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RESEARCH ARTICLE

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Assessing the effect of oxidative enzymes and stem anatomy on adventitious rooting of *Olea europaea* (L.) leafy cuttings

Nikoleta-Kleio Denaxa (Denaxa, NK)[1], Peter A. Roussos (Roussos, PA)[1], Stavros N. Vemmos (Vemmos, SN)[1] and Konstantinos Fasseas (Fasseas, K)[2]

[1]Agricultural University of Athens, Faculty of Crop Science, Laboratory of Pomology, Iera Odos 75, Athens 118 55, Greece. [2]Agricultural University of Athens, Faculty of Crop Science, Laboratory of Electron Microscopy, Iera Odos 75, Athens 118 55, Greece.

Abstract

Aim of study: To assess the role of polyphenol oxidase (PPO), peroxidase (POD) and indole-3-acetic acid oxidase (IAAox) during adventitious rooting (Ar) in semi-hardwood cuttings of the easy-to-root olive cv. 'Arbequina' and the difficult-to-root cv. 'Kalamata'. Simultaneously, a histological study was carried out in both cultivars to investigate the tissue related with Ar development.

Area of study: The rooting experiments were carried out in 'Kostelenos' nurseries (Troizinia, Greece) and in Agricultural University of Athens.

Material and methods: Plant material to set up the experiment was collected from current year shoots from 15-year-old mother plants of 'Arbequina' and 'Kalamata' at three different seasons (summer, autumn and spring). The auxin indole-3-butyric acid (IBA) at 2000 mg L^{-1} was used as rooting inducer.

Main results: Analysis revealed that 'Kalamata' had significantly higher enzymatic activities before experiment onset and during Ar compared to 'Arbequina'. Control cuttings of both cultivars exhibited increased enzymatic activities compared to IBA treated ones. IAAox was on average three times higher in 'Kalamata' than in 'Arbequina' and exhibited significant peaks during Ar. Similar peaks of POD and PPO activities were also detected. Histological analyses in 'Kalamata' revealed a continuous sheath of sclerenchyma ring and increased cortex thickness. Significant cell proliferation occurred in the phloem region in 'Arbequina' 15 days after planting and afterwards the root initials started developing in the secondary phloem from cambial cells.

Research highlights: The differences in enzymatic activities as well as in stem anatomy could partly justify the different rooting ability of both cultivars.

Additional keywords: callus formation; enzymatic activity; IAA oxidase; olive 'Kalamata'; peroxidase; root initials.

Abbreviations used: Ar (adventitious rooting); DAP (days after planting); IAAox (IAA oxidase); IBA (indole-3-butyric acid); POD (peroxidase); PPO (polyphenoloxidase).

Authors' contributions: Conceived and designed the experiments: PAR, SNV and NKD. Performed the experiments, analyzed the data and wrote the paper: NKD. Contributed reagents and materials: SNV, KF and PAR. Supervising the experiment: PAR and SNV. Supervising the anatomical observations: KF. Revision of the manuscript: PAR, SNV and KF. All authors read and approved the final manuscript.

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Correspondence should be addressed to Nikoleta-Kleio Denaxa: nkdenaxa@gmail.com

Introduction

Adventitious root formation is a complex process regulated by a number of endogenous and exogenous factors. Enzymes regulating auxin metabolism represent a key factor that affects adventitious root formation in almost all phases of rhizogenesis (Hartmann *et al.*, 2001). The enzymes that appear most often to be involved in the formation of adventitious roots are (Kavrayan & Aydemir, 2001; Qaddoury & Amssa, 2003; Porfirio *et al.*, 2016a): peroxidase (POD; donor: H_2O_2 oxidoreductase, EC 1.11.1.7), polyphenoloxidase (PPO; monophenol, dihydroxy-L-phenylalanine oxygen oxidoreductase, EC 1.14.18.1) and indole-3-acetic acid oxidase (IAAox).

Peroxidase activity has been suggested to serve as a good indicator of the rooting potential (Rout *et al.*,

2000; Husen, 2012). The initial reports of Van Hoof & Gaspar (1976) related rhizogenesis with a reduction in POD activity. From then on, several researchers described that adventitious rooting in various species occurs after POD activity peaked and followed by a subsequent decrease (Metaxas *et al.*, 2004; Vatulescu *et al.*, 2004; Husen & Pal, 2007). However, the opposite trend has also been observed in some species such as *Castanea sativa* × *C. crenata* (Gonçalves *et al.*, 1998), disputing in part POD's role as a biochemical marker (De Klerk, 1996).

Polyphenoloxidase is another enzyme involved in the initiation and development of adventitious roots (Kose *et al.*, 2011). According to Yilmaz *et al.* (2003), PPO may play a role in the organisation and development of root primordia, as it takes part in cell division and differentiation and it can directly regulate the synthesis of phenolics. Furthermore, Cheniany *et al.* (2010) reported that the changes in PPO activity appeared to be greater in easy-to-root cultivars and suggested the use of PPO as a marker of the onset and duration of the different phases of rooting.

A close relationship between IAAox activity and adventitious rooting has also been reported (Güneş, 2000), as this enzyme is involved in the regulation of IAA levels. According to Vatulescu *et al.* (2004), the reduction of IAAox activity could inhibit rooting when the endogenous level of IAA is supra-optimal, while a sub-optimal concentration usually promotes rooting. There is also a close relationship between POD, PPO and IAAox (Shinshi & Noguchi, 1975). It is generally accepted that some POD isoenzymes possess IAAox activity, as well as PPO activity (Srivastava & van Huystee, 1977). It is believed that PPO does not influence root formation directly, but its effects rather occur through a disturbance in POD activity, revealing a possible inverse relationship between these two enzymes (Cheniany *et al.*, 2010; Porfirio *et al.*, 2016a).

There is a wide variation in rooting ability of semi-hardwood cuttings among olive cultivars (Fontanazza, 1993). The exact reason behind the difficulty of rooting of an olive cultivar is unknown and it is believed that in some cases the differences in the rooting capacity may be due to the anatomical structure of the cuttings. The presence of a continuous sclerenchyma ring as well as the thick cortex in stem cuttings' base may act as a physiological barrier to adventitious root initiation or as a mechanical barrier to the emergence of the newly formed roots and has been negatively correlated with the rooting ability (Ayoub & Qrunfleh, 2008; Amissah *et al.*, 2008). Furthermore, the success of the rooting process has been partially associated with the location of the potential root initiation sites as well as the ability of target cells to be de-differentiated (Avidan & Lavee, 1978; Amissah *et al.*, 2008).

The objectives of this study were: a) to obtain information over the role of the enzymes POD, PPO and IAAox during the different phases of adventitious rooting process in an easy and a difficult-to-root olive cultivar, and b) to examine how their changes are related with differences on adventitious rooting ability. Additional objectives included the identification of the sites of root primordia initiation and the anatomical differences between 'Arbequina' and 'Kalamata' stems. Attention was also given to whether these anatomical differences could be interpreted as a barrier to the initiation and emergence of adventitious roots.

