Tracking biochemical changes during adventitious root formation in olive (*Olea europaea* L.)

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Abstract

The activity of oxidative enzymes and the levels of free auxins were determined during adventitious root formation in olive explants. Rooting trials were performed both with *in vitro*-cultured microshoots of the cultivar 'Galega Vulgar', treated with indole-3-butyric acid (IBA) and with salicylhydroxamic acid (SHAM) + IBA, as well as with semi-hardwood cuttings of the cultivars 'Galega Vulgar' (difficult-to-root) and 'Cobrançosa' (easy-to-root), treated with IBA. The auxin (IBA) was used in all experiments as a rooting promoter, while SHAM was used in micropropagation trials as rooting inhibitor, providing a negative control. Free indole-3-acetic acid (IAA) and IBA concentrations were determined in microshoots, as well as in semi-hardwood cuttings, throughout the rooting period at pre-established time-points. At the same time-points, the enzymatic activity of polyphenol oxidases (PPO), peroxidases (POX), and IAA oxidase (IAAox) was evaluated in the microshoots. Microshoots treated with SHAM+IBA revealed higher POX and IAAox activity, as well as lower PPO activity, than those treated only with IBA. IAA levels were higher in IBA-treated microshoots during induction phase, but lower during early initiation phase. In contrast, free IBA levels were higher in microshoots treated with SHAM+IBA during induction, but lower during initiation. A similar pattern of free auxin levels was observed in semi-hardwood cuttings of the two contrasting cultivars under evaluation. The similarities found on the auxin patterns of microshoots treated with SHAM and those of semi-hardwood cuttings of the difficult-to-root olive cultivar allow considering SHAM a reliable control for when simulation of a difficult-to-root behavior is necessary. The inhibitory effect of SHAM in root formation could be related with 1) the inhibition of alternative oxidase (AOX), leading to a downregulation of phenylpropanoid biosynthetic pathways, which would decrease the concentration of phenolic substrates for PPO; 2) an increase in IAAox activity resulting in lower free IAA levels or; 3) a defective conversion of IBA into IAA.

Keywords: indole-3-acetic acid (IAA); indole-3-butyric acid (IBA); oxidative enzymes; phenylpropanoid biosynthetic pathway; salicylhydroxamic acid (SHAM)

1. Introduction

Olive (*Olea europaea* L.) is one of the main crops in the Mediterranean basin, and its production area is expanding as a result of an increase in olive oil consumption worldwide. Olive trees are mainly propagated by cuttings, a process dependent on the ability to form new adventitious roots. However, some important cultivars display a difficult-to-root behavior (Fouad et al., 1990). A scientific answer able to explain this contrasting performance among cultivars is still unavailable despite all the research done on the subject. Adventitious root formation can be divided in three physiological phases: i) **induction**, comprising molecular and biochemical events and corresponding to a period preceding any visible histological modifications, ii) **initiation**, which starts when the first histological events take place, like root primordia organization, being characterized by the occurrence of small cells with large nuclei and dense cytoplasm and iii) **expression**, that involves the development of the typical dome shape structures, intra-stem growth and emergence of root primordia (Pacurar et al., 2014). In olive, induction phase corresponds to the first 96 h after microshoot treatment, initiation corresponds to the period between 96 - 336 h and is followed by expression of roots thereafter (Macedo et al., 2013).

Among the factors that may influence adventitious rooting (reviewed in Porfirio et al., 2016a), oxidative enzymes and auxins are the most studied and discussed. The involvement of auxins in adventitious rooting has been studied for a long time (Wiesman et al., 1989) and they are extensively used in plant propagation protocols as root-inducing compounds (Preece, 2003). The two main natural auxins are indole-3-acetic acid (**IAA**) and indole-3-butyric acid (**IBA**), quantified by various methods, as recently revised by Porfirio et al. (2016b). Evidence suggests that IAA possibly promotes adventitious rooting through a signaling network similar to that happening in lateral roots, involving auxin response factors (ARF) and other plant hormones (Porfirio et al., 2016a). On the other hand, IBA auxin activity seems to be a result of its conversion into IAA (Korasick et al., 2013) by peroxissomal enzymes (Strader and Bartel,

2011; Zolman et al., 2007, 2008). Although the genotype appears to have a stronger influence on rooting performance, changes in auxin concentration have been associated both with the interdependent phases of the process and with the rooting capacity of a species or cultivar (Ayoub and Qrunfleh, 2008; Nag et al., 2001). According to De Klerk et al. (1995), the high auxin levels needed for the success of induction phase become inhibitory during root expression, possibly because high auxin concentrations inhibit root elongation and promote cellular differentiation (Li et al., 2009a). This ultimately implies that IAA catabolism is mandatory to avoid the inhibition of root development..

Oxidative enzymes have long been related to adventitious root formation. Peroxidases (**POX**) are a group of hemic proteins that catalyze the oxidation of diverse electron donors, such as phenolic compounds and IAA (Bandurski et al., 1995; Hiraga et al., 2001), using hydrogen peroxide (H₂O₂) as oxidative agent (Dawson, 1988). A group of POX isoforms, commonly known as IAA-oxidase (**IAAox**), is considered to be responsible for the enzymatic oxidative decarboxylation of IAA (Ljung et al., 2002) and the activity of this group of enzymes has been largely associated with adventitious rooting (Bansal and Nanda, 1981; Güneş, 2000). In fact, three homologous POX isoforms – PRX33, PRX34 and PRX37 – have already been identified as IAAox isoforms in *Arabidopsis* (Passardi et al., 2006; Pedreira et al., 2011). Polyphenol oxidases (**PPO**) are a group of copper-containing oxidative enzymes that catalyze two different reactions: hydroxylation of monophenols to *o*-diphenols (Mayer, 2006) and oxidation of *o*-diphenols to *o*-quinones (Constabel and Barbehenn, 2008). In addition to mono- and di-phenols, PPO are also capable of degrading other phenolic compounds, structurally more complex, such as anthocyanins and other polyphenols (Jiménez and García-Carmona, 1999), and their involvement in adventitious rooting has also been studied previously (Macedo et al., 2013; Porfirio et al., 2016a).

