The Effect of IBA and Putrescine on Rhizogenesis and Biochemical Changes in Cuttings of *Olea europaea* L. cv. 'Konservalia'

Esmaeil KHALEGHI (✉)
Sara ALAVIPOUR

Summary

This study aimed to investigate the effect of IBA at different concentrations (0, 2000 and 4000 ppm) and putrescine (0, 100 and 200 ppm) on rhizogenesis and the biochemical changes of leafy hardwood cuttings of *Olea europaea* L. This experiment was conducted as a factorial in a complete randomized block design with three replications. The result showed that rhizogenesis was the highest and callus induction was the lowest in the treatment with 4000 ppm IBA. Furthermore, co-treatment with IBA and putrescine increased rhizogenesis and callus induction two-times over their individual treatments. Moreover, biochemical changes of protein, carbohydrate (C), nitrogen (N) percentage, C/N ratio, peroxidase activity and polyphenol oxidase showed significantly different results in two parts of the leaf (bud) and stem bark of the cutting (root and callus). Overall, based on the results, it can be concluded that IBA application at 4000 ppm can be recommended as a suitable treatment for rhizogenesis of hard-to-root 'Konservalia' cultivar.

Key words

carbohydrate, cutting, nitrogen, peroxidase, polyphenol oxidase, rhizogenesis

Department of Horticultural Science, Faculty of Agriculture, Shahid Chamran University of Ahvaz, Ahvaz, Iran

✉ Corresponding author: khaleghi@scu.ac.ir

Received: August 17, 2023 | Accepted: March 26, 2024 | Online first version published: May 1, 2024

Introduction

Olive (*Olea europaea* L.) is one of the most important fruit trees that originated from the Mediterranean area. This tree is important in terms of oil production, therapeutic properties and the food industry (Iran-nejad et al., 2010). Asexual and sexual propagation methods are typically used for olive propagation (Khoshkhoi, 2007). Rhizogenesis in olive cuttings is difficult because there is not enough endogenous auxin in these organs. In other words, they lack the required minimum level of auxin for proper rhizogenesis (Turkoglu and. Durmus, 2005).

Consequently, without hormone treatment, most olive cultivars produce few roots. The 'Konservalia' cultivar is one of the olive cultivars that are difficult to root (Hosseini, et al., 2008). Therefore, they need growth regulators to generate adventitious roots (Denaxa, et al., 2012). There is evidence that plant growth regulators such as auxin have been applied to increase the rhizogenesis of hard-to-root woody plants (Hartmann et al., 2002). Plant growth and organogenesis are greatly affected by auxins, which mainly contribute to regulating cell elongation, cell division and differentiation and extending callus production as well (Akiyoshi et al., 1983; Dietz et al., 1990). Indole-3-butyric acid (IBA), applied to different kinds of hard-to-root plants, is one of the best and most prevalent rhizogenesis agents. Since this agent has a weak auxin effect and is degraded too slowly by the degrading enzymes, it has a long-term effect on rhizogenesis (Copes and Mandel., 2000; Henrique, et al., 2006). Attarzadeh et al. (2015) investigated the effect of different hormone treatments and rhizogenesis-promoting agents on the rhizogenesis of two olive cuttings cultivars (Shiraz and Feishami). They applied IBA hormone (2000, 4000, and 6000 mg L^{-1}), NAA at the concentration of 2000 mg L^{-1}, 2% ascorbic acid and 3.5% peroxide hydrogen. The results presented that 4000 mg L^{-1} IBA for 5 seconds with 3.5% peroxide hydrogen for 30 seconds treatment caused the highest rhizogenesis in olive cuttings after 110 days. Further, it was observed that polyamines were a new class of growth regulators playing major roles in physiological processes such as embryogenesis, cell division, growth and root formation (Lee et al., 2009).

Recently, it has been noted that these compounds can enhance root production and development in hard-to-root plants, and putrescine has shown better reactions compared to other polyamines (Wu et al., 2010). It has been shown for the first time that polyamines can affect rhizogenesis when endogenous polyamine amounts are inadequate (Rugini et al., 1997). This phenomenon can be attributed to the cutting terminals' enhanced peroxidase activity (Rugini, et al., 1990). Investigating the effect of different putrescine concentrations (2 and 4 m mol) and IBA (1500 and 3000 mg L^{-1}) on the rhizogenesis of GF677 hybrid cuttings. Karimi and Yadollahi (2012) observed that IBA with 1500 mg L^{-1} led to improved rhizogenesis, while 3000 mg L^{-1} of IBA had toxic effects on rhizogenesis and cuttings shoots. In addition, the highest root and branch number was propagated in the cutting treated with putresine. They concluded that IBA could be replaced with putrescine to improve rhizogenesis in GF677 hybrid cuttings. It can be clarified that the external application of IBA, polyamines (putrescine) and peroxide hydrogen is effective in accelerating rhizogenesis (Rugini et al., 1990, 1997). Similarly, Wiesman, and Lavee, (1995) identified the positive effects of IBA+ Urea phosphate (UP) and Paclobutrazol on root induction and residues of rooted cuttings compared to IBA treatment. According to Rugini et al. (1990), putrescine with IBA increased rhizogenesis percentage and induced early rhizogenesis and could be presented as promising compound.

There are molecular and biochemical incidents at the root induction stage without visible changes. It is possible to recognize the starting stage by the cell division and the primordium organization in the root (Sebastiani et al., 2002). Several factors influence lateral rhizogenesis, including genotype, mother plants, cutting type, collection date and cutting-related endogenous factors (Tworkoski and Takeda, 2007). The variations of phenolic compounds, sugar and protein that affect root formation have been studied extensively in lateral roots (Kevresan et al., 2007; Satisha et al., 2008).

Early studies documented that the enzymatic activity of polyphenol oxidase and peroxidase enzymes during the rhizogenesis process could be changed (Ludwig-Muller, 2003; Yilmaz et al., 2003).

In organogenesis, polyphenol oxidase contributes to phenol oxidation, providing energy for cell division and differentiation. Peroxidase is a marker of inducing phases and initial stages of root formation in plants (Yilmaz et al., 2003; Kevresan et al., 2007). Zhang et al. (2013) found that physiological parameters such as soluble protein, peroxidase activity, soluble sugar and starch, sugar-to-nitrogen ratio, carbohydrate-to-nitrogen ratio (C/N), IAA level, and IAA to ABA ratio significantly influenced the rhizogenesis of *Chrysanthemum* sp. cuttings. This study was conducted to investigate the effect of different concentrations of IBA (0, 2000 and 4000 ppm) and putrescine (0, 100 and 200 ppm) on the rhizogenesis, callus induction, and biochemical changes in leaf and bark of hardwood cuttings of olive 'Konservalia' cultivar.

Material and Methods

Sample Preparation

'Konservalia' cultivars were prepared from the olive orchard collection of the Horticultural Department, Faculty of Agriculture, Shahid Chamran University of Ahvaz (31° 20' N, 48° 40' E, 22 m above sea level). In early February 2017, ten leafy and healthy cuttings were prepared with equal lengths and diameters of 15 cm for each treatment. The experimental treatments included indole butyric acid (0, 2000, 4000 ppm) and putrescine (0, 100, and 200 ppm).

First, some of the cuttings were immersed in different concentrations of IBA for 30 seconds. Then, some of the cuttings were also treated with different concentrations of putrescine using a micro-sprayer twice with an interval of 24 hours at 8 am and for one minute. Furthermore, some of the cuttings were treated with different concentrations of IBA and putrescine. Distilled water was used as control treatment. After treating the cuttings, they were cultivated in cocopeat and perlite culture medium and were kept for 90 days in the research greenhouse of Shahid Chamran University of Ahvaz under moisture conditions (temperature of 30/25 degrees Celsius and a relative humidity above 80%).

Various indices such as peroxidase enzyme activity and polyphenol oxidase, the content of carbohydrates, proteins and nitrogen in cuttings' stem bark and leaf were measured every 30 days after treatment. At the end of the experiment, callus induction and rhizogenesis were also evaluated.

Chemical Analyses

Carbohydrate

To calculate total soluble sugars, the method by Irrigoyen et al. (1992) was used. The prepared alcoholic extract (100 µL) was poured into sealed 15 mL tubes, and 3 mL of fresh anthrone reagent was added. Then, the tubes were incubated in a water bath for 10 seconds. After cooling, the absorbance of samples (and standards) was read in 625 nm wavelength by UV-VIS spectrophotometer.

Nitrogen

To calculate total nitrogen, the Kjeldahl method was applied (Bradstreet, 1954). Dried leaf and stem skin samples of olive cuttings in each treatment were weighed to 1.0 g and used as samples to measure total nitrogen. The Kjeldahl method consists of three steps: digestion, distillation and titration. Thus, the digestion of the samples was done with sulphuric acid, the distillation of the digestion solution was done with steam and finally the titration of the distillate and calculation of the result were performed.

Protein

To isolate the crude extract, the method presented by Sofo et al. (2004) was used. The purified cold methanol (10 mL) was added to 1 g of the frozen leaf containing liquid nitrogen. The remaining suspension was centrifuged at 0 °C and 10000 rpm for 10 minutes after 30 minutes and the supernatant was extracted. In the second step, 10 mL of purified cold methanol were added to the platelets for the second time and centrifugation was performed again after 30 minutes. After 3 minutes, the remaining platelets were suspended in 5 mL of extraction buffer (containing 50 mM of sodium phosphate buffer and 0.1% PVPP (W/V) at pH = 7) and centrifuged at 4 °C at 10000 g rpm for 10 minutes. Finally, the supernatant was removed for enzymatic analysis. Furthermore, the total soluble protein content was determined based on Bradford, (1979).