Material and methods

Plant material and establishment of rooting experiments

The rooting experiments were carried out in a mist propagation unit at Kostelenos nurseries, located in Troizinia area (Poros), Greece and in Agricultural University of Athens. Two olive cultivars with different rooting ability, 'Arbequina', easy-to-root, and 'Kalamata', difficult-to-root (Fontanazza, 1993), were used. Plant material was collected from current year shoots from 15-year-old mother plants. Sub-apical cuttings with four leaves on, approx. 7-10 cm long and 5 mm in diameter were used. The base of each cutting (approx. 1 cm) was treated for 5 sec with 2000 mg L^{-1} indole-3-butyric acid (IBA) dissolved in a 45% v/v aqueous solution of ethanol, while cuttings dipped for 5 sec in 45% v/v aqueous solution of ethanol served as control. IBA (2000 mg L^{-1}) was selected as the most effective auxin concentration in promoting rooting after the conduction of preliminary experiments (Denaxa *et al.*, 2010). The cuttings were established in plant plugs (Preforma Plug/240 M1413 Vecol, Jiffy, the Netherlands) and were finally put under an automatic mist unit.

The experiment was arranged as a completely randomized design with four replications of four 240 cell propagation trays per replicate (4 replications × 4 propagation trays × 240 cuttings × 2 treatments × 2 cultivars). The trials were carried out in three different seasons; summer (July), autumn (November) and spring (April), as significant seasonal variation in the rooting potential of olive stem cuttings was determined by other researchers (Sebastiani & Tognetti, 2004). The air and plant rooting medium temperature were recorded by a data logger. At the end

of the rooting period (3 months later) the percentage of rooted cuttings and callus formation was recorded.

Extraction and measurement of enzymatic activity

Samples of 40 cuttings per replicate (a total of 160 cuttings per treatment/40 cuttings × 4 replicates) were taken just before planting (day 0) and during Ar (at 1, 3, 5, 7 and 15 days after planting, DAP). The cuttings sampled for enzymes analyses were used as fresh material.

One gram of fresh tissue taken from the basal region (near 1 cm length) of seven stem cuttings (randomly selected out of the initial 40 cuttings per replicate), was homogenized in an Ultra-Turrax with 10 mL of cold phosphate buffer 100 mM pH 7.0 containing 2 mM EDTA, 1% PVP and 1 mM ascorbic acid. The crude extract was centrifuged at 4° C for 10 min at 20,000 x g and the supernatant was collected. All operations were carried out on ice. The total protein content was determined according to Bradford (1976) and bovine serum albumin was used as standard. For the enzymatic activities four different extractions took place per time point and each extraction was assayed twice. The mean value for each time point is the result of eight values (four extractions that were measured twice) and the results expressed as units mg^{-1} protein.

Peroxidase assay

Peroxidase activity was determined spectrophotometrically according to Flurkey & Jen (1978). The reaction mixture consisted of 2.15 mL phosphate buffer 50 mM pH 6.0 containing 0.5% (w/v) guaiacol, 0.25 mL 0.1% (v/v) H_2O_2 and 50 μL of the enzyme extract. POD activity was measured by monitoring the increase in absorbance at 470 nm at time 0 (when enzyme extract was added in the reaction mixture) and 3 min after incubation under room temperature (25±0.5°C). One unit of POD activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 min^{-1} at 470 nm.

Polyphenol oxidase assay

Polyphenol oxidase activity was determined spectrophotometrically according to Flurkey & Jen (1978). The reaction mixture consisted of 2.2 mL of phosphate buffer 50 mM pH 6.2, 0.25 mL of catechol 0.2 M and 50 μL of the enzyme extract. PPO activity was measured by monitoring the increase in absorbance at 420 nm at time 0 (when enzyme extract was added in the reaction mixture) and 2 min after incubation under room temperature (25±0.5°C). One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 per minute at 420 nm.

IAA oxidase assay

IAAox activity was determined spectrophotometrically according to Liu *et al.* (1996). The reaction mixture consisted of 0.2 mL of enzyme extract, 0.78 mL of potassium phosphate buffer 50 mM (pH 6.0), 0.01 mL $MnCl_2$ 5 mM, 0.01 mL 2,4-dichlorophenol (DCP) 5 mM and 0.02 mL of 2.5 g L^{-1} IAA. The reaction mixture stood in dark at 37° C for 30 min. Further, 2 mL Salkowski reagent that consisted on 15 mL of $FeCl_3$ 0.5M dissolved in 500 mL distilled water with 300 mL concentrated H_2SO_4 sp.gr. 1.84 (Meudt & Gaines, 1967) was added. The consumption of IAA was determined by measuring the absorbance at 535 nm after 30 min incubation at room temperature (25±0.5°C). IAAox activity was expressed as μg IAA mg^{-1} protein.

Histological analyses

Samples of 30 cuttings per replicate of each cv 'Arbequina' and 'Kalamata' (a total of 120 cuttings/cv) were randomly taken just before planting and IBA application (day 0) and at 1, 3, 5, 7 15 and 30 DAP with IBA application. Approx. 5 cm of each cutting's base was stored in formalin:alcohol:glacial acetic acid (FAA) (1:18:1 by volume) until use.

Longitudinal and transverse sections (60 μm) of the rooting zone (0.5 cm of the basal region of the cutting) were cut with a cryotome (Leica CM1850, Germany). Tissue samples were embedded in Jung Tissue Freezing Medium (Leica Microsystems Nussloch, Germany), sections were made at -18ºC and immediately examined with an Olympus BX40 microscope equipped with a digital camera (DP71, Olympus 12.5 Mp, Japan). Furthermore, a BP 330-385 exciter filter and a BA 420 barrier filter were used. Observations for the time course of root development were made and digital photos were taken.

Data collection and statistical analysis

A total of 7,680 cuttings per cultivar were used to examine the rooting percentage as well as to analyze the enzymatic activities and to conduct the histological study (a total of 960 cuttings per replicate – 3840 cuttings per treatment). The statistical analysis was performed using JMP 7.0 statistical software (SAS Institute, NC, USA). Data of cuttings exhibiting callus or roots were analyzed as one-way ANOVA. Significant differences were detected using the Student's T-test at α=0.05. Data for antioxidant enzymes activities during

adventitious rooting were analyzed as a Multi-Factor ANOVA with the factors being the cultivar ('Arbequina' and 'Kalamata'), auxin treatment (IBA 2000 mg L^{-1} and control) and DAP (0, 1, 3, 5, 7 and 15 days). Standard errors were calculated from the residual variances of the Multi-Factor ANOVA and used to determine significant differences between means when factors' effect was significant (Denaxa *et al.*, 2014).