The combined involvement of auxin and POX in plant growth and development has been recently described in *Arabidopsis*, where the gene *FtSH4* was suggested to mediate auxin metabolism, transport

or signaling (Zhang et al., 2014).. The *ftsh4-4* mutants showed growth and developmental deficiencies, including lower IAA levels and higher H₂O₂ levels, which were attributed to a higher POX gene expression, activity and isozyme content. A higher-than-normal POX content and activity would affect auxin levels which, in turn, would result in growth defects. This conclusion was corroborated by the fact that in *ftsh4-4* mutants the most highly expressed POX genes were the IAAox isoforms PRX33, PRX34 and PRX37 (Zhang et al., 2014).

In this work, the temporal changes in free auxin levels and oxidative enzymes activity were evaluated during adventitious root formation in microshoots and semi-hardwood cuttings of two Portuguese olive cultivars; **1)** ‘Galega Vulgar’, which usually presents average rooting rates of 5–20 % when semi-hardwood cuttings are used, but can achieve 60–90 % rooting under optimized conditions for *in vitro* culture (Peixe et al., 2010); **2)** ‘Cobrançosa’, which is considered easy-to-root by semi-hardwood cuttings, with common rooting rates higher than 70 % (Santos et al., 2013), being until now recalcitrant to *in vitro* establishment. This work is aiming to compare two contrasting behaviors concerning root formation using two types of plant material (microshoots and semi hardwood cuttings). For semi-hardwood cuttings, this was accomplished by using the two cultivars under evaluation. For micropropagation trials, considering the recalcitrance of the cv. ‘Cobrançosa’ under *in vitro* culture conditions, microshoots of the cultivar ‘Galega Vulgar’ were treated with salicylhydroxamic acid (**SHAM**). SHAM has been shown to inhibit root formation in olive (Santos Macedo et al., 2012), thus providing a negative control by imitating a difficult-to-root cultivar.

2. Materials and Methods

2.1. Reagents

IAA, IBA, SHAM, 4-methylcatechol and *p*-coumaric acid were purchased from Sigma-Aldrich Quimica, S.A., Sintra, Portugal. Agar-agar, D-mannitol, activated charcoal, sodium acetate, 3-methyl-2-benzothiazolinone-hydrazone-hydrochloride, and isopropanol were all supplied by Merck-Portugal, Lisboa, Portugal. Ethylenediamine-tetra-acetic acid and magnesium chloride were purchased from VWR-Portugal, Carnaxide, Portugal. Phenylmethylsulfonyl fluoride was supplied by AppliChem, Darmstadt, Germany. Hydrogen peroxide was purchased from Alfa Aesar GmbH, Karlsruhe, Germany. Formic acid and ammonium hydroxide were supplied by Merck S.A., Germany. Indole-3-propionic acid was purchased from Sigma-Aldrich, MO, USA. [¹³C₆]IAA was supplied by Cambridge Isotopes Laboratories, MA, USA. Acetone (HPLC-grade), hexane (HPLC-grade), methanol (LC/MS-grade), chloroform (HPLC-grade) and sodium chloride (≥99.0% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Butylated hydroxytoluene (BHT) was purchased from MP Biomedicals (Solon, OH, USA). Hydrochloric acid (HCl) (36.5 – 38.0% purity) was purchased from J.T. Baker (Center Valley, PA, USA). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for GC derivatization (≥99.0% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material, rooting procedures, and culture conditions

In vitro-cultured microshoots and semi-hardwood cuttings were used as initial explants for the rooting trials. *In vivo* trials were performed using semi-hardwood cuttings of a single clone of the olive cultivar ‘Galega Vulgar’ and a single clone of cv. ‘Cobrançosa’, while the *in vitro* experiments were achieved using only a clone of the cultivar ‘Galega Vulgar’.

***In vitro* rooting experiments:** Microshoots established *in vitro* since 2005 and maintained to date according to the protocol proposed by Peixe et al. (2007). Rooting treatments and culture conditions were adapted from Macedo et al. (2013). In brief, explants with four-to-five nodes were prepared from *in vitro*-cultured microshoots, and all leaves, except for the upper four, were removed. The base of each explant (approx. 1.0 cm) was submitted to a 10 s quick-dip treatment either in a sterile solution of 14.7 mM IBA or a sterile solution of 14.7 mM IBA plus 100 mM SHAM (concentration optimized by Santos Macedo et al., 2012). The explants were then inoculated, *in vitro*, in 500 ml glass flasks containing 75 ml semi-solid olive culture medium (OM), devoid of plant growth regulators and supplemented with 7 g L⁻¹ commercial agar-agar, 30 g L⁻¹ D-mannitol and 2 g L⁻¹ activated charcoal (Rugini, 1984). Medium pH was adjusted to 5.8 prior to sterilization in an autoclave (20 min at 121°C). All cultures were kept in a plant growth chamber at 24°C/21°C (± 1°C) day/night temperatures, with a 15 h photoperiod, under cool-white fluorescent lights at a photosynthetically active radiation (PAR) level of 36 µmol m⁻² s⁻² at culture height.

***In vivo* propagation:** Semi-hardwood cuttings (12-15 cm from the middle region of year growing sprouts) of the two cultivars under evaluation were collected from field grown plants (nursery mother-plant field with 10 years after planting). To induce rooting, the base of each cutting (approx. 1.0 cm) was submitted to a 10 s quick-dip treatment in a non-sterile solution of 17.15 mM IBA. After IBA treatment the cuttings were transferred into a water-cooling greenhouse and planted on a rooting bench with bottom heating. The greenhouse air temperature was maintained at 22-24°C and the rooting substrate at 26-28°C. Water loss through transpiration was reduced by removing all leaves except the 4 on the top and by automatically sprinkling water at regular intervals throughout the rooting assay. Relative humidity was maintained at 70-80% throughout the rooting period.