Peroxidase Enzyme

The peroxidase enzyme activity was measured by Hemeda, and Kelin (1990). 3 ml of reaction solution: 50 mM of sodium-potassium phosphate (pH = 6.6), 1% guaiacol, 0.3% peroxide hydrogen, and 600 µl of crude extract. Peroxidase enzyme activity was determined based on guaiacol oxidation at 470 nm wavelength and 26.6 mM^{-1} cm^{-1} as the quenching coefficient.

Polyphenol Oxidase Enzyme

Polyphenol oxidase enzyme activity was assayed based on Kar and Mishra (1976). 3 ml of the reaction solution, 25 m of sodium potassium phosphate (pH = 6.8), 10 m of Pyrogallol, and 500 µL of crude extract. Enzyme activity was expressed as a quenching coefficient based on the change in color intensity produced by purpurogallin at 420 nm wavelength and 12 mM^{-1} cm^{-1}.

Statistical Analysis

An analysis of variance was performed based on the Kolmogorov-Smirnov test after normalizing all recorded characteristics using SAS 9.1 software. The percentages of callus induction and rhizogenesis reported in the final experiment were calculated based on complete randomized block design and biochemical properties recorded during time based on factorial test by repeated measuring with three replications. Duncan's multiple range test was applied at a 5% significant level to compare the means.

Results and Discussion

The results revealed that the highest rhizogenesis percentage and the lowest callus induction were recorded in cuttings treated with 4000 ppm IBA. Furthermore, spraying with different concentrations of putrescine and submersion in different IBA concentrations, individually and simultaneously, led to enhanced root and callus formation in the studied cutting compared with the control where only 20% of the cuttings produced callus after 90 days. Still, the cuttings treated with 4000 ppm IBA achieved 80% rhisogenesis and those treated with 4000 ppm IBA plus 100 ppm putrescine, achieved 100 % rhizogenesis, respectively. In addition, rhizogenesis and callus induction of 40% were observed in cuttings treated with 4000 ppm IBA and 200 ppm putrescine (Table 1). Furthermore, the results exhibited that the simultaneous application of IBA and putrescine doubled the total sum of rhizogenesis and callus induction of cuttings compared with the individual treatment with putrescine (Table 1).

Hartman and Kaster (1975) claim that callus formation and rhizogenesis are frequently independent. The formation of calluses is recognized, however, as the precursor of lateral roots in some species.

Table 1. Treatment effect on some rooting and callus percentage of olive (*Olea europaea* L. cv. 'Konservalia') cuttings

Treatment	Rooting (%)	Callus (%)
Control	0 ±0 ^{e}	20 ± 5.98 ^{c}
IBA 2000 mg/l	20 ± 5.77 ^{d}	20 ± 5.69 ^{c}
IBA 4000 mg/l	100 ± 0 ^{a}	0 ± 0 ^{d}
Put. 100 mg/l	0 ± 0 ^{e}	40 ± 5.32 ^{b}
Put. 200 mg/l	20 ± 5.78 ^{d}	20 ± 5.36 ^{c}
IBA 2000 mg/l + Put. 100 mg/l	40 ± 5.12 ^{c}	40 ± 5.68 ^{b}
IBA 2000 mg/l + Put. 200 mg/l	40 ± 5.45 ^{c}	40 ± 5.98 ^{b}
IBA 4000 mg/l + Put. 100 mg/l	80 ± 5.69 ^{b}	0 ± 0 ^{d}
IBA 4000 mg/l + Put. 200 mg/l	40 ± 5.23 ^{c}	60 ± 5.79 ^{a}

Note: The same letters within a column represent a significant difference at $P < 0.05$ level

Further experiments showed that rhizogenesis was enhanced when semi-hardwood cuttings of Frantoio and Gentile di Marino cultivars of olive were treated with IBA at 4000 ppm and hydrogen peroxide at 4000 ppm (Sebastiani and Tognetti, 2004). Similarly, Lazaj et al. (2015) applied different concentrations of 2000, 4000 and 6000 ppm to improve the rhizogenesis of the olive 'Kalinjot' cultivar. They observed that 4000 ppm IBA led to the highest rate of rhizogenesis, number of the root, length, and fresh and dried weight of root compared with others. It seems that successful rhizogenesis is not only evaluated by rhizogenesis percentage but also by the number of propagated roots (Hartmann et al., 2002).

In the early stages of rhizogenesis, Rugini et al. (1997) observed a negligible but significant increase in the root number of olive explants treated with putrescine (putrescine degradation by Δ- Pyrroline pathway, which is associated with H_2O_2 production). External application of IBA was effective on internal IAA concentration. While external IBA may decrease IAA biosynthesis, it is more likely that external IBA changes directly into IAA. In addition, a part of the constant IAA peak can be attributed to the conversion of IAAsp to IAA (Epstein and Ludwig-Muller. 1993). There was, however, a difference in the concentration variation of IAAsp between species during the root induction phase (Heloir et al., 1996). Similarly, Aslmoshtaghi and Shahsavar (2010) studied rhizogenesis of hard-to-root cuttings of olive cultivars 'Tokhmkabki' and 'Roghani' over a period of 4 months and found that putrescine could positively influence rhizogenesis percentage and root quality.

The results of table 2 showed that interaction between time × IBA× putrescine × sampling had a significant effect on carbohydrates at the level of 1%.

Table 2. Repeated measure analysis on chlorophyll content, total soluble carbohydrate and proline of 'Konservalia' olive cuttings

Source of variation	Degree of freedom	Mean of squares					
		Carbohydrate (C)	Nitrogen (N)	C/N	Protein	Proxidase	PPO
Within- Subjects Effects							
Time	2	402.614**	0.510**	2242.686*	136.267	0.0003	0.0222
Time × Block	4	3.194	0.008	444.547	181.719	0.0007	0.0061
Time × IBA	4	591.245**	0.298**	900.146	185.173	0.0027*	0.0221*
Time × Putrescine	4	47.484**	0.378**	223.742	400.718**	0.0037**	0.0076
Time × Sampling	2	655.911**	0.024*	1073.909	385.506	0.0031*	0.0171
Time × IBA × Putrescine	8	527.374**	0.217**	290.576	249.661	0.0025**	0.0104
Time × IBA × Sampling	4	271.408**	0.156**	683.913	476.806**	0.0014	0.0063
Time × Putrescine × Sampling	4	332.497**	0.354**	982.231	276.925	0.0027*	0.0070
Time×IBA × Putrescine × Sampling	8	357.222**	0.263**	869.494	209.949	0.0021*	0.0116
Error (Time)	68	7.515	0.006	478.266	130.514	0.0008	0.0068
Between- Subjects Effects							
Block	2	48.177**	0.009	518.468	147.365	0.0038**	0.0051
IBA	2	400.895**	0.114**	920.216	33.999	0.0042**	0.0197
Putrescine	2	410.665**	0.284**	423.660	94.294	0.0066**	0.0100
Sampling	1	1294.183**	28.288**	17401.280**	1252.256**	0.0018	0.0159
IBA × Putrescine	4	795.960**	0.131**	965.964	273.555	0.0010	0.0076
IBA × Sampling	2	17.329	0.061**	553.590	597.613*	0.0025*	0.0046
Putrescine × Sampling	2	407.484**	0.461**	1183.421	655.763*	0.0031*	0.0251*
IBA × Putrescine × Sampling	4	1323.858**	0.395**	2115.524**	157.319	0.0006	0.0074
Error	34	7.322	0.006	460.137	134.129	0.0007	0.0070

Note: * and ** indicate statistical significance at the $P < 0.05$ and 0.01 level, respectively

The carbohydrate content in the cuttings' leaves (54.01 mg g^{-1}) was higher than in stem bark (49.07 mg g^{-1}) at the beginning of the experiment. Over time, there have been significant changes in the carbohydrate content of the leaves and stem bark (Table 3). In the control cuttings, the carbohydrate content of the leaves decreased after 60 days of cultivation. However, after 90 days, this parameter in the leaf increased significantly again. In contrast, the trend of carbohydrate change in bark was different. Accordingly, the carbohydrate content of the stem bark was increased after 30 days of cuttings culture and then decreased until the end of the experiment. In the control treatment at the end of the experiment, this parameter was 69.10 mg g^{-1} in the leaf, whereas it was 50.39 mg g^{-1} in the bark. The carbohydrate content in leafy cuttings treated with 2000 ppm IBA decreased after 60 days of the experiment when putrescine was applied at 100 and 200 ppm concentrations, but there was an increase in carbohydrate content after 90 days at the same treatment. In contrast, putrescine treatment (100 and 200 ppm) increased carbohydrate content in the leaf of treated cuttings with 4000 ppm IBA after 60 days of cutting cultivation and then reduced this parameter after 90 days. Moreover, after 30 to 90 days of cuttings cultivation, the carbohydrate content of the bark was almost constant. When cuttings were treated with 2000 ppm IBA and putrescine, the carbohydrate content of the bark decreased compared to the 30th day on the 60th and 90th days after cultivation, while this trait increased in cuttings treated with 100 ppm putrescine (Table 3).