Results

Rooting ability of olive cuttings

The rooting ability of the two olive cultivars differed significantly on each experimental season (Fig. 1). 'Arbequina' exhibited the highest rooting percentage, while the majority of 'Kalamata' cuttings either rotted or produced only callus. Exogenous application of 2000 mg L^{-1} IBA in semi-hardwood cuttings significantly increased rooting percentage in the easy-to-root cv 'Arbequina' irrespective of season (Fig. 1B). However, in the difficult-to-root cv 'Kalamata', IBA treatment was ineffective. Rooting percentage of 'Arbequina' cuttings when treated with IBA was highest in the summer (76%) followed by autumn and spring with (40% and 37% respectively) (Fig. 1A-B). On the other hand, 'Kalamata' cuttings presented only 1% rooted cuttings in spring and summer while this number increased to 3% in autumn, irrespective of auxin treatment (Fig. 1A-B).

Analysis of antioxidant enzymes during adventitious rooting

Polyphenol oxidase activity

PPO increased in both cultivars during spring and summer, while in autumn its values were lower (Fig. 2). In both periods, higher values were observed in the difficult-to-root 'Kalamata' respect to 'Arbequina' (on average 1.5 times higher) (Fig. 2A-B). Significant differences were also detected between control and IBA treated cuttings. Generally, control cuttings exhibited higher enzymatic activity values than those IBA-treated.

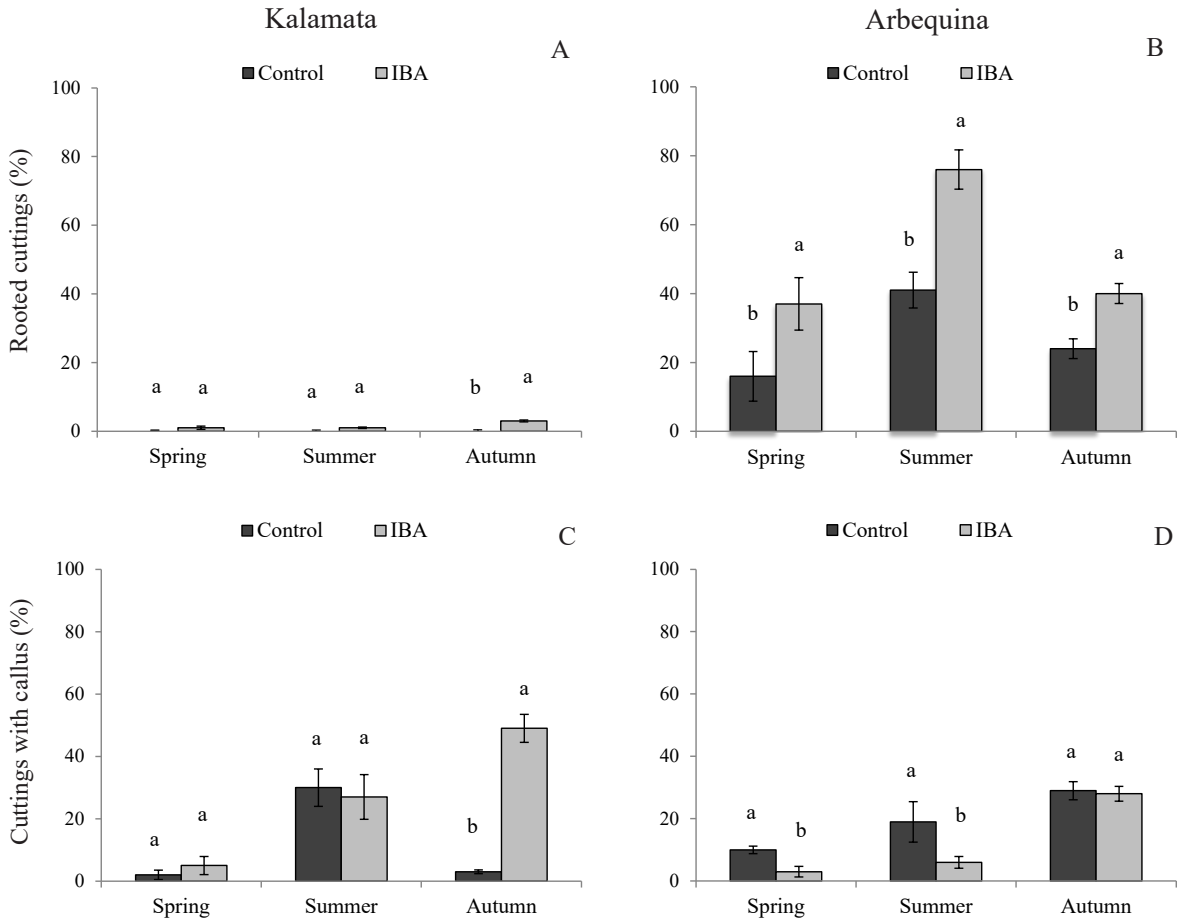

Figure 1. The effect of treatment (IBA 2000 mg L^{-1} and control) and season on rooting ability of 'Kalamata' (A, C) and 'Arbequina' (B, D) olive cuttings. Different lowercase letter above each column within the same season denotes statistically significant differences between treatments according to Student's T-test at α=0.05 (*n*=20).