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115 **2.3. Sample collection**

116 During *in vitro* rooting, ten segments from the basal portion (approx. 1 cm from the base) of the
117 explants were collected in triplicate at 4, 8, 24, 48, 96, 144, 192, 240, 336, 432, 528, 624 and 720 h after
118 auxin treatment. In addition, ten segments were collected in triplicate before auxin treatment (0 hours
119 after treatment) and were used as control samples. A total of 840 explants were collected. All samples
120 were flash frozen in liquid nitrogen and stored at -80°C for subsequent enzyme assays.

121 Samples for auxin quantification by gas chromatography / mass spectrometry (GC/MS) were collected
122 similarly, but using double amount of explants, i.e., each sample consisted of twenty segments of plant
123 tissue, totaling 1680 explants.

124 In rooting trials with semi-hardwood cuttings a similar procedure was used for sample collection.
125 However, in this case, the sampling material consisted of a ring-bark of approximately 1 cm-long,
126 including the bottom node of the cutting and was obtained by girdling of the cutting base.

127 These rooting trials also included control samples, taken at the time of collection from the mother plants
128 (1 hour before treatment). In total, 900 cuttings were used. Furthermore, in this case, each replicate
129 used for auxin analysis consisted in 10 segments of plant tissues.

130 A graphic representation of the experimental design used in sample collection is shown in **Fig. 1**.

Fig. 1. Schematic representation of the experimental design used for sample collection. (A) Sample collection for enzymatic activities in microshoots; (B) Sample collection for auxin quantification in microshoots; (C) Sample collection for auxin quantification in semi-hardwood cuttings.

2.4. Extraction of oxidative enzymes

The collected material (ten segments per replicate per time-point) was ground and homogenized in a mortar with liquid nitrogen. Approximately 50 mg of sample was transferred into a 1.5 ml microtube for extraction. Samples were extracted using 1.0 ml of extraction buffer containing 50 mM sodium acetate, 2.0 mM ethylenediamine-tetra-acetic acid (EDTA), 1.0 mM magnesium chloride and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) at pH 5.5. Each extract was vigorously vortexed for 15 s and centrifuged (10,000 x g) at 4°C for 20 min. The supernatant was divided into 4 aliquots and used as crude enzyme extract for quantification of enzyme activity (PPO, POX and IAAox) and total protein quantification. All aliquots were stored at -80°C before use.

2.5. Measurement of soluble peroxidases (POX) and polyphenol oxidases (PPO) activities

Total soluble PPO and POX activities were determined based on Kar and Mishra (1976), Tzika et al. (2009) and Macedo et al. (2013), with modifications.

PPO activity: 100 μ L of crude extract was added to 900 μ L of a buffer solution containing 45 mM sodium acetate, 2 mM 3-methyl-2-benzothiazolinone-hydrazone-hydrochloride (MBTH) and 20 mM 4-methylcatechol at pH 5.5. Soluble PPO activity was determined by measuring the change in absorbance at 490 nm during 1 min using a Beckman DU®530 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA). Enzyme activity was expressed in terms of $\Delta\text{Abs}_{490} \text{ min}^{-1} \text{ protein (mg)}^{-1}$.

POX activity: 100 μ L of crude extract was added to 900 μ L of a buffer solution containing 45 mM sodium acetate, 2.0 mM MBTH, 20 mM 4-methylcatechol and 1.0 mM H_2O_2 at pH 5.5. Soluble POX activity was determined by measuring the change in absorbance at 490 nm during 1 min using a Beckman DU®530 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA). Enzyme activity was expressed in terms of $\Delta\text{Abs}_{490} \text{ min}^{-1} \text{ protein (mg)}^{-1}$.

2.6. Measurement of soluble IAA oxidase (IAAox) activity

IAAox activity was measured using an adaptation of the methods of Güneş (2000) and Nag et al. (2001). The crude extracts were incubated with a buffer containing a fixed amount of IAA and the activity of IAAox was determined indirectly by measuring the remaining amount of residual IAA after the incubation period.

Briefly, 100 μ L of crude extract was added to 650 μ L of a buffer solution containing 12.8 mM sodium acetate, 0.5 mM H_2O_2 , 0.1 mM *p*-coumaric acid and 0.1 mM IAA at pH 5. The mixtures were incubated at 30°C for 5 min. The reaction was stopped by adding 300 μ L of *n*-butanol : formic acid (15:1). A control

corresponding to null activity, where the reaction was immediately stopped at 0 min incubation time by adding *n*-butanol : formic acid (15:1), was added for comparison. Samples were centrifuged at 3000 rpm for 1 min and the upper organic phase was used for further quantification of IAAox activity. IAAox activity was measured indirectly through quantification of residual IAA by high performance thin layer chromatography (HPTLC).

HPTLC: The organic fractions containing residual IAA were applied in silica gel plates (LiChrospher® 0.2 mm, 20 x 10 cm, Merck, Portugal) as 6 mm bands using a semi-automated device (Linomat 4, CAMAG, Muttentz, Switzerland). The plates were previously activated for 15 min at 70°C. By applying known amounts of IAA standard along with the samples, a calibration curve was built in each plate to allow for IAA quantification. After 20 min of pre-conditioning, the plates were eluted with a mobile phase consisting of *n*-butanol : isopropanol : ammonium hydroxide : water (2.5 : 10 : 1 : 1, v/v) in a horizontal developing chamber (CAMAG, Muttentz, Switzerland) using a solvent migration distance of 50 mm. To remove residual ammonia completely, the plates were dried at 110°C on a TLC Plate Heater III (CAMAG, Muttentz, Switzerland) for 2 min, and then cooled to room temperature. Once cooled, the plates were inspected under UV light at 254 nm (Dual wavelength UV lamp, CAMAG, Muttentz, Switzerland) for confirmation of IAA bands (**Supplementary Figure S1**). Plates were scanned (TLC Scanner 2, CAMAG, Muttentz, Switzerland) under monochromatic light in fluorescence mode and residual IAA was quantified by classical densitometry using CATS software version 3.20 /1998 (CAMAG, Muttentz, Switzerland). IAAox activity was expressed in terms of residual IAA (ng) protein (mg)⁻¹.