Results showed that 100 ppm putrescine could lead to increased carbohydrate content in the leaves exposed to 2000 ppm IBA compared with cuttings with no putrescine treatment. Further, applying 100 and 200 ppm putrescine enhanced carbohydrate content in the leaves treated with 4000 ppm IBA. Furthermore, in the cuttings treated with 2000 and 4000 ppm IBA, putrescine application (100 and 200 ppm) enhanced carbohydrate content in the bark (Table 4).

Carbohydrates play an important role in the plant as a source of energy and are mainly involved in the process of rhizogenesis (Druege, et al., 2000). Leaves and seedlings are the main organs for photosynthesis and carbohydrate production, which can affect the function of rhizogenesis of cuttings by different auxin levels and carbohydrate resources (Porfirio et al., 2016 a).

Table 3. Effect of time × Sampling × IBA × Putrescine on carbohydrate (mg g^{-1} F.W.) of olive (*Olea europaea* L. cv.'Konservalia') cuttings

Sampling	IBA (ppm)	Putrescine (ppm)	Days after planting			
			0	30	60	90
Leaf	0	0	54.01±1.82 $^{g-j}$	55.150±3.050 ^{ghi}	50.850±0.440 $^{i-m}$	69.100±3.620 ^{bc}
		100		54.730±1.110 ^{ghi}	75.676±4.210 ^{a}	56.452±1.055 ^{fgh}
		200		35.865±0.660 $^{t-w}$	51.680±0.501 $^{h-k}$	22.381±0.524 ^{xy}
	2000	0		36.923±2.807 $^{s-v}$	46.476±1.051 $^{l-p}$	71.013±0.966 ^{b}
		100		58.195±4.342 ^{efg}	54.615±0.712 ^{ghi}	60.335±2.293 ^{def}
		200		37.987±0.591 ^{rst}	31.017±0.409 ^{w}	58.430±0.854 ^{efg}
	4000	0		21.036±1.120 ^{y}	49.426±0.601 $^{j-m}$	32.316±1.093 ^{vw}
		100		34.767±0.229 $^{t-w}$	56.092±1.049 ^{fgh}	48.782±0.493 $^{k-o}$
		200		56.093±0.586 ^{fgh}	67.664±1.299 ^{bc}	49.464±0.523 $^{j-m}$
Stem bark	0	0	49.07±1.55 $^{j-n}$	55.960±0.60 ^{fgh}	51.300±0.910 $^{h-l}$	50.350±2.650 $^{i-m}$
		100		15.990±0.290 ^{z}	36.699±0.568 $^{t-v}$	57.096±3.478 ^{fg}
		200		64.823±1.822 ^{cd}	43.896±0.213 ^{opq}	41.225±1.029 ^{qrs}
	2000	0		37.115±0.336 $^{r-v}$	26.074±0.483 ^{x}	37.608±1.036 $^{r-v}$
		100		57.059±1.696 ^{fg}	45.865±0.787 $^{m-q}$	48.574±1.116 $^{k-o}$
		200		37.437±0.710 $^{r-v}$	44.141±0.688 $^{n-q}$	62.380±3.362 ^{de}
	4000	0		44.047±0.583 $^{n-q}$	41.112±1.184 ^{qrs}	33.916±0.440 $^{t-w}$
		100		55.941±0.849 ^{fgh}	42.115±0.689 ^{qpr}	42.058±3.025 ^{qpr}
		200		32.486±0.812 ^{uvw}	37.077±0.460 $^{r-v}$	47.532±1.430 $^{k-o}$

Note: The same letters represent a significant difference at $P < 0.05$ level

Table 4. Effect of interaction sampling × IBA × putrescine on carbohydrate, nitrogen and C/N of olive (*Olea europaea* L. cv.'Konservalia') cuttings

Sampling	IBA (ppm)	Putrescine (ppm)	Carbohydrate (mg/g F.W)	Nitrogen (%)	Carbohydrate/Nitrogen (C/N)
Leaf	0	0	58.368±1.973 ^{b}	2.212±0.0006 ^{a}	29.117±1.035 ^{h}
		100	62.285±1.457 ^{a}	1.680±0.0014 ^{h}	37.063±0.722 ^{e}
		200	36.642±0.415 ^{h}	1.829±0.0004 ^{e}	19.865±0.231 ^{k}
	2000	0	51.471±0.378 ^{c}	1.944±0.0052 ^{b}	29.760±0.231 ^{h}
		100	57.715±2.130 ^{b}	1.468±0.0013 i	39.999±1.327 ^{d}
		200	42.478±0.139 ^{e}	1.891±0.0134 ^{c}	23.104±0.179 ^{j}
	4000	0	34.259±0.697 ^{i}	1.750±0.004 ^{g}	19.043±0.424 ^{k}
		100	46.547±0.360 ^{d}	1.786±0.0028 ^{f}	26.457±0.207 ^{i}
		200	57.740±0.635 ^{b}	1.859±0.004 ^{d}	32.283±0.364 ^{g}
Stem bark	0	0	52.538±0.875 ^{c}	0.954±0.0008 ^{m}	55.322±1.033 ^{a}
		100	36.598±1.126 ^{h}	1.127±0.0006 ^{k}	34.284±1.203 ^{f}
		200	49.981±0.844 ^{d}	0.907±0.1126 ^{n}	55.105±3.267 ^{a}
	2000	0	33.599±0.402 ^{i}	0.788±0.001 ^{lm}	46.155±0.649 ^{c}
		100	50.499±0.989 ^{cd}	1.020±0.0012 ^{l}	49.724±0.982 ^{b}
		200	47.987±1.456 ^{d}	1.046±0.022 ^{l}	47.560±1.323 ^{c}
	4000	0	39.692±0.585 ^{f}	1.158±0.0017 ^{j}	34.515±0.507 ^{f}
		100	46.705±0.900 ^{d}	0.876±0.004 ^{lm}	55.889±0.784 ^{a}
		200	39.032±0.324 ^{g}	1.021±0.0074 ^{l}	38.997±0.469 ^{d}

Note: The same letters within a column represent a significant difference at $P < 0.05$ level

Olive cuttings of some cultivars also show that leaf presence influences root growth (De Oliveira et al., 2003). Bartolini, et al. (1996) present that the increased amount of carbohydrates may be attributed to the branch formation in cuttings and root growth. They claim that high carbohydrate content is crucial for the initiation and process of rhizogenesis.

During this process, carbohydrates provide energy and serve as necessary nutrition for new and developing cells. Hartmann et al. (2002) noted that starch hydrolysis during rhizogenesis and diffusion of soluble sugars in the bark tissues could be used for root growth. Generally, it is well characterized that the distribution and transfer of carbohydrates in the stem cuttings of olives have a great positive impact on the rhizogenesis of the cuttings (Denaxa et al., 2012). The results of total carbohydrate and rhizogenesis potential of cuttings also prove that other biochemical, physiological and anatomical, or endogenous auxins amount and cofactors also probably can be involved in lateral rhizogenesis of the cutting (Denaxa et al., 2012). Aslmoshtaghi and Shahsavar (2010) identified numerous differences in the rhizogenesis potential of cuttings in one species in an experiment to evaluate the correlation between monthly rhizogenesis changes and internal factors such as soluble sugar, starch content and the total amount of phenolic compounds in olive cuttings. The availability of carbohydrates and their distribution to the top (terminal part) of the cutting may be an important factor in the rhizogenesis potential of olive cuttings. Initially, the cuttings of 'Roghani' cultivar showed the highest concentrations of soluble sugars and starch, but after 60 days, the result was completely different.

Furthermore, their results showed that carbohydrates, fructose, galactose, and a high C/N ratio were directly related to high rhizogenesis in olive cuttings. Carbohydrate is an energy source for cell division during rhizogenesis (Reuveni and Raviv, 1981). According to Anderson et al. (2010), the variation in respiration rate is also consistent with the pattern of soluble sugars, nonstructural carbohydrates, and biomass accumulation in big roots, suggesting that roots save carbohydrates during respiration. In addition, pelargonium cuttings have been shown to grow new roots with lower levels of soluble sugars because of respiration (Druege and Kadner, 2008). The carbohydrate content of cuttings' leaves was higher than that of the stem bark in some experimental treatments as the final product of photosynthesis. This issue can be attributed to photosynthesis and

carbohydrate production in the leaf (Druege et al., 2000). There is a challenging relationship between carbohydrates and lateral root formation in cutting. On the other hand, some researchers believe that rhizogenesis potential is determined by the amount of carbohydrate in cuttings, whether they contain free sugars or stored carbohydrates for rhizogenesis as energetic compounds, as well as their cell structure, which is essential for permodium induction (Bartolini et al., 2008; Aslmoshtaghi et al., 2014). On the other hand, some researchers report no correlation between rhizogenesis and carbohydrate content in terminal parts of cutting (Cruz et al., 2003).

Nitrogen

According to the results, it was found that interaction between time × IBA× putrescine × sampling had a significant effect ($P < 0.01$) on the nitrogen content (Table 2). The highest percentage of nitrogen (2.212%) was observed in the leaf of untreated cuttings (control), while the lowest value (0.788%) was achieved in the stem bark of the cuttings treated with 4000 ppm IBA (Table 4). The leaf nitrogen content decreased in the cuttings treated with 2000 ppm IBA and untreated cuttings (0 ppm IBA) when 100 and 200 ppm putrescine were applied compared with no putrescine application.