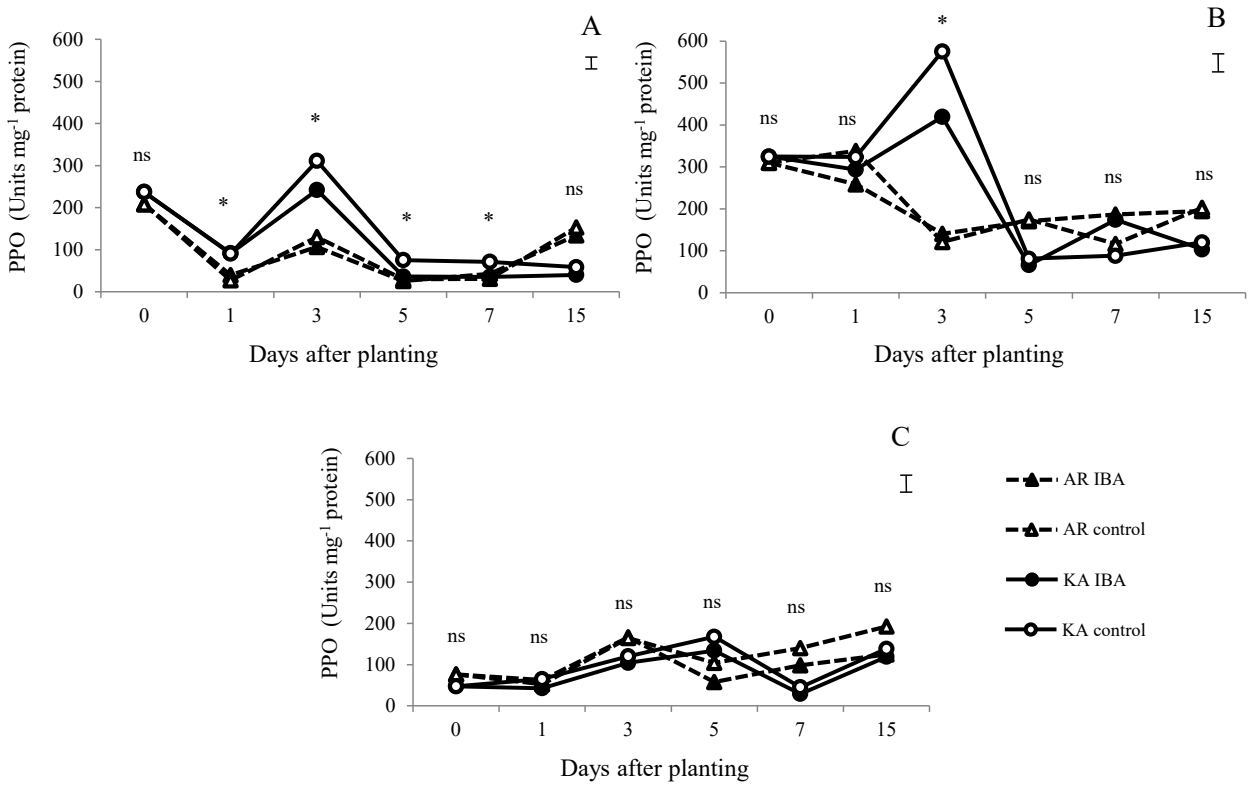

Figure 2. Effect of cultivar and IBA treatment on cuttings' PPO activity during A) spring, B) summer and C) autumn experimental season. AR, olive cv. 'Arbequina'; KA, olive cv. 'Kalamata'. Asterisks (*) denote the presence of statistically significant differences at each specific time point. The vertical bar at the right side of each diagram is the SE of the Multi-Factor ANOVA, which indicates statistically significant differences at α=0.05 (*n*=8).

In spring, PPO activity of both cultivars exhibited high values at day 0 regardless of auxin treatment, then increased again at 3 DAP, this increase being more pronounced in 'Kalamata' cuttings. At this phase of rhizogenesis (3 DAP), PPO was almost doubled in 'Kalamata' compared to 'Arbequina' in both control and IBA-treated cuttings. Thereafter, in 'Kalamata' the enzymatic activity declined gradually, while in 'Arbequina' it increased again 15 DAP (Fig. 2A). In summer, 'Kalamata' PPO activity peaked 3 DAP and declined thereafter (Fig. 2B). The detected increase at day 3 was more pronounced in control cuttings. In 'Arbequina', PPO declined significantly 3 DAP and afterwards increased slightly in both IBA-treated and control cuttings until 15 DAP (Fig. 2B). In autumn, Kalamata's enzymatic activity peaked 5 DAP, then decreased significantly 7 DAP and increased again 15 DAP. In 'Arbequina' the enzymatic activity reached its maximum 3 and 15 DAP (Fig. 2C).

Peroxidase activity

Overall, 'Kalamata' cuttings had significantly higher POD activity compared to 'Arbequina' throughout all seasons (Fig. 3). In average, POD activity of 'Kalamata' cuttings was 3 times higher in spring, 2 times higher in summer and 2.5 times in autumn compared to 'Arbequina'. Furthermore, the enzymatic activity was much more increased in spring and summer, while in autumn it presented lower activity. Significant differences were also detected between control and IBA treated cuttings. Generally, control cuttings of 'Kalamata' exhibited higher enzymatic activity values than IBA treated, while in 'Arbequina' control cuttings presented higher POD activity only in autumn.

In spring, POD activity of 'Kalamata' cuttings peaked 3 and 5 DAP, then decreased 7 DAP and afterwards a second significant increase was detected at day 15 (Fig. 3A). At this phase of rhizogenesis the enzymatic activity of 'Kalamata' cuttings increased sevenfold and fourfold respectively compared to 'Arbequina'. In summer, Kalamata's enzyme activity exhibited a large increase 3 DAP and then 15 DAP it reached the highest level (Fig. 3B). The increase in POD activity of 'Kalamata' cuttings at day 3 was seven times higher in IBA treated cuttings and 5.5 times in control compared to 'Arbequina'. Finally, in autumn, in 'Kalamata', POD activity increased significantly 5 DAP, then decreased and peaked again 15 DAP (Fig. 3C). In 'Arbequina'

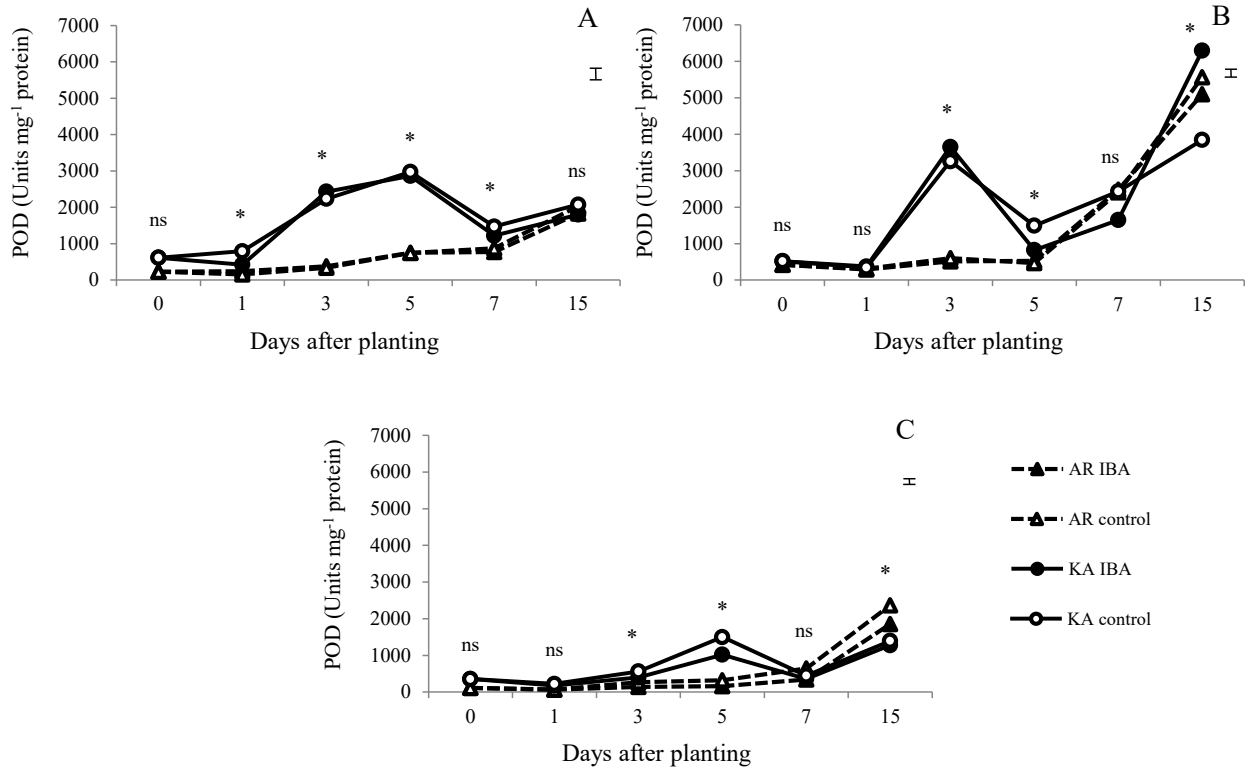

Figure 3. Effect of cultivar and IBA treatment on cuttings' POD activity during A) spring, B) summer and C) autumn experimental season. AR, olive cv. 'Arbequina'; KA, olive cv. 'Kalamata'. Asterisks (*) denote the presence of statistically significant differences at each specific time point. The vertical bar at the right side of each diagram is the SE of the Multi-Factor ANOVA which indicates statistically significant differences at α=0.05 (*n*=8).