2.7. Measurement of total protein content

Total protein concentration was determined using the bicinchoninic acid assay (BCA assay kit, Sigma-Aldrich Quimica, S.A., Sintra, Portugal), according to manufacturer recommendations.

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192 **2.8. Auxin quantification by gas chromatography/mass spectrometry (GC/MS)**

193 The collected material (twenty microshoot segments and ten semi-hardwood segments per replicate per
194 time-point) was lyophilized in preparation for auxin extraction. In the case of microshoot samples, auxin
195 extraction and quantification was performed using composite samples as a consequence of the low
196 mass of each individual replicate. Each sample was ground and homogenized in a mortar with liquid
197 nitrogen. About 100 mg of the powdered plant tissue was transferred into a solvent-rinsed 5 ml screw-
198 cap glass tube and extracted according to the protocol described below, using [¹³C₆]IAA and IPA as
199 internal standards for IAA and IBA, respectively. The resulting methanolic extracts were further
200 submitted to dispersive liquid-liquid microextraction (DLLME) followed by microwave derivatization
201 (MAD). Finally, free IAA and IBA quantification was performed by GC/MS-SIM, as described below.

202 Briefly, 3 ml of 80% methanol containing 1 mM BHT (stored at 4 °C before use) was added to each
203 sample to eliminate oxidation processes, and extraction was performed by end-over-end shaking in the
204 dark at 4°C overnight. After extraction, each tube was centrifuged (Beckman-Coulter Allegra 6R) at 3000
205 rpm, 4°C for 10 min with the supernatant being transferred into a solvent rinsed conical glass tube. The
206 residual pellet was re-extracted with 1 ml of methanol for 1 h under the same conditions as described
207 above. Subsequently, the extracts were combined, dried under a stream of nitrogen, redissolved with
208 420 µL of methanol and diluted with water to a final volume of 3 ml. The extract was prevented from
209 being exposed to light at all stages of extraction. The samples were further purified by DLLME by adding
210 0.450 g of NaCl to the aqueous sample and adjusting the pH to 4 with 100 mM HCl. A solvent mixture
211 containing 200 µL of chloroform (CHCl₃) (extractant) and 1 ml acetone (disperser) was injected into the
212 sample via a glass syringe forming a cloudy solution. The mixture was briefly shaken manually, sonicated
213 in ice for 1 min and centrifuged at 3000 rpm for 10 min at 4°C. After centrifugation, the lower organic

layer was collected with a glass syringe (Hamilton, Reno, NV, USA) and transferred into a conical amber GC vial (ThermoScientific, Rockwood, TN, USA). Then, the samples were subjected to MAD, using 100 μ L of BSTFA and by heating the tightly capped vials at 630 watts (W) for 5 min in a commercially available microwave oven (Hamilton Beach P70B20AP-G5W). After cooling, excess reagent was evaporated under a mild stream of N₂ and, immediately after drying, the derivatized samples were dissolved with 100 μ L hexane for subsequent GC/MS analysis.

Samples were analyzed using a 7890A GC system interfaced to a 5975C MSD quadrupole spectrometer (Agilent Technologies, Wilmington, DE, USA), which was equipped with an electron impact (EI) ionization source. The GC was equipped with a 7693 autosampler (Agilent Technologies, Wilmington, DE, USA) and the analysis was performed by a ZB-1 capillary column (Phenomenex, 30 m $\times$ 0.250 mm with 0.25 μ m film thickness df). The injected volume was set at 2 μ L in splitless mode for 1 minute. The front inlet injector temperature was 250°C, and the transfer line temperature was 280°C. The ion source temperature was set at 250°C. The oven conditions used were the following: initial temperature of 80°C held for 2 min, temperature was ramped to 140°C at 20°C/min and held for 2 min, temperature was ramped to 200°C at 2°C/min and held for 5 min and finally, temperature was ramped to 250°C at 30°C/min and held for 10 min. A post-run at 270°C for 5 min was included to completely clean the column. Helium was the carrier gas flowing at 1 ml/min. Samples were analyzed both in full scan and selected ion monitoring (SIM) modes.

2.9. Statistical analysis

Temporal changes in enzyme activities and in auxin levels of semi-hardwood cuttings were analyzed by one-way ANOVA followed by post-hoc Tukey HSD test. Significant differences were considered at $p < 0.05$. Temporal changes in auxin levels of microshoots were analyzed by Student's *t*-tests. Differences

between treatments and between cultivars at specific time-points were analyzed by Student's *t*-tests. Significant differences were considered at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). All analyses were performed using R Studio software package (version 0.98.1083).

3. Results

3.1. Rooting performance of microshoots and semi-hardwood cuttings

As expected, during *in vitro* culture trials IBA treatment promoted rooting of olive microshoots, whereas SHAM had an inhibitory effect on the formation of adventitious roots (**Fig. 2**). Nevertheless, no visual negative traits were observed on growth and nutritional status of the microshoots treated with SHAM (**Fig. 2A and 2B**). SHAM also had no effect on calli formation (**Fig. 2C**), considering that root development was preceded in all microshoots by calli formation at the site of treatment. The inhibitory effect of SHAM in adventitious root formation of olive microshoots was described in terms of rooting percentage and number of roots per microshoot. Microshoots treated only with IBA yielded significantly higher rooting rates and average number of roots per plant than microshoots treated with SHAM + IBA (**Fig. 2D and 2E**).

Fig. 2. Inhibitory effect of SHAM on adventitious root formation in olive microshoots. (A) Microshoots collected 30 days after treatment with 14.7 mM IBA; (B) Microshoots collected 30 days after treatment with 14.7 mM IBA + 100 mM SHAM; (C) Effect of SHAM on calli formation; (D) Effect of SHAM of rooting percentage; (E) Effect of SHAM on number of roots per microshoot. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Data from trials performed with semi-hardwood cuttings of the two selected olive cultivars confirmed their characteristic rooting performance 60 days after treatment: 'Galega Vulgar' showed 4% of rooted cuttings (difficult-to-root) and 'Cobrançosa' presented 60% of rooted cuttings (easy-to-root). The experiments were performed in winter, usually the worst period of the year for rooting, aiming to observe the maximal expression of the cultivars features regarding adventitious root formation.