Based on the Table (5), it was found that the nitrogen content in the leaf (1.73%) was higher than in the stem bark (1.596%) at the beginning of the experiment. The highest nitrogen percentage was observed in the cuttings' leaves of control (3.01%), 2000 ppm IBA+200 ppm putrescine (2.22%), and 4000 ppm IBA+ 200 ppm putrescine (2.19%) treatments after 30, 60, and 90 days of cultivation, respectively. It is noteworthy that the nitrogen content in the leaves of all treated cuttings was significantly higher than that of the stem bark (Table 5). These results exhibited that the nitrogen percentage in the leaf was higher than in the stem bark. By contrast, putrescine application with 100 and 200 ppm increased nitrogen percentage in the leaves treated with 4000 ppm IBA. Furthermore, 100 ppm putrescine in untreated cuttings (without IBA treatment) or treated cuttings with 2000 ppm IBA increased stem bark nitrogen content (Table 5).

Table 5. Effect of time × sampling × IBA × putrescine on nitrogen (%) of olive (*Olea europaea* L. cv.'Konservalia') cuttings

Sampling	IBA (ppm)	Putrescine (ppm)	Nitrogen			
			0	30	60	90
Leaf	0	0	1.730±0.0005 $^{i-l}$	3.014±0.0017 ^{a}	2.068±0.0017 ^{de}	1.554±0.0017 ^{mno}
		100		1.421±0.0014 ^{opq}	1.991±0.0014 ^{ef}	1.627±0.0014 $^{k-n}$
		200		1.970±0.0012 ^{efg}	1.844±0.0017 ^{ghi}	1.673±0.0014 $^{j-m}$
	2000	0		2.323±0.0025 ^{b}	2.133±0.0011 ^{cd}	1.376±0.0145 ^{pq}
		100		1.743±0.0011 ^{ijk}	1.223±0.0014 ^{rst}	1.437±0.0014 ^{opq}
		200		1.596±0.0011 ^{lmn}	2.229±0.0011 ^{bc}	1.850±0.0404 ^{ghi}
	4000	0		1.504±0.0023 ^{nop}	2.073±0.0014 ^{de}	1.673±0.0023 $^{j-m}$
		100		1.910±0.0115 ^{fgh}	1.637±0.0014 $^{k-n}$	1.812±0.0014 ^{hij}
		200		1.389±0.0012 ^{pq}	1.991±0.0014 ^{ef}	2.197±0.0014 ^{bcd}
Stem bark	0	0	1.596±0.0005 ^{lmn}	1.039±0.0008 $^{u-x}$	0.983±0.0024 $^{w-y}$	0.839±0.0008 ^{yz}
		100		1.141±0.0020 ^{tuv}	1.296±0.0012 ^{qrs}	0.943±0.0014 ^{xyz}
		200		0.901±0.0023 ^{xyz}	1.014±0.0008 $^{v-x}$	0.807±0.3351 ^{z}
	2000	0		0.717±0.001 ^{lz}	1.037±0.0011 $^{v-x}$	0.611±0.0011 ^{z}
		100		1.187±0.0011 st	0.985±0.0017 ^{wxy}	0.890±0.0008 ^{xyz}
		200		1.230±0.0068 ^{rst}	0.767±0.0017 ^{z}	1.140±0.0014 ^{tuv}
	4000	0		1.153±0.0025 ^{tu}	1.347±0.0014 ^{qr}	0.973±0.0014 ^{wxy}
		100		0.694±0.0017 ^{z}	0.932±0.0017 ^{xyz}	1.004±0.0020 $^{v-y}$
		200		1.115±0.0015 $^{t-w}$	1.037±0.0011 $^{v-x}$	0.912±0.0221 ^{xyz}

Note: The same letters represent a significant difference at $P < 0.05$ level

Druege et al. (2000) stated that the increased amount of nitrogen production decreased the starch level, while it increased the carbohydrate concentration in leaves of *Chrysanthemum* sp. cuttings. The effect of nitrogen tissue on the start and development of stem cuttings depends on numerous factors, such as carbohydrate availability, C/N ratio and the interaction among endogenous hormones. During rhizogenesis, nitrogen seems to play a role in the synthesis of nucleic acids and proteins.

On the other hand, the distribution of nitrogen during rhizogenesis in stem cuttings has been reported to differ among cultivars (Hartmann et al., 2002). Similarly, Izadi et al. (2016) found that rhizogenesis potential does not depend on nitrogen levels in leaves and stems. This element is also required for the synthesis of various nitrogenous compounds, but the positive effects of nitrogen on rhizogenesis can also be considered for carbohydrate content, allocation, distribution and metabolism (Porfirio et al., 2016 b; Dag et al., 2012). During the first 30 days of treatment with IBA and putrescine, the nitrogen content in the terminal part of the cuttings was reduced. After that, it increased, while the nitrogen content in the stem bark decreased significantly in the treated cuttings (IBA +Putrescine). This phenomenon can be explained by the distribution and transfer of nitrogen from the bark to the terminal part of the cutting and its subsequent consumption during rhizogenesis (Table 5).

Moreover, nitrogen assimilation to amino acids may explain the variation and increased percentage of nitrogen in leaves and seedlings in this experiment (Huber et al., 1996). Scagel (2004) found that *Rosa chinensis* Jacq. cuttings showed greater lateral rhizogenesis when nitrogen-containing compounds were present, rather than carbohydrates at the beginning. It is reported that the number and the length of roots of *Chrysanthemum* cutting (*Dendranthema* × *grandiflorum* (Ramat.) Kitamura) have a positive and significant correlation with the initial nitrogen content of cuttings (Druege et al., 2000). On the other hand, the nitrogen transfer for amino acid synthesis and finally the protein of the cells is important for rhizogenesis. This shows that frequent amino acid transfers to other cuttings are essential for increased protein synthesis during the formation of the lateral root (Ahkami et al., 2009; Druege and Kadner, 2008).

Carbohydrate to Nitrogen Ratio (C/N ratio)

The results of Table 2 showed that the simple effect of time ($P < 0.05$) and the sampling ($P < 0.01$) as well as the interaction between IBA × putrescine × sampling had a significant effect on C/N ratio ($P < 0.01$). The highest C/N ratio was recorded in the stem bark of cuttings treated with 200 ppm putrescine and 4000 ppm IBA (55.889), while the lowest value was reported in the leaf treated with 200 ppm putrescine (19.865) and 4000 ppm IBA (19.043) (Table 4). Furthermore, a higher C/N ratio was observed in stem bark compared to the leaf in all auxin and putrescine treatments. Izadi et al. (2016) found that although the C/N ratio of the mother plant was important for the rhizogenesis ability of olive cuttings, this proportion was not significant in the rhizogenesis potential of cuttings. Conversely, Hambrick et al. (1991) reported that there was a high correlation between C/N ratio and the rhizogenesis percentage of *Rosa multiflora* Thunb. cuttings. In addition, the evidence has shown that limitation in rhizogenesis is associated with low carbohydrate reservoirs and low C/N ratio (Druege et al., 2000).

Protein

The variance analysis revealed that interaction effect of time and putrescine and three- way effects between time, sampling and IBA were significant on cuttings' protein content ($P \leq 0.01$). In addition, there was a significant effect of IBA and putrescine interaction on protein content ($P \leq 0.01$) (Table 2). Furthermore, at the beginning of the experiment, the IBA treatment resulted in a higher protein content in the leaves (39.214 mg g^{-1}) compared with the bark (31.689 mg g^{-1}) (Table 6).

Over time, this parameter decreased significantly in both leaves and stem bark compared to the beginning of the experiment. It is noteworthy that the protein content of the leaf was higher than that of the stem bark at all IBA treatment levels over time.

Further, after 90 days of planting cuttings, the highest amount of protein was recorded in the leaf of the cuttings treated with 2000 ppm IBA (55.76 mg g^{-1}), while the lowest protein content (13.71 mg g^{-1}) was associated with the bark of control (Table 6).

After 30 days, the protein content was significantly reduced at each three putrescine treatment levels. This parameter displayed a declining trend after 60 and 90 days of cuttings treatment with 100 and 200 ppm putrescine (Table 7).

According to the results, protein content in leaves decreased with higher putrescine concentration, while the same parameter increased in stem bark. The highest amount of protein was obtained in the leaves of untreated cuttings with putrescine (26.56 mg g^{-1}) which indicated a significant difference in the protein content of stem bark treated with 100 and 200 ppm putrescine (Table 8, 9). When IBA and putrescine were used simultaneously, these proteins appear to be involved in callus induction and the rhizogenesis process (Aslmoshtaghi et al., 2014).