POD activity reached its maximum 15 DAP, during all experimental seasons (Fig. 3A-C).

IAA oxidase activity

IAAox activity was significantly higher in 'Kalamata' compared to 'Arbequina' (Fig. 4A-C). Furthermore, IAAox was significantly higher in control of both cultivars during spring (Fig. 4A) and autumn (Fig. 4C) in almost all the rhizogenesis phases, with few exceptions.

In spring and autumn, IAAox activity increased significantly 5 DAP in 'Kalamata', with this increase being more pronounced in control cuttings and decreased thereafter (Fig. 4A and C). The maximum IAAox activity detected in 'Kalamata' in spring was 5 times higher in control cuttings (Fig. 4A) compared to 'Arbequina' and even 8.7 times higher in both IBA and control cuttings in autumn (Fig. 4C). In summer, IAAox activity showed a great peak 3 DAP in 'Kalamata' and declined thereafter, while in 'Arbequina' it did not exhibit any significant change during the experimental period (Fig. 4B). At day 3 the increase in IAAox activity in 'Kalamata' eightfolded compared to 'Arbequina'. It is noteworthy the fact that the maximum value of IAAox activity in 'Kalamata' in autumn was almost one tenth of that determined in summer and spring and this coincided with the highest rooting percentage and percentage of cuttings with callus observed in autumn in this cultivar (Fig. 1). Furthermore, in 'Arbequina' IAAox was highest at day 0 and then gradually declined in spring (Fig. 4A), while this enzyme activity exhibited a small increase at 3 DAP in summer and autumn (Fig. 4B and C).

Anatomical observations of adventitious root formation in olive stems

The stem anatomy of 'Arbequina' and 'Kalamata' cuttings was similar with regard to tissue organization and cell types (Fig. 5A-D). Control cuttings showed the normal stem organization of an epidermis with the early stages of phellem formation, the cortex, the phloem which was surrounded by the sclerenchyma ring, formed by groups of phloem fibers, the vascular cambium and the pith (Fig. 5A-D). However, transversal sections of the base of 'Kalamata' made at day 0 (Fig. 5B), showed a continuous sheath of sclerenchyma ring, as well as an increase in cortex thickness compared

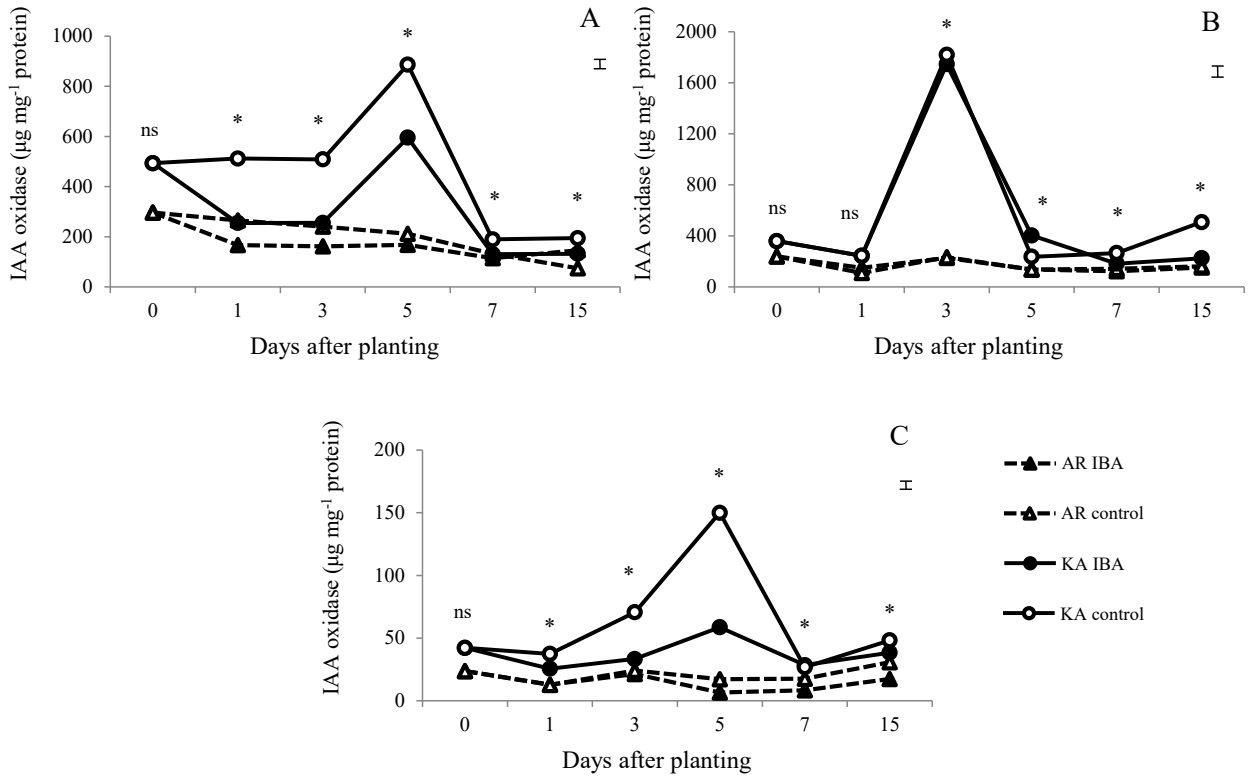

Figure 4. Effect of cultivar and IBA treatment on cuttings' IAA oxidase activity during A) spring, B) summer and C) autumn experimental season. AR, olive cv. 'Arbequina'; KA, olive cv. 'Kalamata'. Asterisks (*) denote the presence of statistically significant differences at each specific time point. The vertical bar at the right side of each diagram is the SE of the Multi-Factor ANOVA which indicates statistically significant differences at α=0.05 (*n*=8).

to 'Arbequina' cuttings (Fig. 5D). Furthermore, no significant seasonal differences in stem anatomy were found in both cultivars (data not presented).