3.2. Evaluation of activities of oxidative enzymes

The activities of several oxidative enzymes were evaluated during adventitious rooting. Although a similar pattern was observed in microshoots treated only with IBA and those treated with SHAM + IBA (Fig. 3), significant differences in the activity of particular enzymes were detected between treatments (Fig. 4).

Fig. 3. Effect of SHAM treatment on activity levels of oxidative enzymes during adventitious root formation in olive microshoots. Activity levels of PPO (A), POX (B) and IAAox (C) were measured on microshoots treated with IBA (left) and SHAM + IBA (right). Different lower-case letters correspond to statistically significant differences ($p < 0.05$).

In microshoots treated only with IBA (Fig. 3), PPO activity varied significantly throughout adventitious root formation. Thus, such activity increased initially up to a maximum at 24 h, then proceeded to a

278 minimum at 144 h, that was followed by a new significant increase (68%) at 528 h. POX activity
279 decreased significantly until 144 h, increased until 192 h and decreased again at 240 h, maintaining a
280 somewhat constant level until 720 h. IAAox activity was nearly constant during the first 48 h of
281 induction. Then, it showed a significant increase (30%) at 96 h and increased to a maximum thereafter.
282 At 336 h and onwards, no residual IAA was detected, indicating very high IAAox activity levels.

283 On the other hand, microshoots treated with SHAM + IBA (**Fig. 3**), showed a nearly constant PPO activity
284 throughout root formation. In this case POX activity also decreased significantly during root formation,
285 at a more constant pace than in IBA treatment and it stabilized after 192 h. IAAox activity also increased
286 throughout adventitious rooting. Whereas such an increase was not significant until 48 h, two significant
287 increases were observed at 96 h and 192 h. Finally, the maximum IAAox activity was reached at 432h (no
288 detectable residual IAA), exactly 96 hours later than in IBA treatment, remaining stable until the end of
289 the trial, at 720 h.

290 Differences were observed between treatments regarding each enzyme activity (**Fig. 4**). PPO activity
291 levels were typically higher in microshoots treated only with IBA, except at 144 h when this trend was
292 reversed. Indeed, significant differences between treatments were identified at 8, 24, 48, 144, 528, 624
293 and 720 h (**Fig. 4A**). On the contrary, differences between treatments in terms of POX and IAAox
294 activities were less identified. In the case of POX activity, significant differences were only found at 48,
295 144 and 624 h. While at 48 and 144 h activity levels were significantly higher in microshoots treated with
296 SHAM + IBA, microshoots treated with IBA had higher POX activity at 624 h (**Fig. 4B**). Significantly higher
297 IAAox activity was also found in SHAM + IBA microshoots at 96, 192 and 240 h (**Fig. 4C**).

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Fig. 4. Effect of SHAM treatment on individual enzyme activities. (A) PPO activity, (B) POX activity, (C) IAAox activity (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The indicated rooting stages were previously determined by Macedo et al. (2013). The timepoints shown here do not indicate the length of each phase.

3.3. Evaluation of free auxin levels

3.3.1. Free auxin levels in microshoots

Temporal changes in free IAA and IBA were evaluated throughout adventitious root formation in microshoots treated with IBA and with SHAM + IBA (Fig. 5). In both treatments, auxin levels increased drastically in the first hours after treatment and tended to decrease with time. Concerning IBA-treated microshoots, IAA levels reached a peak at 8 h, decreased significantly to a plateau between 24 and 48 h and decreased again until the end of the rooting period (Fig. 5A). In turn, IBA levels showed a similar trend although not so linear: they reached a peak earlier than IAA levels (at 4 h), decreased until 24 h and then increased again at 48 h. After this lower increase, IBA levels decreased until 96 h and a new increase was observed between 96 and 192 h. From this point onwards IBA levels decreased significantly to a minimum at 720 h (Fig. 5B). A similar trend was observed for IBA levels in microshoots treated with SHAM + IBA (Fig. 5D). In contrast, IAA levels in these microshoots showed a very different pattern than

in microshoots treated with IBA alone. In SHAM + IBA treatment, IAA levels also reached a peak at 8 h and decreased markedly at 24 h, but increased again significantly (247%) at 48 h to a level close to that of 4 h, decreasing after this point until the end of the rooting assay (Fig. 5C).

Fig. 5. Changes in free auxin levels during adventitious root formation in olive microshoots treated with IBA (left) and with SHAM + IBA (right). (A) IAA levels in microshoots treated with IBA; (B) IBA levels in microshoots treated with IBA; (C) IAA levels in microshoots treated with SHAM + IBA; (D) IBA levels in microshoots treated with SHAM + IBA. Different lower-case letters correspond to statistically significant differences ($p < 0.05$).

The treatments were markedly different in terms of auxin levels (Fig. 6). In microshoots treated only with IBA, while IAA levels were consistently higher, the reverse was observed for IBA levels, at least during the first stages of root formation. During the first 24h, IAA levels in IBA-treated shoots were 100 – 200 % higher than IAA levels in shoots treated with SHAM + IBA (a representative chromatogram is

shown in **Supplementary Fig. S2**). However, at 48 h this difference was no longer observed, as a result of a marked increase of IAA levels in SHAM + IBA treatment. Actually, at 96 and 144 h IAA levels were significantly higher in SHAM + IBA microshoots (**Fig. 6A**). After this point no differences were observed between treatments in terms of IAA levels. By contrast, IBA levels were significantly higher in SHAM + IBA microshoots until 96 h, especially at 8 h ($317 \pm 6 \mu\text{g/g}$ compared with $130 \pm 1 \mu\text{g/g}$). From 144 to 240 h this trend was reversed and from 336 to 720 h IBA levels decreased progressively to a minimum in both treatments (**Fig. 6B**).