Peroxidase Enzyme

According to the results of Table 2, it was observed that the interaction between time × IBA × putrescine × sampling had a significant effect on peroxidase enzyme ($P < 0.05$). Based on the results (Table 8), it was clarified that the peroxidase activity significantly declined in leaf and stem bark of cuttings treated with 2000 ppm IBA compared to the untreated cuttings, while this enzyme activity enhanced as much as 53.12% in the leaves exposed to 4000 ppm IBA compared with control cuttings whereas this enzyme activity decreased by 18.18% in the stem bark of cuttings treated with 4000 ppm IBA rather than control. Moreover, no significant difference was found between the leaf and stem bark of untreated cuttings and samples treated with 2000 ppm IBA in terms of peroxidase activity. The peroxidase activity was 0.049 and 0.027 μmol min^{-1} g^{-1} F.W. in the leaf and stem bark of cuttings treated with IBA (4000 ppm), respectively. By increasing putrescine concentration, peroxidase enzyme content was increased in stem bark and leaf (Table 9). Accordingly, the highest value was observed in the leaves treated with 200 ppm, which increased to 51.51% compared with untreated cuttings.

Further, the results revealed statistically significant peroxidase activity in leaf and stem bark at all putrescine concentrations, and this increase was higher in leaves than in stem bark with increasing putrescine concentration. However, peroxidase activity was lower in leaf than in stem bark at a putrescine concentration of 100 ppm.

The lowest peroxidase activity (0.017 $min^{-1} g^{-1}$ F.W.) was associated with the stem barks untreated with putrescine (Table 9).

The results revealed that the peroxidase activity in cuttings' leaves at the start of the experiment (0.008 μmol $min^{-1} g^{-1}$ F.W.) was four times higher than the activity of this enzyme in stem bark (0.002 μmol $min^{-1} g^{-1}$ F.W.) (Table 10). Although this enzyme content was enhanced in the leaf and stem bark of all treated cuttings (IBA and purescine after 30 days of cuttings cultivation), it was reduced in the untreated cuttings (Table 10).

The highest peroxidase activity was recorded in the stem bark of treated cuttings with 200 ppm putrescine (0.091 μmol $min^{-1} g^{-1}$ F.W.), the leaves treated with 4000 ppm IBA (0.155 μmol $min^{-1} g^{-1}$ F.W.) and 200 ppm putrescine (0.076 μmol $min^{-1} g^{-1}$ F.W.) in 30, 60 and 90 days after cuttings cultivation.

Additionally, significant changes in peroxidase activity were observed in the leaf and stem bark of cuttings treated with different concentrations of putrescine and IBA (Table 10).

Table 6. Effect of time × sampling × IBA on protein (mg/g F.W.) of olive (*Olea europaea* L. cv.'Konservalia') cuttings

Sampling	IBA (ppm)	Days after planting			
		0	30	60	90
Leaf	0	39.214±1.966 [a]	25.774±1.205 [c]	21.066±1.415 [d]	23.347±1.774 [cd]
	2000		23.654±1.400 [cd]	21.999±1.861 [d]	32.342±5.900 [ab]
	4000		15.931±2.235 [ef]	21.891±0.845 [d]	22.649±1.157 [d]
Stem bark	0	31.689±1.576 [b]	13.642±1.282 [f]	18.932±3.438 [de]	14.500±0.357 [f]
	2000		15.687±0.640 [e]	13.937±0.942 [f]	15.738±0.584 [e]
	4000		35.968±4.221 [ab]	14.155±1.324 [ef]	16.049±0.486 [e]

Note: The same letters represent a significant difference at $P < 0.05$ level

Table 7. Effect of time × sampling × IBA on protein (mg/g F.W.) of olive (*Olea europaea* L. cv.'Konservalia') cuttings

Putrescine (ppm)	Days after planting			
	0	30	60	90
0	35.452±2.025 [a]	16.221±1.742 [d]	18.822±1.241 [c]	25.677±3.605 [b]
100		23.124±1.904 [b]	16.035±1.026 [d]	18.356±0.705 [c]
200		25.983±4.080 [b]	21.133±1.885 [bc]	18.280±0.765 [c]

Note: The same letters represent a significant difference at $P < 0.05$ level

Table 8. Effect of sampling × IBA on protein and proxidase activity of olive (*Olea europaea* L. cv.'Konservalia') cuttings

Sampling	IBA (ppm)	Protein (mg/g F.W.)	Proxidase activity (μmol/min/g F.W.)
Leaf	0	23.396±0.797 [a]	0.032±0.006 [ab]
	2000	25.998±2.437 [a]	0.033±0.009 [ab]
	4000	20.157±0.689 [b]	0.020±0.004 [c]
Stem bark	0	15.691±1.209 [c]	0.021±0.002 [c]
	2000	15.120±0.540 [c]	0.049±0.009 [a]
	4000	22.057±5.347 [ab]	0.027±0.002 [b]

Note: The same letters within a column represent a significant difference at $P < 0.05$ level

Table 9. Effect of sampling × putrescine on protein, proxidase and polyphenol oxidase activity of olive (*Olea europaea* L. cv.'Konservalia') cuttings

Sampling	Putrescine (ppm)	Protein (mg/g F.W.)	Proxidase activity (µmol/min/g F.W.)	Poly phenol oxidase activity (µmol/min/g F.W.)
Leaf	0	26.566±2.563 ^{a}	0.033±0.010 ^{b}	0.105±0.005 ^{ab}
	100	21.826±0.491 ^{b}	0.017±0.002 ^{c}	0.097±0.007 ^{ab}
	200	21.159±0.455 ^{b}	0.050±0.006 ^{a}	0.089±0.004 ^{b}
Stem bark	0	13.914±0.459 ^{d}	0.017±0.003 ^{c}	0.086±0.004 ^{b}
	100	16.516±1.116 ^{c}	0.028±0.004 ^{b}	0.109±0.012 ^{a}
	200	22.439±5.274 ^{ab}	0.036±0.007 ^{b}	0.155±0.037 ^{a}

Note: The same letters within a column represent a significant difference at $P < 0.05$ level

Table 10. Effect of time × sampling × IBA × putrescine on proxidase activity of olive (*Olea europae*a L. cv.'Konservalia') cuttings

Sampling	IBA (ppm)	Putrescine (ppm)	Proxidase activity (µmol min^{-1} g^{-1} F.W.)			
			Days after planting			
			0	30	60	90
Leaf	0	0	0.008±0.0002 ^{gh}	0.007±0.0003 ^{h}	0.051±0.015 $^{b-h}$	0.010±0.0003 ^{gh}
		100		0.012±0.0003f ^{gh}	0.028±0.0021 $^{c-h}$	0.006±0.001 ^{h}
		200		0.089±0.0138 ^{b}	0.007±0.0010 ^{h}	0.076±0.0012 ^{bc}
	2000	0		0.010±0.0003 ^{gh}	0.014±0.0031 $^{e-h}$	0.025±0.0012 $^{c-h}$
		100		0.013±0.0003 ^{fgh}	0.008±0.0010 ^{h}	0.023±0.0153 $^{c-h}$
		200		0.049±0.0375 $^{b-h}$	0.029±0.0017 $^{c-h}$	0.009±0.0033 ^{gh}
	4000	0		0.016±0.0003 $^{d-h}$	0.155±0.0703 ^{a}	0.014±0.0029 $^{e-h}$
		100		0.030±0.0083 $^{c-h}$	0.029±0.0025 $^{c-h}$	0.005±0.0003 ^{h}
		200		0.054±0.0036 $^{b-h}$	0.068±0.0020 $^{b-f}$	0.071±0.0054 ^{bcd}
Stem bark	0	0	0.002±0.0002 ^{h}	0.009±0.0005 ^{gh}	0.008±0.00 ^{h}	0.039±0.0301 $^{b-h}$
		100		0.011±0.0006 ^{fgh}	0.024±0.0065 $^{c-h}$	0.066±0.0500 $^{b-g}$
		200		0.091±0.0666 ^{b}	0.016±0.0008 $^{d-h}$	0.034±0.0073 $^{c-h}$
	2000	0		0.011±0.0043 ^{fgh}	0.010±0.0001 ^{gh}	0.017±0.0068 $^{d-h}$
		100		0.023±0.0011 $^{c-h}$	0.034±0.0018 $^{c-h}$	0.005±0.0003 ^{h}
		200		0.024±0.0014 $^{c-h}$	0.024±0.0017 $^{c-h}$	0.043±0.0080 $^{b-h}$
	4000	0		0.024±0.0095 $^{c-h}$	0.024±0.0012 $^{c-h}$	0.012±0.0024 ^{fgh}
		100		0.008±0.0005 ^{h}	0.012±0.0037 ^{fgh}	0.071±0.0030 ^{bcd}
		200		0.013±0.0003 ^{fgh}	0.043±0.0056 $^{b-h}$	0.033±0.0018 $^{c-h}$

Note: The same letters represent a significant difference at $P < 0.05$ level

Plant peroxidases are hemic proteins involved in broad physiological processes such as auxin metabolism. In many conditions, IAA hydrolysis is predominantly non-decarboxylation oxidation. However, enzymatic oxidative decarboxylation of IAA can also occur by a group of peroxidase isoforms called IAAox, which is associated with lateral rhizogenesis (Porfirio et al., 2016 b). Several studies have documented that during the rhizogenesis process of *Castanea sativa*, IAA oxidase activity is reduced (Mato and Vieitez, 1986), while the activity of indole acetic acid oxidase was reduced in the rhizogenesis of populus' cuttings (Güneş, 2000). Plant peroxidases were discovered to affect auxin metabolism and cell wall lignification in the presence of phenol (Rout, 2006). Three investigated sections, including cuttings treated with IBA and putrescine individually and in combination, showed remarkable changes in peroxidase activity over time. It has been reported that the peroxidase activity increases during cell division and primordium formation, assisting the essential cofactors triggering rhizogenesis (Ganez, 2000). Rout (2000) also explained that *Camellia sinensis* L. cuttings treated with IBA exhibited higher peroxidase activity than the control. In the initial stages of rhizogenesis, peroxidase activity is a good indication of successful rhizogenesis.