Observations for the first seven days after IBA application showed no detectable anatomical changes in both cultivars (data not presented). By 15 DAP a significant cell proliferation (callus growth) occurred in the phloem region of 'Arbequina' cuttings. The root primordia were visible at the secondary phloem from cambial cells (Fig. 6A-C). On the other hand, in 'Kalamata' cuttings 15 DAP a cell differentiation occurred in the cortex outside the sclerenchyma ring resulting in callus formation (Fig. 7A-B). However, 30 DAP and after callus tissue formed at the base of the cuttings, the sites of initiation of root primordia were identified in the secondary phloem from cambial cells (Fig. 8). In both cultivars, the root primordium that grew outward forced its way between fiber strands, crushed the sclerenchyma ring and reached the outer cortex, attended by progressive differentiation of the vascular system (Fig. 6A-B and Fig. 8). Under optimum conditions, the already described anatomical changes lead to external callus formation (Fig. 7C) as well as root emergence and development (Figs. 6D and 7D).

Discussion

The presence of high concentrations of auxin during the de-differentiation of the cells is of utmost importance for cuttings' rooting (Sagee *et al.*, 1992; Gaspar *et al.*, 1997). Nonetheless, in the later stages of growth and development of root primordia, high concentrations of auxin may be inhibitory thus its reduction is necessary (Gaspar *et al.*, 1997). Therefore certain enzymes (especially POD, PPO and IAAox) can contribute directly or indirectly to the regulation of auxin concentration during Ar (Hartmann *et al.*, 2001). However, there is very little information so far regarding the role of oxidative enzymes in adventitious rooting in olive. Macedo *et al.* (2013) and Porfirio *et al.* (2016b) have investigated and related the role of PPO and POD activities mainly in olive microcuttings, while Aslmoshtaghi & Shahsavar (2016) are among the few researchers who studied the enzyme activities in olive semi-harwood cuttings.

Pretreatment with auxin improved rooting in 'Arbequina' (76% in summer, 40% in autumn and 37% in spring), while the difficult-to-root 'Kalamata' presented only 3% rooting (Fig. 1). The very low rooting ability

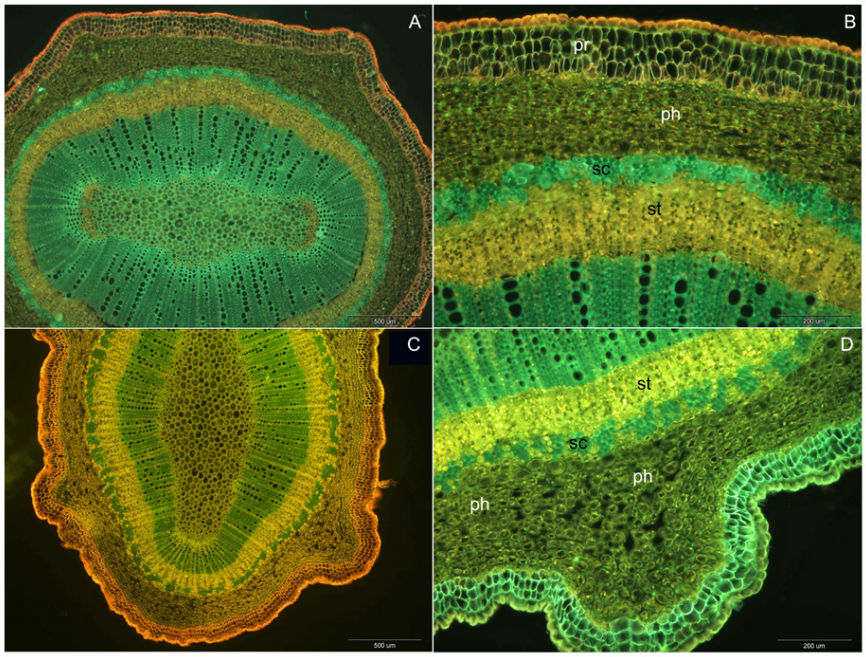

Figure 5. Transverse sections at the rooting zone of *Olea europaea* (L.) stem cuttings before IBA application in 'Kalamata' (A and B) and in 'Arbequina' (C and D) cultivars. pr: periderm; ph: photosynthetic parenchyma; sc: sclerenchyma; st: storage parenchyma. Scale bars: A and C = 500 µm; B and D = 200 µm.

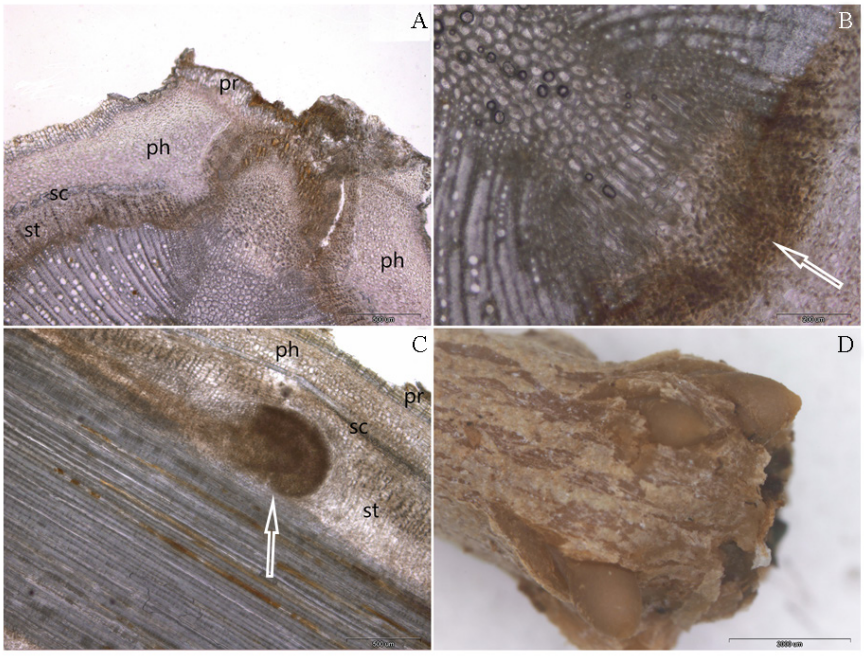

Figure 6. Transverse section at the rooting zone of *Olea europaea* (L.) cv 'Arbequina' stem cutting 15 DAP, showing an organized root primordium with an apical meristem protruding between bundles of phloem fibers in the secondary phloem (A); transverse section of the organized root primordium in 'Arbequina' attended by progressive differentiation of the vascular system (B) (the arrow pointing to the growing root primordium); longitudinal section at the rooting zone of 'Arbequina' cutting 15 DAP showing an organized root primordium (C) (the arrow pointing to the growing root primordium); root emergence in the base of cutting (D). pr: periderm; ph: photosynthetic parenchyma; sc: sclerenchyma; st: storage parenchyma. Scale bars: A, B and C = 500 µm; D = 200 µm.

