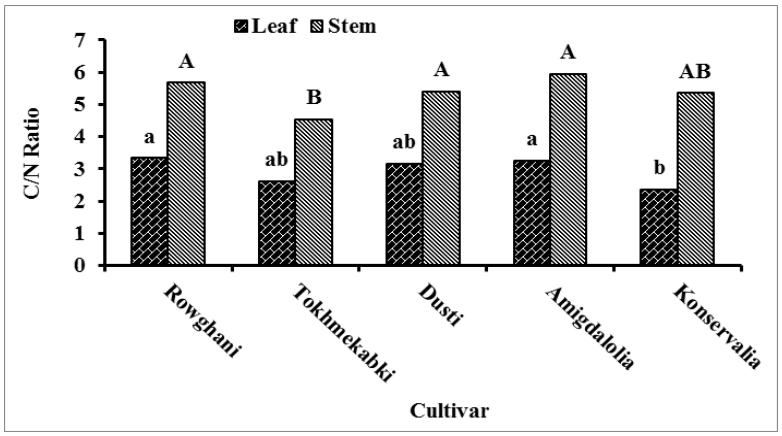

Fig. 10. Leaf and stem C/N ratio of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at $P \leq 0.05$ using LSD test.

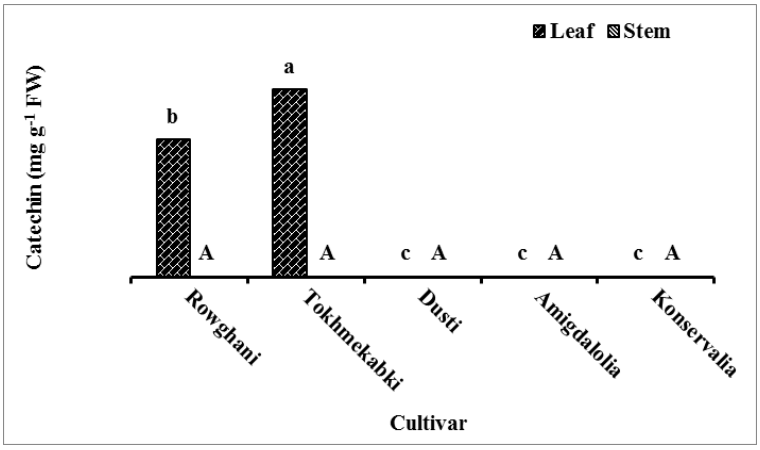

Fig. 11. Leaf and stem catechin contents of five olive cultivars. Columns followed by the same capital or small letters do not differ significantly at $P \leq 0.05$ using LSD test.

Results of regression analysis by stepwise method for leaf and stem variables and also their total (leaf and stem with together) are shown in Tables 1, 2 and 3 respectively. Data in Tables 1, 2 and 3 show intercept, probably values (Pr) and parameters estimated from these variables. Based on this method, variables entered into the model (step by step) and ultimately selected the variables having the most effect on the dependent variable (rooting percentage). The correlation coefficients (R^2) of the equation were 0.99, 0.76 and 0.69 for leaf, stem and their total (leaf and stem with together), respectively.

Table 1. Stepwise regression analysis of leaf variables.

Parameters	Parameters estimated	Probably values
Intercept	43.17	0.011
Gallic acid	-0.089	0.0148
Vanillic acid	-2.106	0.0076
Narengin	0.715	0.0046
Caffeic acid	-3.94	0.0123

Table 2. Stepwise regression analysis of stem variables

Parameters	Parameters estimated	Probably values
Intercept	5.718	0.016
Gallic acid	-0.178	0.023

Table 3. Stepwise regression analysis of total (leaf+ stem) variables.

Parameters	Parameters estimated	Probably values
Intercept	2.834	<0.0001
Gallic acid	-0.078	0.0023
Carbohydrates	-0.023	0.0378

Discussion

The effects of tissue nitrogen contents on root initiation and development in stem cuttings depend on several factors such as carbohydrate availability, C/N ratio and interactions between endogenous hormones. It is believed that the function of nitrogen in root formation in cuttings is related to its role in the synthesis of nucleic acids and proteins. On the other hand, nitrogen redistribution in stem cuttings has been reported to be different among different cultivars during the rooting process (Hartman *et al*., 2001). Results of current study indicated that rooting capacity of olive cultivars was not related to nitrogen contents of their leaves or stems. Since, no differences were found among five olive cultivars for N content of their stem and no specified relation was found between changes in leaf N contents and rooting percentage of the cuttings (Figs. 1 and 2). The idea which rulled out the involvement of nitrogen contents in rooting ability can be further supported.Nitrogen is needed for the synthesis of different nitrogenous compounds, but the promotive effects of nitrogen in advantious root formation may also be considered when it has a close influence on carbohydrate contents, allocation, partitioning and metabolism (Porfírio *et al*., 2016; Dag *et al*., 2012; Hambrick *et al*., 1991; Druege *et al*., 2000; Scheible *et al*., 1997; Blazich, 1988).