The results of analysis of variance in Table 2 showed that the interaction between time × IBA × putrescine × sampling had a significant effect on the polyphenol oxidase enzyme activity ($P < 0.05$). According to our findings, the polyphenol oxidase activity in leaves was found to decrease when putrescine concentration was increased to 200 ppm. However, it was increased in stem bark (Table 9). The highest polyphenol oxidase activity (0.155) was observed in the stem bark of cuttings treated with 200 ppm putrescine. Although no significant difference was found between polyphenol oxidase activity in the leaf and stem bark of control cuttings and samples treated with 100 ppm, this enzyme content in stem bark of untreated cuttings was 1.85 times higher than the barks treated with 200 ppm putrescine (Table 9). Similarly, in other experiments, polyphenol oxidase activity was found to be increased in cuttings of 'Soltani', round and seedless, 'Yaolva', 'Insisi' and 'Tarakia ikern' grape varieties after cuttings cultivation until rhizogenesis of the cuttings, while it was reduced after rhizogenesis process. Generally, based on the results, it is considered that the polyphenol oxidase enzyme does not affect the processes after rhizogenesis. In contrast, polyphenol oxidase is effective in dividing cells, differentiating cells and initiating rhizogenesis in early stages. In addition, Coban (2007) reported that the polyphenol oxidase activity in treated plants was reduced. Then the iation amount was increased in rhizogenesis induction during gene expression again and rhizogenesis initiation. The results of this experiment were consistent with those of others, showing the presence of numerous variations in the activity of polyphenol oxidase for rhizogenesis of easy-to-root cultivars such as *Phoenix dactylifera*, *Vitis* sp. and *Juglans regia* L. (Qaddoury and Amssa, 2003; Satisha et al., 2008; Cheniany et al., 2010). According to (Table 11), it was revealed that the polyphenol oxidase enzyme activity was increased significantly in the cuttings untreated with IBA and treated with 2000 and 4000 ppm IBA after 30 days of cuttings cultivation.

Accordingly, the highest level of polyphenol oxidase activity (0.185 μmol min^{-1} g^{-1} F.W.) was recorded in cuttings treated with 2000 ppm IBA, while the lowest activity (0.082 μmol min^{-1} g^{-1} F.W.) was obtained in the samples subjected to 4000 ppm IBA. Nevertheless, after 60 days of cuttings culture, polyphenol oxidase activity significantly increased in the cuttings treated with 4000 ppm IBA, while it decreased in untreated cuttings and samples treated with 2000 ppm IBA. Although over time, 90 days after cultivation, polyphenol oxidase activity decreased in the untreated cuttings and the cuttings treated with IBA, no significant difference was found between the treatment levels. Polyphenol oxidase is a key factor in cell division, differentiation and primordium development (Huystee and Cairns, 1982). Besides, Rout (2006) noted that this enzyme activity increased during induction and rhizogenesis initiation in *Camellia sinensis* L. cuttings, while it was reduced during the expression phase. In addition, other study indicated that this enzyme content was reduced in grape cuttings with poor rhizogenesis during induction and trigger of rhizogenesis. By contrast, its content increased during rhizogenesis and expression (Satisha et al., 2008). Likewise, Yilmaz et al. (2003) documented a correlation between polyphenol oxidase activity and rhizogenesis in grape cuttings and presented that this enzyme activity was lower in hard-to-root cuttings than in easy-to-root cuttings during rhizogenesis. Tehranifar et al. (2014) investigated the activity of peroxidase, poly oxidase (polyphenol oxidase), phenolic compounds and total sugar (TS) during rhizogenesis of Berberis's cuttings treated with IBA and IBA + H_2O_2. They demonstrated that peroxidase activity was initially reduced, whereas it was increased after 50 days of cultivation in cuttings treated with IBA + H_2O_2. In addition, they argued that during rhizogenesis, the polyphenol oxidase activity in untreated cuttings was lower than in treated ones. Furthermore, this enzyme significantly decreased after 60 to 80 days of cultivation. On the other hand, the treated cuttings showed a higher concentration of phenolic compounds compared

Table 11. Effect of time × IBA on polyphenol oxidase activity (μmol/min/g F.W.) of olive (*Olea europaea* L. cv. 'Konservalia') cuttings

IBA (ppm)	Days after planting			
	0	30	60	90
0		0.113±0.009 [b]	0.082±0.004 [d]	0.084±0.003 [d]
2000	0.063±0.0001 [e]	0.185±0.058 [a]	0.108±0.010 [b]	0.093±0.003 [c]
4000		0.082±0.003 [d]	0.135±0.013 [a]	0.081±0.004 [d]

Note: The same letters represent a significant difference at $P < 0.05$ level

with the control. The increased polyphenol oxidase activity can be associated with lignification processes, phenol metabolism (Batish et al., 2008), or H_2O_2 levels (Porfirio et al., 2016a). Santos Macedo et al. (2009) explained that the treatment of olive cultivars with IBA could transcribe the AOX gene, eliciting the phenylpropanoids biosynthesis (Sircar et al., 2012) and the increasing concentration of monophenolic compounds as a natural substrate of polyphenol oxidase. On the other hand, auxin can increase the apoplastic production of reactive oxygen species (ROS), which promotes cell wall expansion through polysaccharide development and protein hydrolysis (Schopfer et al, 2002). To counter the effects of oxidative stress on the plant, phenolic compounds with antioxidant properties are produced by plants. Thus, substrates of polyphenol oxidase accumulate and facilitate polyphenol oxidase activity (Porfirio et al., 2016b). The proceeding polymerizations improve the compounds. The product polymers, such as the precursor of lignin (which is essential for cell wall synthesis and construction) (Hiraga et al., 2001; Vanholme et al., 2010) become sensitive to the metabolism by polyphenol oxidase. In other words, AOX acts on these compounds after initial activity in the last phase of induction or at an early stage. In addition, IAA hydrolysis produces ROS by IAAox in this phase (Schopfer et al., 2002), which can elicit the production of phenolic compounds and enhance the activity of polyphenol oxidase. Szecskó et al. (2004) interpreted that the season-dependent reaction of cuttings to the rhizogenesis could be due to root-inducing factors associated with some enzyme activity such as peroxidase, polyphenol oxidase, or phenolic content. Further, various studies have shown that the phenolic compounds content and polyphenol oxidase activity can cause a seasonal pattern. Gur et al. (1988) and Bassuk and Howard, (1981) claimed that there is a positive relationship between rhizogenesis and elevated polyphenol oxidase activity and phenolic compounds content in hard-to-root cuttings of apples. By contrast, Szecskó et al. (2004) expressed that there was no direct and significant correlation between rhizogenesis percentage with poly phenol oxidase and phenol content in hardwood and hard-to-root cuttings of Prunus. Overall, plant hormones, enzymes, and phenolic compounds are assumed to play key roles in the endogenous control mechanism of rhizogenesis in olives. Moreover, endogenous phenolic compounds differentially affect rhizogenesis of cuttings (Lee et al., 2009).

Conclusion

Our results exhibited that the application of IBA and putrescine during the callus formation and rooting phases of 'Konservalia' cultivar olive cuttings increased the rhizogenesis and callus generation percentage by changing the amounts of protein, carbohydrate, nitrogen and peroxidase and polyphenol oxidase enzymes in the stem bark and leaf. Accordingly, the combined use of IBA and putrescine promoted rooting and callus formation of the cuttings compared to the individual use of putrescine. Also, it was found that co-application of IBA and putrescine treatment positively influenced rooting time. The highest percentage of root formation and the lowest percentage of callus formation were recorded in the IBA treatment at a concentration of 4000 ppm. As for IAA treatment, the protein and carbohydrate content and nitrogen percentage in the leaf were higher than that of the stem bark, whereas the results of C/N ratio were different. The C/N ratio and enzyme activity of polyphenol oxidase and peroxidase in cuttings treated with 4000 ppm decreased and then increased significantly. The highest C/N ratio, nitrogen percentage, polyphenol oxidase and peroxidase activity levels were observed in the cuttings treated with 200 ppm putrescine. Based on the analysis of the biochemical characteristics of the examined cuttings in leaves, stem bark, and roots, the study concluded that the process of callus formation and rooting of olive cuttings might be affected by changes in enzymatic and biochemical characteristics that occur over time and in different parts of the cutting depending on its performance. Therefore, the application of proper external concentrations of hormones such as IBA and putrescine plays a very important role in accelerating these processes.

Acknowledgements

This project was supported by Shahid Chamran University of Ahvaz (SCU). We thank the anonymous reviewer for his/her most helpful comments.