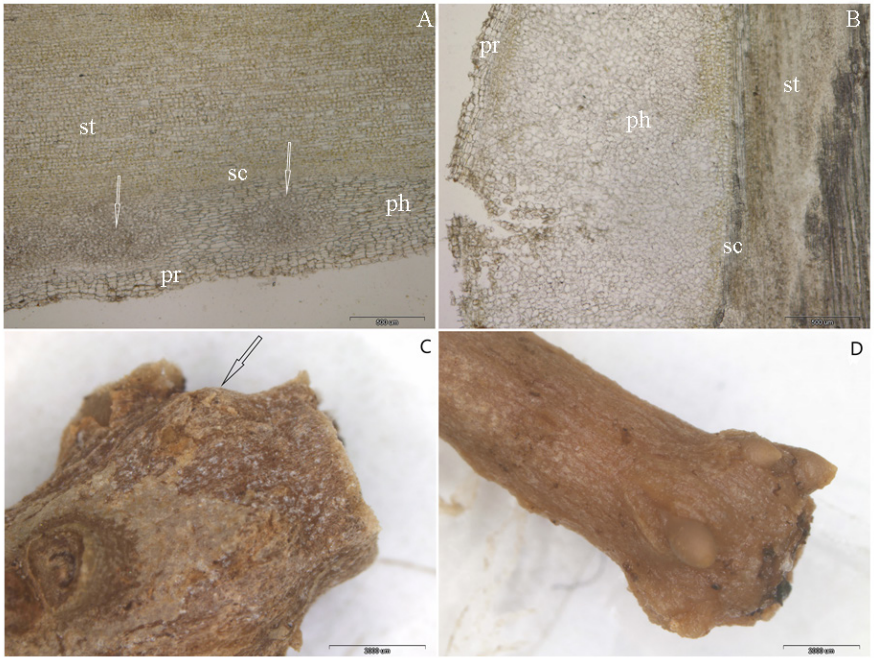

Figure 7. Longitudinal sections at the rooting zone of *Olea europaea* (L.) cv 'Kalamata' stem cuttings showing response to the application of IBA 2000 mg L^{-1}. At 15 DAP callus formation occurred in the photosynthetic parenchyma outside the sclerenchyma ring of cutting (A) (the arrows pointing to the formation of callus tissue); callus cells in the photosynthetic parenchyma 15 DAP (B); callus formation close to the basal end of the cutting's surface as pointing by the arrow (C); root emergence in the base of cutting (D). pr: periderm; ph: photosynthetic parenchyma; sc: sclerenchyma; st: storage parenchyma. Scale bars: A and B = 500 µm; C and D = 200 µm.

of 'Kalamata' is in agreement with the results of Avidan & Lavee (1978) and Wiesman & Lavee (1995), and this was the reason why this cultivar was characterised by the researchers as a difficult-to-root. The present results showed that the auxin treatment was unable to stimulate root initiation even a massive callus was formed in the majority of 'Kalamata' cuttings. The failure of auxin to stimulate rooting in 'Kalamata' suggests that other physiological and/or biochemical factors and/or the different anatomical structure might also be involved in this process.

Many biochemical studies showed a close relationship between PPO, POD, IAAox and adventitious rooting (Ríos *et al.*, 1997; Tchinda *et al.*, 2013). In the

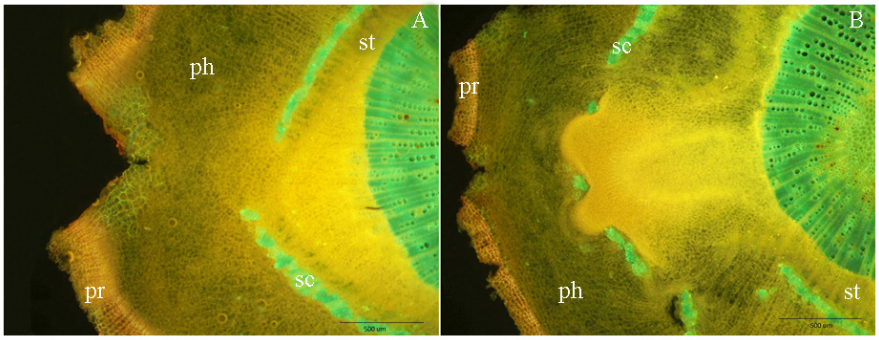

Figure 8. Transverse sections at the rooting zone of olive cv 'Kalamata' stem cuttings 30 DAP showing an organized root primordium with an apical meristem protruding between bundles of phloem fibers in the secondary phloem at different section depths: A, proximal; B, distal section from cutting's base. The continuous sclerenchyma ring was displaced by the proliferation in the phloem. pr: periderm; ph: photosynthetic parenchyma; sc: sclerenchyma; st: storage parenchyma. Scale bars: A and B = 500 µm.

present study, the difficult-to-root 'Kalamata' cuttings exhibited generally higher enzymatic activities at day 0 and during the rhizogenesis phases compared to 'Arbequina' (Figs. 2, 3 and 4). It is noteworthy the fact that PPO and POD activities were, in average, two times higher in 'Kalamata' compared to 'Arbequina', while for the same cultivar IAAox activity presented even an eightfold increase during the initiation phase (3-5 DAP). Ludwig-Müller (2003) also found that the activity of the above enzymes was higher in difficult-to-root species compared to easy-to-root ones. Therefore, the particular high activity of the above enzymes may inhibit the rooting of 'Kalamata' cuttings and could partly justify its recalcitrancy to root. Bansal & Nanda (1981) came to a similar conclusion about the role of these enzymes in the rooting capacity of various forest species.

It is noteworthy that in both cultivars a similar pattern of enzymatic activities was observed between control and IBA treated cuttings (Figs. 2-4). These observations are in agreement with Tchinda *et al.* (2013), as well as with Qaddoury & Amssa (2004), who found that in the control cuttings POD and IAAox activities followed the same pattern as in IBA treated. Data also showed that control cuttings exhibited higher enzymatic activities, but in some cases the detected differences were not statistically significant. The present results appear to contradict the findings of other researchers, where IBA treated leafy cuttings of *Ricinodendron heudelotii* (Tchinda *et al.*, 2013) as well as date palm offshoots (Qaddoury & Amssa, 2004) exhibited higher enzymatic activities. Generally, PPO, POD and IAAox presented increased activities in both cultivars during spring and summer, while in autumn the enzymatic activities decreased (Figs. 2-4). However, it is difficult to establish any relation between the above observed seasonal enzymatic activities and rooting in both cultivars.

PPO, POD, IAAox activity underwent significant changes during the rooting period (RP) (Figs. 2-4). Similar changes in the enzymatic activities have been reported for various plant species such as almond (Caboni *et al.*, 1997), poplar (Güneş, 2000) and vine cuttings (Kose *et al.*, 2011). The determination of POD activity in the basal region of 'Kalamata' cuttings during RP showed that the enzyme activity peaked within 3-5 DAP (Fig. 3A-C). The increase in this enzyme activity was relatively high compared to 'Arbequina' and since peroxidases are known to be involved in IAA catabolism (Caboni *et al.*, 1997; Gaspar *et al.*, 1997), it seems that POD negatively affected the auxin balance at meristematic regions, that corroborate with previous reports made in different species (*Arbutus unedo*, *Taxus baccata* and peach rootstock GF-677) (Metaxas *et al.*, 2004; Molassiotis *et al.*, 2004).