3.3.2. Free auxin levels in semi-hardwood cuttings

Changes in free IAA and IBA levels were evaluated over the rooting period (**Fig. 7** and **Fig. 8**). In ‘Galega Vulgar’ cuttings, IAA levels tended to increase significantly during the first 24 h, decreased to a transient minimum at 48 h and increased again to a peak at 144 h. After this point, IAA levels decreased to a minimum at 192 h and remained relatively constant until 624 h, when a new increase was observed up to 720 h (**Fig. 7A**). Contrarily, in ‘Cobrançosa’ cuttings, IAA levels increased to a maximum at 24h and decreased steeply at 48 h, continuing to decrease until 192 h. Between 240 h and 720 h IAA levels increased significantly, reaching a new peak at 528 h (**Fig. 7C**).

IBA levels described a peak at 4 h in both cultivars, decreasing sharply until 48 h. After this point, in ‘Galega Vulgar’ levels remained low until 624 h, increasing significantly until 720 h (**Fig. 7B**). In turn, in ‘Cobrançosa’ cuttings three statistically significant transient peaks were observed at 96, 336 and 528 h, and IBA levels decreased after this point (**Fig. 7D**).

Fig. 7. Changes in free IAA and IBA levels during rooting of semi-hardwood cuttings. (A) IAA levels of ‘Galega Vulgar’ cuttings; (B) IBA levels of ‘Galega Vulgar’ cuttings; (C) IAA levels of ‘Cobrançosa’ cuttings; (D) IBA levels of ‘Cobrançosa’ cuttings. Different lower-case letters correspond to statistically significant differences ($p < 0.05$). n.q. = not quantified.

Several differences in auxin levels were found between cultivars. IAA levels were significantly higher in ‘Galega Vulgar’ cuttings in early induction phase and also during initiation. Conversely, IAA levels were higher in ‘Cobrançosa’ cuttings during late induction and expression phases (**Fig. 8A**). In contrast, IBA levels were higher in ‘Galega Vulgar’ cuttings during induction phase (4 – 8 h) while ‘Cobrançosa’ cuttings had equal or higher IBA levels than ‘Galega Vulgar’ cuttings during initiation and early expression. Only at the end of the evaluated rooting period (624 – 720 h) did this trend reversed (**Fig. 8B**).

367

368 **4. Discussion**

369 **4.1. Rooting performance as affected by treatments and cultivars**

370 Confirming previous results on this subject (Santos Macedo et al., 2009, 2012), the treatment of olive
371 microshoots with SHAM significantly reduced the rooting percentage and the average number of roots
372 per microshoot, therefore inhibiting the formation of adventitious roots. Also in agreement with
373 previous results obtained in olive (Santos Macedo et al., 2009, 2012), the inhibitory effects of SHAM did
374 not affect calli percentage because in both treatments, calli formation always preceded root
375 development at the site of treatment. These results suggest the inhibitory effect of SHAM is likely
376 related with the later stages of adventitious root induction rather than with cell dedifferentiation. SHAM
377 has been suggested to suppress adventitious rooting in olive by inhibiting AOX activity (Santos Macedo
378 et al., 2009, 2012), which could lead to an increased production of reactive oxygen species (ROS), as
379 documented for tobacco, soybean and pea (Maxwell et al., 1999; Popov et al., 1997; Van Aken et al.,
380 2009). However the exact action of SHAM in adventitious rooting is not well understood and other
381 molecular mechanisms may be involved in the process, as discussed below.

382

383 **4.2. Temporal changes in oxidative enzymes activity**

384 Monophenols, such as SHAM, have been reported to stimulate the enzymatic degradation of IAA by
385 IAAox *in vitro* as well as in maize stems (Grambow and Langenbeck-Schwich, 1983; Lee, 1980) and this
386 effect is dependent on the type of monophenol. *p*-substituted monophenols (as the case of *p*-coumaric
387 acid, used here in the determination of IAAox activity) are described to be more active in IAAox
388 stimulation than *m*- and *o*-monophenols (Lee, 1980). Although the mechanism controlling this effect

hasn't been clarified, phenolic co-factors may act as electron donors allowing recycling of the Fe³⁺-IAAox isoform during IAA degradation (Pedreño et al., 1990). Therefore, *m*-monophenols like SHAM could possibly promote enzymatic IAA catabolism by IAAox, leaving less free IAA available for root formation and ultimately inhibiting rooting. Indeed, we observed that microshoots treated with SHAM + IBA had significantly lower amounts of residual IAA, indicative of a higher IAAox activity, during initiation (at 96, 192 and 240 h). Similar results were found in *Populus* sp., where recalcitrant cuttings had higher IAAox activity throughout the rooting process and IAAox activity reached its peak during root emergence (Güneş, 2000). On the other hand, IAAox activity increased faster in IBA-treated microshoots: at 336 h no residual IAA was detected while in SHAM+IBA-treated microshoots this only happened at 432 h. This could indicate higher levels of IAA during expression phase, which have been described to be detrimental to root formation in apple microcuttings (De Klerk et al., 1995), hindering or delaying the rooting process in SHAM+IBA-treated explants.

IAAox are described as a group of POX isoforms responsible for the enzymatic, H₂O₂-dependent, oxidative degradation of IAA (Ljung et al., 2002). POX are also responsible for the oxidation of many other phenolic compounds, such as lignin precursors (Hiraga et al., 2001). During root induction and early initiation (Macedo et al., 2013), difficult-to-root microshoots treated with SHAM + IBA showed higher POX activity, in agreement with results from Güneş (2000), Faivre-Rampant et al. (1998, 2000) and Ludwig-Müller (2003). Considering that total POX activity was measured in this work, this result is likely related with the increased IAAox activity observed also in SHAM+IBA-treated microshoots. POX are described to have an extremely high isozymic variety (Siegel, 1993), which is reflected in a broad diversity of functions (Passardi et al., 2005). In fact, several reports described changes in the number of POX isoforms during rooting of peach rootstock GF-677, *Nothofagus* sp., *Ebenus cretica* and *Vitis vinifera* (Molassiotis et al., 2004; Pastur et al., 2001; Syros et al., 2004; Vatulescu et al., 2004). Therefore, total POX activity may decrease during rooting, although specific isoforms, such as IAAox, increase their

413 activity to control IAA levels and facilitate the development of new adventitious roots by stimulating
414 lignin formation and cross-linking of cell wall components (Passardi et al., 2004, 2005). This would also
415 explain why IBA-treated explants have higher POX activity during expression phase, since more roots are
416 produced in response to this treatment, in agreement with Tonon et al. (2001). A decrease in POX
417 activity during root formation, also described by Tartoura et al. (2004) and Fekete et al. (2002), could be
418 a result of the root-inducing treatment itself. IBA treatments significantly decreased POX activity in
419 mung bean seedlings (Li et al., 2009b) and naphthaleneacetic acid (NAA) has been described to have a
420 suppressive effect on POX gene expression in soybean hypocotyls (Chen et al., 2002). This suppressive
421 effect could be a results of auxin responsive elements that are regulated by exogenously applied auxins,
422 as suggested by results from *Cinnamomum kanehirae* (Cho et al., 2011).