CRediT authorship contribution statement

Esmaeil Khaleghi: Conceived the project and supervised the work, contributed to the editing of the manuscript. **Sara Alavipour:** Investigation, performed the experiments, analyzed the data and drafted the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Ahkami A. M., Lischewski S., Haensch K. T., Porfirova S., Hofmann J., Rolletschek H., Melzer M., Franken P., Hause B., Druege U., Hajirezaei M. R. (2009). Molecular Physiology of Adventitious Root Formation in *Petunia* Hybrid Cuttings: Involvement of Wound Response and Primary Metabolism. New Phytol 181: 613-625. doi: 10.1111/j.1469-8137.2008.02704.x

Akiyoshi D. E., Morris R. O., Hinz R., Mischke B. S., Kosuge T., Garfinkel D. J., Gordon M. P., Nester E. W. (1983). Cytokinin/Auxin Balance in Crown Gall Tumors Is Regulated by Specific Loci in the T-DNA. Proc Natl Acad Sci USA 80: 407–411. doi: 10.1073/pnas.80.2.407

Anderson L. J., Derner J. D., Polley H. W., Gordon W. S., Eissenstat D. M., Jackson R. B. (2010). Root Response along a Subambient to Elevated CO_2 Gradient in a C-3-C-4 Grassland. Glob Change Biol 16 (1): 454-468. doi: 10.1111/j.1365-2486.2009.01975.x

Aslmoshtaghi E., Shahsavar A. R. (2010). Endogenous Soluble Sugars, Starch Contents and Phenolic Compounds in Easy – and Difficult – to – Root Olive Cuttings. J Biol Environ Sci 4 (11): 83-86.

Aslmoshtaghi E., Shahsavar A. R. Taslimpour M. R. (2014). Effects of IBA and Putrescine on Root Formation of Olive Cuttings. Agric Conspec Sci 79 (3): 191-194.

Attarzadeh M., Aboutalebi A. Attarzadeh M. (2015). Effect of Different Hormonal Treatments and Rooting-Cofactors on Rooting of Olive Cultivars (Fishomi and Shiraz). J Soil Plant Interactions 7 (25): 49-57.

Bartolini G., Pestelli P., Toponi M. A. Monte G. D. (1996). Rooting and Carbohydrate Availability in *vitis 140 Ruggeri* Stem Cuttings. Vitis 35 (1): 11-14.

Bassuk N. L. Howard B. H. (1981). A Positive Correlation between Endogenous Root-inducing Cofactor Activity in Vacuum-Extracted Sap and Seasonal Changes in Rooting of M26 Winter Apple Cuttings. J Hort Sci 56 (4): 301-312.

Batish D. R., Singh H. P. Kaur S., Kohli R. K., Yadav S. S. (2008). Caffeic Acid Affects Early Growth, and Morphogenetic Response of Hypocotyl Cuttings of Mung Bean (*Phaseolus aureus*). J Plant Physiol 165: 297-305. doi: 10.1016/j.jplph.2007.05.003

Bradstreet R. B. (1954). Kjeldahl Method for Organic Nitrogen. Anal Chem 26 (1): 185-187. doi: 10.1021/ac60085a028

Bradford M. M. (1979). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dry Binding. Anal Biochem 72: 248-254. doi: 10.1016/0003-2697(76)90527-3

Cheniany M., Ebrahimzadeh H., Masoudi-nejad A., Vahdati K. Leslie C. (2010). Effect of Endogenous Phenols and Some Antioxidant Enzyme Activities on Rooting of Persian walnut (*Juglans regia* L.). Afr J Plant Sci 4: 479-487.

Coban H. (2007). Determination of Polyphenol Oxidase Activity during Rooting in Cutting of Some Grape Varieties (*Vitis vinifera* L.). Asian J Chem 19(5): 4020-4024.

Copes D. L. Mandel N. L. (2000). Effects of IBA and NAA Treatments on Rooting Douglas–Fir Stem Cuttings. New For 20: 249-257. doi: 10.1023/A:1006752717350

Cruz A., Perez B., Moreno J. M. (2003). Plant Stored Reserves Do not Drive Resprouting of the Lignotuberous Shrub *Erica australis*. New Phytol 157: 251–261.

Dag A., Erel R., Ben-Gal A., Zipori I., Yermiyahu U. (2012). The Effect of Olive Tree Stock Plant Nutritional Status on Propagation Rates. Hort Sci 47 (2): 307-310. doi: 10.21273/HORTSCI.47.2.307

De Oliveira A. F., Pasqual M., Chalfun N. N. J., Regina M. A., Rincón C. D. R. (2003). Influência do número de nós em estacas semilenhosas de oliveira (*Olea europaea* L.) no enraizamento sob câmara de nebulização. Ciência e Agrotecnologia 27: 332–338. doi: 10.1590/S1413-70542003000200012 (in Portuguese)

Denaxa N. K., Vemmos S. N. Roussos P. A. (2012). The Role of Endogenous Carbohydrates and Seasonal Variation in Rooting Ability of Cuttings of an Easy and a Hard-to-Root Olive Cultivars (*Olea europaea* L.). Sci Hort 143: 19-28. doi: 10.1016/j.scienta.2012.05.026

Dietz A., Kutschera U., Ray P. M. (1990). Auxin Enhancement of mRNAs in Epidermis and Internal Tissues of the Pea Stem and Its Significance for Control of Elongation. Plant Physiol 93: 432–438. doi: 10.1104/pp.93.2.432

Druege U., Kadner R. (2008). Response of Post-Storage Carbohydrate Levels in Pelargonium Cuttings to Reduced Air Temperature during Rooting and the Relationship with Leaf Senescence and Adventitious Root Formation. Postharvest Biology and Technology 47 (1): 126-135. doi: 10.1016/j.postharvbio.2007.06.008

Druege U., Zerche S., Kadner R., Ernst M. (2000). Relation between Nitrogen Status, Carbohydrate Distribution and Subsequent Rooting of *Chrysanthemum* Cuttings as Affected by Pre-Harvest Nitrogen Supply and Cold Storage. Ann Bot 85: 687–701. doi: 10.1006/anbo.2000.1132

Epstein E. and Ludwig-Muller J. (1993). Indole-3-Butyric Acid in Plants: Occurrence, Synthesis, Metabolism and Transport. Physiol Plant. 88: 382–389. doi: 10.1111/j.1399-3054.1993.tb05513.x

Güneş T. (2000). Peroxidase and IAA-Oxidase Activities during Rooting in Cuttings of Three Poplar Species. Turk J Bot 24: 97–101

Gur A., Gad A. E. Haas E. (1988). Rooting of Apple Rootstock Clones as Related to Phenols and Their Oxidation. Acta Hortic 227: 160-166. doi: 10.17660/ActaHortic.1988.227.25

Hambrick C. E., Davies J. F. T., Pemberton H. B. (1991). Seasonal Changes in Carbohydrate/ Nitrogen Levels during Field Rooting of *Rosa multiflora* 'Brooks 56' Hardwood Cuttings. Sci Hortic 46: 137-146.doi: 10.1016/0304-4238(91)90099-K

Hartmann H.T., Kester D. E., Davies J. J. T. (2002). Plant Propagation. Principle and Practices, 5th ed. Prentice-Hall, New Jersey.

Heloir J. F., Kevers C. Hausman J.F. Gaspar T. (1996). Changes in the Concentration of Auxins and Polyamines during Rooting of *in vitro* Propagated Walnut Shoots. Tree Physiol 16: 515–519. doi: 10.1093/treephys/16.5.515

Hemeda H. M. Kelin B. P. (1990). Effects of Naturally Occurring Antioxidants on Peroxidase Activity of Vegetables Extracts. J Food Sci 55(1): 184-185. doi: /10.1111/j.1365-2621.1990.tb06048.x

Henrique A., Campinhos E. N., Ono E. O., dePinho S. Z. (2006). Effect of Plant Growth Regulators in the Rooting of *Pinus* Cuttings. Braz Arch Biol Technol 49 (2): 28-38. doi: 10.1590/S1516-89132006000300002

Hiraga S., Sasaki K., Ito H., Ohashi Y. Matsui H. (2001). A Large Family of Class III Plant Peroxidases. Plant Cell Physiol 42: 462-468. doi: 10.1093/pcp/pce061

Hosseini S. M., Nourmohammadi Z., Hosseini-Mazinani S.M., Sadeghi A., Esmati A. (2008). Effect of Different Media on Rooting Abilities of Two Iranian Olive Cultivars. Acta Hortic 791: 209-212. doi: 10.17660/ActaHortic.2008.791.29

Huber S.C., Kaiser W. M. (1996). Regulation of C/N Interactions in Higher Plants by Protein Phosphorylation. In: Signal Transduction in Plant Growth and Development (Verma D. P. S., ed.). Wien, New York: Springer-Verlag, pp. 87-112.