It is well known that POD activity has a fundamental role in root initiation and changes in the activity of this enzyme have been used as a predictive marker of the rooting process (Hartmann *et al.*, 2001; Metaxas *et al.*, 2004). In 'Arbequina', POD activity increased significantly 15 DAP in all experimental seasons, as was also observed in 'Kalamata' cuttings (Fig. 3A-C). An increase in POD activity 15 DAP has been also described in other species like date palm (*Phoenix dactylifera*) (Qaddoury & Amssa, 2003) and apple rootstock MM106 (Naija *et al.*, 2008). According to Porfirio *et al.* (2016b) this increase coincides with the expression phase in olive, confirming the well-known function of POD in cell wall formation and modification (Molassiotis *et al.*, 2004; Konieczny *et al.*, 2014). This increase in POD activity in the present study corresponded to the observed histological changes leading to callus or root formation in 'Arbequina' cuttings (Fig. 6D) as was also observed in olive microcuttings of the 'Galega vulgar' (Macedo *et al.*, 2013). Nonetheless, this POD function seems to occur only in 'Arbequina' as in 'Kalamata' cuttings no root formation was observed.

IAAox activity in 'Kalamata' exhibited the same pattern of increase as POD with maximum enzyme activity 3 DAP in summer and 5 DAP in spring and autumn, followed by a subsequent decrease (Fig. 4A and C). On the other hand, IAAox in the easy-to-root 'Arbequina' decreased during the induction phase (from day 0 to day 1) (Fig. 4). These results are in agreement with those of Husen (2012) and Wiesman *et al.* (1988), who pointed out that the low IAAox activity during the induction phase seems to be responsible for the better adventitious rooting by preserving the source of free auxin. According to Mato & Vieitez (1986) and Basak *et al.* (2000), it appears that low IAAox activity favors rooting in *Castanea sativa* and other species, as was also observed in 'Arbequina' cuttings. Generally, the low enzyme activity in 'Arbequina' compared to 'Kalamata', is likely to regulate endogenous auxin concentration to appropriate levels, in order to trigger the rooting initiation phase and subsequently the rooting expression phase.

Polyphenoloxidase is another enzyme whose activity is in close relationship with root formation, as it may oxidize auxin during growth and development of root primordia (Qaddoury & Amssa, 2003; Tchinda *et al.*, 2013). The results of this study showed that PPO activity of 'Kalamata' cuttings reached its maximum level between 3 and 5 DAP (Fig. 2). The increase in this enzyme activity coincided with the maximum POD and IAAox activities observed for 'Kalamata' cuttings (Figs. 3 and 4). Therefore, PPO may act synergistically to IAAox and POD during the initiation of rhizogenesis (3 to 5 DAP). In 'Arbequina', PPO activity generally

increased 15 DAP during all experimental seasons (Fig. 2A-C). In the following days, callus or root formation was observed in the stem of the 'Arbequina' cuttings (Fig. 6D). Similar changes have been reported in vine and *Ricinodendron heudelotii* (Yilmaz *et al.*, 2003; Kose *et al.*, 2011; Tchinda *et al.*, 2013). Consequently, PPO activity may play a significant role in formation of root primordia in 'Arbequina' cuttings as has been also found in walnut by Cheniany *et al.* (2010). Nevertheless Aslmoshtaghi & Shahsavar (2016) found no clear relationship between PPO activity and root initiation in olive cuttings.

The anatomical observations of this study showed that cells divisions occurred mainly close to the basal end of the cutting surface, as was previously observed by other researchers (Jasik & De Klerk, 1997; Hartmann *et al.*, 2001). In 'Kalamata' cuttings the cell differentiation occurred in the cortex outside the sclerenchyma ring from cells of the photosynthetic parenchyma, resulting in a swelling of the basal part of the cutting and contributing to callus formation, even excessive, which did not lead to root primordia (Fig. 7A-B). These results confirm that callus formation is a characteristic of the difficult-to-root olive cultivars, as has been also reported by Ayoub & Qrunflesh (2008) for the olive cv 'Nabali'. On the other hand, most of the 'Arbequina' cuttings rooted without callus formation while few formed callus (Fig. 6D). These results confirm firstly that in the easy-to-root cultivar, root formation may occur without callus formation and secondly that callusing and adventitious rooting are independent, even though both involve cell division (Hartmann *et al.*, 2001).

The origin of adventitious roots on stem cuttings has been reported to be located in various tissues and it varies from one species to another (Naija *et al.*, 2008; Agulló-Antón *et al.*, 2014). In the present study the origin of root initials in both cultivars was the cambial zone (Figs. 6A-C and 8A-B), as was previously observed in other olive cuttings of cvs. 'Wetaken' (Bakr *et al.*, 1977), 'Manzanillo', 'Mission' and 'Hamed' (Salama *et al.*, 1987).

Several anatomical studies have suggested a correlation between difficulty in rooting and the presence of a thick cortex and a pericyclic sclerenchyma layer (Avidan & Lavee, 1978; Amissah *et al.*, 2008; Porfirio *et al.*, 2016a). In the present study, in the cuttings of the difficult-to-root 'Kalamata', a noticeable thicker cortex and a continuous sclerenchyma ring were observed in comparison with the easy-to-root 'Arbequina' (Fig. 5A-D). The above two anatomical barriers may reduce the absorption and movement of exogenous applied auxin in the inner layers of 'Kalamata' cuttings, resulting in a weak auxin stimulus, not enough to induce de-differentiation of the cambial cells into root initials. This speculation was further supported by the fact that in 'Kalamata' cuttings 15 DAP, a cell differentiation occurred in the cortex outside the sclerenchyma ring resulting in callus formation (Fig. 7A-B), while the cambial cells remained inactivated, as if the auxin stimulus never reached the cambial cells and only triggered the outer layers.

However, further anatomical observations during the rooting period at 30 DAP revealed that, once the cambial cells de-differentiated and the root initials formed in 'Kalamata', the continuous sclerenchyma ring did not constitute an impenetrable barrier as it was easily penetrated by the pressure of the growing primordium (Fig. 8). The present findings support Bakr *et al.* (1977), Avidan & Lavee (1978) and Fabbri (1980) observations, according to whom the difficulty in adventitious rooting of olive cuttings was not related to the anatomical structure of the cutting. Thus, it can be suggested that the inability of 'Kalamata' cuttings to form root primordia is not due to the presence of sclerenchyma ring acting as a physical barrier to root emergence but a barrier that difficults exogenous applied auxins to reach the sites where root primordia are supposed to be induced and further differentiated.

Based on the above, it can be concluded that the adventitious rooting ability of 'Arbequina' and 'Kalamata' is affected by the combined interaction of auxin, enzymatic activities and the anatomical structure, rather than by a single factor alone. In fact, in 'Kalamata' the high enzymatic activities determined during the rhizogenesis period combined with this cultivar's tendency to form a massive callus, without redifferentiation to root primordia, is a more likely reason for the poor rooting percentages.

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