423 A reversed behavior between POX and IAAox has already been reported in *Zea mays* (Beffa et al., 1990)
424 and *Populus tomentosa* (Jinyao et al., 2001). After chromatographic purification of maize extracts, Beffa
425 et al. (1990) described that fractions containing a high IAAox activity showed a low POX activity and vice
426 versa. Higher POX activity has also been related with lower rooting ability in *Arbutus unedo*, *Taxus*
427 *baccata* and peach rootstock GF-677 (Metaxas et al., 2004; Molassiotis et al., 2004). Furthermore, SHAM
428 can act as a substrate for some POX (Gumiero et al., 2010), which would also contribute to higher POX
429 activity in SHAM+IBA-treated microshoots. Unlike previous reports from other species such as *Casuarina*
430 *equisetifolia* and *Asparagus* sp. (Gaspar et al., 1992; Rout et al., 1996), a clear relationship between POX
431 activity and rooting ability couldn't be established from our results, which had already been described
432 by other authors (Güneş, 2000). Nevertheless, the results presented here confirm previous work also
433 describing a significant decrease in POX activity in the first 24h after IBA treatment, a period included in
434 the induction phase in olive (Macedo et al., 2013).

435 The biggest changes in enzyme activity were observed in PPO activity. While in SHAM+IBA-treated
436 microshoots no major changes were detected during root formation, in IBA-treated explants PPO
437 activity significantly increased during induction, decreased during initiation and increased again during
438 expression. These results are in agreement with results from Qaddoury and Amssa (2003), Satisha et al.
439 (2008) and Cheniany et al. (2010), who observed a larger magnitude of changes in PPO activity in easy-
440 to-root cultivars of *Phoenix dactylifera*, *Vitis* sp. and *Juglans regia*. Sharp increases of PPO activity and
441 higher PPO activity have also been related with enhanced rooting in other species (*Bruguiera parviflora*,
442 *Cynometra iripa*, *Excoecaria agallocha*, *Heritiera fomes*, *Thespesia populnea*, *Eucalyptus urophylla*)
443 (Basak et al., 2000; Li et al., 1999). The increased PPO activity during rooting, previously described in
444 olive (Macedo et al., 2013), could be associated with lignification processes and/or phenolic metabolism
445 (Batish et al., 2008), or could be related to H₂O₂ levels. H₂O₂ has been suggested to work as signaling
446 molecule, acting downstream in the auxin signaling pathway, mediating auxin responses prior to
447 adventitious rooting in cucumber (Li et al., 2007, 2009a). Li et al. (2009c) reported an increase in
448 endogenous H₂O₂ levels in mung bean seedlings after IBA treatment and removal of the primary root,
449 suggesting that IBA may induce rooting indirectly through a pathway involving H₂O₂. Further evidence
450 showed that H₂O₂ treatments, which enhanced adventitious rooting, stimulated PPO activity in
451 *Chrysanthemum* (Liao et al., 2010), possibly through activation of AOX (Santos Macedo et al., 2009). IBA
452 treatments promote AOX gene transcription in olive (Santos Macedo et al., 2012), which in turn can
453 stimulate phenylpropanoid biosynthesis (Sircar et al., 2012; Vogt, 2010) leading to an increased
454 concentration of monophenolic compounds, which are natural substrates of PPO. This would also
455 explain why in microshoots treated with SHAM, an AOX inhibitor, no visible changes in PPO were
456 detected. Alternatively, auxin could promote the apoplastic production of ROS that increase cell wall
457 extensibility by promoting the breakdown of polysaccharides and proteins (Schopfer et al., 2002). In
458 response to the increased generation of ROS, the plant could produce more phenolic compounds with

antioxidant properties to control the oxidative burst and the accumulation of these PPO substrates would then stimulate an increase in PPO activity.

4.3. Temporal changes in free auxin levels

Significant fluctuations in IAA and IBA levels were found throughout adventitious rooting in explants treated with IBA and with SHAM + IBA. As a result of the root inducing treatment, free IBA levels increased steeply during the first 4 h in both treatments suggesting that the inhibitory effect of SHAM is not related with IBA. In fact, IBA levels in microshoots treated with SHAM + IBA were higher than in microshoots treated with IBA alone. Also in both treatments, IBA levels decreased significantly at 24 h and increased again up to a transient peak at 48 h. During this period, IAA levels had a similar increase in the first hours after treatment, peaking at 8h. This delay in the peak of auxin levels points to a conversion of IBA into IAA, as described to happen in olive (Epstein and Lavee, 1984) and other species such as *Arabidopsis* (Ludwig-Müller et al., 2005; Strader et al., 2011). Thus, as proposed by some authors (Korasick et al., 2013; Strader and Bartel, 2011), the root inducing effect of IBA treatments is likely to happen indirectly through an increase in IAA levels during induction phase, which has been described to be a requirement for successful adventitious root formation in apple microcuttings (De Klerk et al., 1995). However, the high IAA concentrations essential for induction phase, become inhibitory during initiation. Interestingly, higher IAA levels were found in IBA-treated microshoots during induction but not during early initiation: actually at 96 and 144 h IAA levels were higher in SHAM+IBA-treated microshoots. Moreover, in IBA-treated microshoots, IAA levels decreased progressively after 8 h until the end of the rooting period, while in explants treated with SHAM + IBA a notorious peak was observed at 48 h. Furthermore, SHAM-treated microshoots had higher IBA levels, yet lower IAA levels, than IBA-treated explants. All these results suggest that root inhibition by SHAM + IBA treatment is partly caused by

excessive auxin levels, in agreement with De Klerk et al. (1995). Curiously, in contrast to IAA levels, IBA levels were lower in SHAM+IBA-treated microshoots during initiation phase, which may indicate that a defective IBA-IAA conversion could also be one of the causes for rooting impairments in these explants.