Huystee R. B., Cairns W. L. (1982). Progreno and Prospects in the Use of Peroxidase to Cell Development. Phytochem 21: 1843-1847. doi: 10.1016/0031-9422(82)83001-X

Iran-nejad A., Watan-pour Azghandi A., Rahnama H., Jaliani N., Zorgipour R. (2010). Improvement of Rooting and Adaptation of Yellow Olive Tissue Culture Seedlings Using *Agrobacterium rhizogenes* and *Trichoderma harzianum* Fungus. Seedling Seed Agric J 26-26 (1): 85-93.(in Persian)

Irigoyen J. J., Emerich D. W., Sanchez-Diaz M. (1992) Water Stress Induced Changes in Concentrations of Proline and Total Soluble Sugars in Nodulated Alfalfa (*Medicago sativa*) Plants. Physiol Plant 84: 55-60. doi: 10.1111/j.1399-3054.1992.tb08764.x

Izadi M., Shahsavari A. R., Mirsoleimani A. (2016). Relation between Leaf and Stem Biochemical Constituents and Rooting Ability of Olive Cuttings. Int J Hort Sci Technol 3(2): 231-242. doi: 10.22059/ijhst.2016.62922

Kar M., Mishra D. (1976). Catalase, Peroxidase and Polyphenoloxidase Activities during Rice Leaf Senescence. Plant Physiol 57: 315-319. doi: 10.1104/pp.57.2.315

Karimi S., Yadollahi A. (2012). Using Putrescine to Increase the Rooting Ability of Hardwood Cuttings of the Peach × Almond Hybrid GF677. J Agrobiol 29 (2): 63–69. doi:10.2478/v10146-012-0010-6

Kevresan S., Kovacevic B., Cirin-Novta V., Kuhajda K., Kandrac J., Pavlovic K., Grbovic L. (2007). Biochemical Changes in Cuttings of *Robinia pseudoacacia* after Treatment with Naphthenate. J Serb Chem Soc 72: 953-959. doi:10.2298/JSC0710953K

Khoshkhoi M. (2007). Plant Propagation: Basics and Methods. Shiraz University Press, 373 p.

Lazaj A., Rama P., Vrapi H. (2015). The Interaction with Season Collection of Cuttings, Indol Butyric Acid (IBA) and Juvenility Factors on Root Induction in *Olea europaea* L. (Cultivar "Kalinjot"). Int Refereed J Eng Sci (IRJES) 4 (3): 32-38

Lee O.H., Lee B.Y., Lee j., Lee H.B. (2009). Assessment of Phenolics-Enriched Extract and Fractions of Olive Leaves and Their Antioxidant Activities. Bioresource Technol 100: 6107-6113.doi: 10.1016/j.biortech.2009.06.059

Ludwig-Muller J. (2003). Peroxidase Isoenzymes as Markers for the Rooting Ability of Easy-to-Root and Difficult-to-Root *Gravillea* Species and Cultivars of *Protea obstusifolia* (Proteaceae). *In vitro* Cell Dev Biol Plant 39: 377-383.doi:10.1079/IVP2003423

Mato M., Vieitez A. (1986). Changes in Auxin Protectors and IAA Oxidases during the Rooting of Chestnut Shoots *in vitro*. Physiol Plant 66: 491–494.doi: 10.1111/j.1399-3054.1986.tb05956.x

Porfirio S., Calado M.L., Noceda C., Cabrita M.J., Da Silva M.G., Azadi P. Peixe A. (2016 a). Tracking Biochemical Changes during Adventitious Root Formation in Olive (*Olea europaea* L.). Sci Hort 204: 41-53. doi:10.1016/j.scienta.2016.03.029

Porfirio S., Gomes da Silva M. D. R., Cabrita M. J., Azadi P. Peixe A. (2016 b). Reviewing Current Knowledge on Olive (*Olea europaea* L.) Adventitious Root Formation. Sci Hort 198: 207-226. doi: 10.1016/j.scienta.2015.11.034

Qaddoury A., Amssa M. (2003). Endogenous Phenolic Contents: Peroxidase and Polyphenoloxidase Activities in Date Palm *(Phoenix dactylifera* L.) Offshoots Related to Rooting Ability. Acta Physiol Plant 25: 417-421. doi:10.1007/s11738-003-0024-1

Reuveni O., Raviv M. (1981). Importance of Leaf Retention to Rooting Avocado Cuttings. J Amer Soc Hort Sci 106 (2): 127-130. doi:10.21273/JASHS.106.2.127

Rout G. R. (2006). Effect of Auxins on Adventitious Root Development from Single Node Cuttings of *Camellia sinensis* L. Kuntze and Associated Biochemical Changes. Plant Grow Regul 48: 111-117. doi:10.1007/s10725-005-5665-1

Rugini E., Di Francesco G., Muganu M., Astolfi S., Caricato G. (1997). The Effects of Polyamines and Hydrogen Peroxide on Root Formation in Olive and the Role of Polyamines as an Early Marker for Rooting Ability. In: Biology of Root Formation and Development (Altman S, Waisel D., eds.). Plenum Publishing Co. New York. pp. 65–73.

Rugini E., Politi V., Bignami C., Agazio M. D. Grego S. (1990). Effect of Polyamine Treatments on Rooting Cutting of Three Olive Cultivars. Acta Hortic 286: 97-100. doi: 10.17660/ActaHortic.1990.286.17

Santos Macedo, E., Cardoso, H. G. Hernandez, A., Peixi, A. A. Polidoros, A., Ferreira, A., Cordeiro, A., Arnholdt-Schmitt, B. 2009. Physiologic Responses and Gene Diversity Indicate Olive Alternative Oxidase as a Potential Source for Markers Involved in Efficient Adventitious Root Induction. Physiol Plant 137: 532-552. doi:10.1111/j.1399-3054.2009.01302.x

Satisha J., Raveendran P., Rokade N. D. (2008). Changes in Polyphenol Oxidase Activity during Rooting of Hardwood Cuttings in Three Grape Rootstocks under Indian Condition. S Afr J Enol Vitic 29: 94-97. doi:10.21548/29-2-1442

Scagel C. F. (2004). Changes in Cutting Composition during Early Stages of Adventitious Rooting of Miniature Rose Altered by Inoculation with Arbuscular Mycorrhizal Fungi. J Amer Soc Hort Sci 129 (5): 624-634. doi: 10.21273/JASHS.129.5.0624

Schopfer P., Liszkay A., Bechtold M., Frahry G., Wagner A. (2002). Evidence that Hydroxyl Radicals Mediate Auxin-Induced Extension Growth. Planta 214: 821-828. doi: 10.1007/s00425-001-0699-8

Sebastiani L., Tognetti R., Di paolo P., Vitagliano C. (2002). Hydrogen Peroxide and Indole-3-Butyric Acid Effects on Root Induction and Development in Cuttings of *Olea europaea* L. (cv. Frantoio and Gentile di Larino). Adv Hort Sci 16: 7-12

Sebastiani L., Tognetti R. (2004). Growing Season and Hydrogen Peroxide Effects on Root Induction and Development in *Olea europaea* L. (cvs 'Frantoio' and 'Gentile di Larino') Cuttings. Sci Hortic 100: 75–82. doi: 10.1016/j.scienta.2003.08.008

Sircar D., Cardoso H. G., Mukherjee C., Mitra A., Arnholdt-Schmitt B. (2012). Alternative Oxidase (AOX) and Phenolic Metabolism in Methyl Jasmonate-Treated Hairy Root Cultures of *Daucus carota* L. J Plant Physiol 169: 657-663. doi: 10.1016/j.jplph.2011.11.019

Sofo A., Dichio B., Xiloyannis C., Masia A. (2004). Lipoxygenase Activity and Praline Accumulation in Leaves and Roots of Olive Tree in Response to Drought Stress. Physiol Plant 121: 56-58. doi: 10.1111/j.0031-9317.2004.00294.x

Szecskó V., Hrotkó K., Stefanovits-Bányai E. (2004). Phenolic Compounds, Bud Dormancy and Rooting Ability of Plum Hardwood Cuttings. Acta Hortic 658: 679-687. doi: 10.17660/ActaHortic.2004.658.104

Tehranifar A., Mahmoody Tabar S., Selahvarzi Y., Balandary A., Kharrazi M. (2014). Biochemical Changes in Barberries during Adventitious Root Formation: The Role of Indole-3-Butyric Acid and Hydrogen Peroxide. Span J Agric Res 12 (2): 477-485. doi: 10.5424/sjar/2014122-3889

Turkoglu N., Durmus M. (2005). A Study on Root Formation of Four Olive Varieties by Application of Hormone. Asian J Plant Sci 4 (5): 455-457. doi: 10.3923/Ajps.2005.455.457

Tworkoski T., Takeda F. (2007). Rooting Response of Shoot Cuttings from Three Peach Growth Habits. Sci Hort 115: 98-100. doi: 10.1016/j.scienta.2007.08.004

Vanholme R., Demedts B., Morreel K., Ralph J., Boerjan W. (2010). Lignin Biosynthesis and Structure. Plant Physiol 153: 895-905. doi: 10.1104/pp.110.155119

Wiesman Z., Lavee S. (1995). Enhancement of IBA Stimulatory Effect on Rooting of Olive Cultivar Stem Cuttings. Sci Hort 62: 189-198. doi:10.1016/0304-4238(95)00772-L

Wu Q. S., Zou Y. N., He X. H. (2010). Exogenous Putrescine, not Spermine or Spermidine, Enhances Root Mycorrhizal Development and Plant Growth of Trifoliate Orange (*Poncirus trifoliata*) Seedlings. Int J Agric Biol 12: 576– 580.

Yilmaz H., Taskin T., Otludil B. (2003). Polyphenol Oxidase Activity during Rooting in Cuttings of Grape (*Vitis vinifera* L.) Varieties. Turk J Bot 27: 495-498.

Zhang J., Chen S., Liu R., Jiang J., Chen F., Fang W. (2013). *Chrysanthemum* Cutting Productivity and Rooting Ability Are Improved Grafting. Sci World J 2013: 1-7. doi: 10.1155/2013/286328

aCS89_13