It is well known that mobilization and translocation of carbohydrates in the olive stem cuttings generally have a positive effect on rooting ability of the cuttings (Aslmoshtaghi and Shahsavar, 2010; Denaxa *et al*., 2012). However, results obtained in the present study indicated that the rooting ability of the cuttings was not related to their total soluble sugar contents. These findings are in agreement with those reported by Tsipouridis *et al*. (2006). The absence of relationship between total sugares and rooting ability of the cuttings suggests that other biochemical, physiological and anatomical factors and/or the amounts of endogenous auxins and rooting co-factors might also be involved in adventious root formation in stem cuttings (Fouad *et al*. 1989; Denaxa *et al*. 2010; Denaxa *et al*., 2012; Del-Rio *et al*., 1991). Although many reserchers have reported that exogenous auxin application to the bases of the cuttings can increase carbohydrate and soluble sugare concentretion and hydrolizing enzyme on the cutting sites, the special role of soluble sugars in rooting has not been clarified yet (Ragonezi *et al*., 2010).

Although C/N ratio is important at the time of taking cutting from mother plants and their rooting ability, in this study the (soluble carbohydrate)/N ratio was not significantly related to rooting ability of the cuttings (Fig. 10). In agreement with our results, Hambrick *et al*. (1991) reported that in *Rosa multiflora* cuttings, starch/N and (total carbohydrate)/N ratios were more closley correlated with rooting than (soluble carbohydrates)/N.

Due to roles of carbohydrates as the source of energy and their requirements for macromolecules synthesis during the root formation process, some studies have focused on the changes of carbohydrates contents during rhizogenesis of woody cuttings (Denaxa *et al*., 2012; Murai *et al*., 1999; Pellicer *et al*., 2000; Welander, 1994). Hartman *et al*. (2001) described changes in carbohydrate levels during root initiation and development. They hypothesized that starch hydrolysis during rhizogenesis and release of soluble sugars in stem tissue can be used for root development. In our study, the time of cutting preparation (spring) did not seem to be a good time and does not coincide with the starch hydrolysis process in the studied olive cultivars. Usually, starch is hydrolyzed at the end of winter and the resulting sugars are consumed by vegetative growth whereas in the summer these soluble sugars are provided by photosynthesis. These findings are in agreement with the results of Denaxa *et al*. (2012). They found that the maximum rooting percentage of ‘Arbequina’ was

achieved in summer (76%) while the lowest rooting percentage was observed in spring (37%). In their study, the highest rooting percentage of the cuttings in summer coincided with the highest initial soluble sugars and the lowest starch concentrations in their tissues.

The rooting potential of the five studied olive cultivars in our study have positive correlations with stem total phenol, catechin and vanillic acid concentrations but have negative correlation with leaf narengin and gallic acid contents (Figs. 7 and 8). Other phenolic compounds had no effects on the rhizogenesis of the cuttings for the studied cultivars. Previous studies have also shown that several phenolic compounds induce the effects of auxin on rooting by inhibiting the deactivation of IAA by IAA-oxidase (Faust, 1989; Haissig, 1986; Padney and Pathak, 1981; Wilson and van Staden, 1990) or by IAA decarboxylation (Wilson and van Staden, 1990). However, some of these compounds have inhibitory effects on root formation through inducing IAA oxidation or decarboxylation (De Klerk *et al*., 2011) or through acting as precursors for lignin formation (Haissig, 1986; Liu *et al*., 1996).

On the other hand, several phenolic compounds have no regulatory effects on the auxin content in plant tissues and as a result have no influence on root formation. Our study revealed that the individual phenolic compounds might have stimulatory or inhibitory effects on root formation. Fogaca and Fett-Neto (2005) investigated the role of auxin and its modulators in the adventitious root formation of two Eucalyptus species and found that tannic acid and phloroglucinal had no significant effects on the rooting response of the two Eucalyptus species. Our study revealed that individual phenolic compounds might have stimulatory or inhibitory effects on root formation. Positively correlation was found between total phenol content of stem and rooting ability. This indicates that the balance between individual phenolic compounds and other endogenous compounds (such as auxins) is more important than their absolute concentrations alone. It has been reported that meta- and ortho- diphenols and polyphenols can inhibitauxin decarboxylation and as a result can promote rooting of cuttings but monophenols usually act as co-factors of IAA-oxidase and enhance IAA decarboxylation, consequently inhibit root formation (Bandurski *et al*., 1995; De Klerk *et al*., 2011).

In conclusion, it is hard to determine the involvement of total phenolic contents or individual phenolic compounds in root formation process of olive cuttings because they can fully mask the effect of each other. However, results of stepwise regression analysis suggested that leaf and stem gallic acid content in all cultivars had the highest negative correlation with the rooting ability of cuttings. Such negative correlations were found with lower intensities for leaf vanillic acid, narengin and caffeic acid contents.

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