The temporal changes of IAA levels also correspond to changes in IAAox activity, as an inverse relationship between IAAox activity and IAA levels was found. IAAox activity was lower during induction (when IAA levels were higher) and increased thereafter reaching a maximum during expression phase, when IAA levels decreased to a minimum. Although the changes in IAA levels observed in SHAM+IBA-treated microshoots did not perfectly correspond to changes in IAAox, the possibility of IAA conjugates controlling auxin levels cannot be excluded. In fact, Tartoura et al. (2004) showed that the levels of conjugated IAA have a reverse trend to those of free IAA levels, increasing during expression phase when free IAA levels decrease to a minimum. These authors actually suggest that conjugates, rather than IAAox, are responsible for regulating IAA levels during the primary stages of adventitious rooting of *Vigna radiata* cuttings. Indeed auxin conjugates have a key role in the regulation of auxin levels (reviewed in Korasick et al. (2013); Ludwig-Müller, 2011). Consequently, it must be considered the possibility that SHAM+IBA-treated microshoots may have different levels of conjugated IAA and/or IBA and that this affects IAA levels more than IAAox activity. Surely, quantification of conjugated auxin levels would definitely help clarifying this matter and it is an important area of future work.

4.4. Relationship between data from microshoots and semi-hardwood cuttings

Several similarities were found between the results obtained with *in vitro*-cultured microshoots and those obtained with semi-hardwood cuttings. In semi-hardwood cuttings, free IAA and IBA levels also increased during the first hours after IBA treatment, likely as a result of auxin absorption by the cuttings. A peak of free IAA was observed at 144 h during initiation and this peak was even higher than the one at

24 h during induction. This resembles the evolution of IAA levels in SHAM+IBA-treated microshoots, where rooting was also inhibited. On the contrary, IAA levels in 'Cobrançosa' cuttings increased to a peak at 24 h and decreased during initiation phase, resembling microshoots treated with IBA alone which displayed high rooting rates. Similarly, IBA levels were higher in 'Galega Vulgar' during induction and lower during initiation, a pattern also observed between IBA and SHAM + IBA treatments. Moreover, at 528 h a peak in auxin levels was found in 'Cobrançosa' cuttings but not in 'Galega Vulgar' cuttings. However, the meaning of this peak is currently unknown.

5. Conclusions

To the best of our knowledge, this was the first attempt to evaluate the molecular mechanisms involved on the adventitious root formation process in *O. europaea*. In fact, whereas the role of oxidative enzymes and auxins is broadly described in the literature, the results tend to be species- or genotype-dependent and studies approaching this subject in olive are scarce.

Although further work is needed to help explaining the precise mechanisms involved in adventitious root formation, especially by integrating knowledge on its molecular basis with its genetic control, the data presented and its interpretation seem to allow proposing an integrated perspective of the molecular pathways which may putatively regulate the process in olive (**Fig. 9**). Root-inducing treatments commonly used in propagation procedures are usually based on the exogenous application of auxins such as IBA (IBA_{exo}), which have been proposed to promote AOX gene transcription (Santos Macedo et al., 2012). The resulting increase in AOX activity may lead to two consecutive metabolic consequences: i) indirect stimulation of the biosynthesis of phenylpropanoids (Sircar et al., 2012), many of which derivatives are substrates of PPO and/or POX, and ii) direct decrease of H₂O₂ levels which may negatively affect POX activity at substrate level, allowing phenylpropanoids to be more available for

528 other metabolic pathways such as accumulation of monophenolics. This would facilitate PPO action and
529 the consequent polymerization of the resulting products. Resulting polymers, such as precursors of
530 lignin (which is necessary for cell wall synthesis and expansion) (Hiraga et al., 2001; Vanholme et al.,
531 2010) are susceptible to be metabolized by POX enzymes, which could ultimately act on these substrates
532 once H₂O₂ levels rebound after the initial AOX activity decreases, at the end of induction or early
533 initiation. Moreover, IAA degradation by IAAox during this phase, also generates ROS (Schopfer et al.,
534 2002), which may stimulate the production of antioxidant phenolic compounds, also increasing PPO
535 activity.

536 On the other hand, exogenously applied IBA can be directly converted into free IAA (Epstein and Lavee,
537 1984), which can then be conjugated with sugars or aminoacids for storage (Ludwig-Müller, 2011). Our
538 results indicate that differences in conversion and/or conjugation of IBA and IAA may explain different
539 rooting behaviors.

540 Finally, SHAM may inhibit adventitious rooting in different ways: i) as a POX substrate (Gumiero et al.,
541 2010), increasing the activity of these enzymes; ii) stimulating IAA degradation by enhancing IAAox
542 activity (Lee, 1980); or iii) inhibiting AOX during induction phase, as proposed by Santos Macedo et al.
543 (2009, 2012). Comprehending the exact role of SHAM, as well as evaluating the changes in conjugated
544 auxins during adventitious rooting are fundamental pieces of information necessary to complete this
545 puzzling subject.

Fig. 9. Schematic representation of molecular pathways putatively involved in olive adventitious root formation.

It was also possible to infer from data analysis that SHAM treatments in *in vitro* cultured microshoots can imitate a difficult-to-root cultivar and thus provide a negative control for comparative studies on adventitious root formation of olive cuttings. Bearing in mind that studies involving semi-hardwood cuttings are currently detrimental, considering the highly random response associated with this type of plant material, *in vitro* studies can be performed instead, to compensate for this high variability.

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