UNDERSTANDING THE ROLE OF AUXINS AND OXIDATIVE ENZYMES ON ADVENTITIOUS ROOT FORMATION IN OLIVE (*OLEA EUROPAEA* L.) CULTIVARS

Sara Porfírio

Tese apresentada à Universidade de Évora

para obtenção do Grau de Doutor em Ciências Agrárias

ORIENTADORES: *Marco Diogo Richter Gomes da Silva*
Augusto António Vieira Peixe
Maria João Pires de Bastos Cabrita
Parastoo Azadi

ÉVORA, MAIO 2016

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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But a PhD is (definitely!) not only traveling and having fun. I was always told that a PhD demands a lot of work and effort from the candidate, and now I can testify on how truthful this statement is. I was also told that a PhD is not about being smart, it's about being stubborn, and stubbornness is a crucial characteristic of a PhD student as one thing I've learned is that grad school has the power to make you extremely aware of your weaknesses at times. Nevertheless, it's by recognizing those weaknesses that we are able to grow professionally and scientifically, and in the end the rewards we get from it are certainly worth it.

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Abstract

Olive (*Olea europaea* L.), one of the main crops in the Mediterranean basin, is mainly propagated by cuttings, a classical propagation method that relies on the ability of the cuttings to form adventitious roots. While some cultivars are easily propagated by this technique, some of the most interesting olive cultivars are considered difficult-to-root which poses a challenge for their preservation and commercialization. Therefore, increasing the current knowledge on adventitious root formation is extremely important for species like olive. This research focuses on evaluating the role of free auxins and oxidative enzymes on adventitious root formation of two olive cultivars with different rooting ability - 'Galega vulgar' (difficult-to-root) and 'Cobrançosa' (easy-to-root). In this context, free auxin levels and enzyme activities were determined in *in vitro*-cultured 'Galega vulgar' microshoots and in semi-hardwood cuttings of cvs. 'Galega vulgar' and 'Cobrançosa'.

To attain this goal, an analytical method for the quantification of free indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) was developed, which is based on dispersive liquid-liquid microextraction followed by microwave derivatization (DLLME-MAD) and gas chromatography-mass spectrometry (GC/MS) analysis. The developed method was validated in terms of linearity, recovery, limit of detection (LOD) and limit of quantification (LOQ) and proved to be useful in the analysis of two very different types of plant tissues. The results from auxin quantification in olive samples point at a relationship between free auxin levels and rooting ability of both microshoots and semi-hardwood cuttings. A defective IBA-IAA conversion, resulting in a peak of free IAA during initiation phase, seems to be associated with low rooting ability.

Likewise, differences in the activity of oxidative enzymes also appear to be related with rooting ability. Higher polyphenol oxidases (PPO) activity is likely related with an easy-to-root behavior, while the opposite is true for peroxidases (POX) (including IAA oxidase (IAAox)) activity. A possible hypothesis for adventitious root formation in olive microcuttings is presented herein for the first time. Free auxins, oxidative enzymes, alternative oxidase (AOX) and reactive oxygen species (ROS) are some of the factors that may be involved in this highly complex physiological process. Interestingly, while temporal changes in auxin levels were similar between microshoots and semi-hardwood cuttings, the conclusions obtained from enzyme activity results in microshoots didn't translate to semi-hardwood tissues, showing the emerging need for adaptation of classical agronomical research studies to modern techniques.

Resumo

Procurando compreender o papel das auxinas e enzimas oxidativas na formação de raízes adventícias em cultivares de oliveira (Olea europaea L.)

A oliveira (*Olea europaea* L.) é uma das principais culturas da bacia Mediterrânica e é propagada maioritariamente por estacaria, um processo altamente dependente da capacidade das estacas para formar raízes adventícias. Enquanto algumas cultivares são fáceis de propagar desta forma, algumas das cultivares de oliveira mais interessantes são consideradas difíceis de enraizar, o que dificulta a sua preservação e comercialização e torna extremamente importante aprofundar o conhecimento sobre o enraizamento adventício desta espécie. Este trabalho foca-se na avaliação do papel das auxinas livres e das enzimas oxidativas na formação de raízes adventícias em duas cultivares de oliveira com diferente capacidade de enraizamento - 'Galega vulgar' (difícil de enraizar) e 'Cobrançosa' (fácil de enraizar). Neste contexto, determinaram-se os níveis de auxinas livres e as actividades de enzimas oxidativas em microestacas de 'Galega vulgar' cultivadas *in vitro* bem como em estacas semi-lenhosas das cvs. 'Galega vulgar' e 'Cobrançosa'. Para tal foi necessário desenvolver uma metodologia analítica para a quantificação de ácido indol-3-acético (IAA) e ácido indol-3-butírico (IBA), baseada em microextracção dispersiva líquido-líquido (DLLME) seguida de derivatização em microondas (MAD) e análise por cromatografia gasosa acoplada a espectrometria de massa (GC/MS). O método desenvolvido foi validado em termos de linearidade, recuperação, limite de detecção (LOD) e limite de quantificação (LOQ), e mostrou-se eficaz na análise de dois tipos de tecidos vegetais bastante diferentes. Os resultados da análise de auxinas em amostras de oliveira apontam para uma possível relação entre os níveis de auxinas livres e a capacidade de enraizamento, tanto em microestacas como em estacas semi-lenhosas. Uma conversão IBA-IAA deficiente, que resulta num pico de IAA durante a fase de iniciação, parece estar associada à baixa capacidade de enraizamento. Por outro lado, a capacidade de enraizamento também parece estar relacionada com diferenças na actividade de enzimas oxidativas. Comportamentos fáceis de enraizar estão associados a actividade mais elevada das polifenoloxidases (PPO), enquanto o oposto é verdade para a actividade das peroxidases (POX) (incluindo a IAA oxidase (IAAox)). Neste trabalho propõe-se pela primeira vez uma possível explicação para o enraizamento adventício em microestacas de oliveira. Auxinas livres, enzimas oxidativas, oxidase alternativa (AOX) e espécies reactivas de oxigénio (ROS) são alguns dos factores envolvidos neste processo fisiológico altamente complexo. Curiosamente, enquanto as alterações temporais nos níveis de auxinas foram semelhantes entre microestacas e estacas semi-lenhosas, o mesmo não se observou relativamente à actividade enzimática, o que mostra a necessidade de adaptação dos estudos agronómicos tradicionais às técnicas correntes.

Abbreviations

[C4mim][PF6]	1-butyl-3-methylimidazolium hexafluorophosphate
2,4-D	2,4-dichlorophenoxyacetic acid
2D-GC	Two-dimensional GC
2D-HPLC	Two-dimensional HPLC
4-APBA	4-aminophenylboronic acid
4-Cl-IAA	4-chloroindole-3-acetic acid
ABA	Abscisic acid
ABCB proteins	Class of ATP-binding cassette (ABC) transporters
ABP1	Auxin-Binding Protein 1
ACC	1-aminocyclopropane-1-carboxylic acid
AEMP	2-(2-aminoethyl)-1-methylpyrrolidine
AM	Arbuscular mycorrhizae
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
anti-IAA	Monoclonal antibodies against IAA
AOX	Alternative oxidase
APF	6-Oxy-(acetyl piperazine) fluorescein
ARF	Auxin Response Factor
ASE	Accelerated solvent extraction
AuNPs	Gold nanoparticles
AUX1/LAX proteins	AUXIN1/Like AUXIN1 proteins
BCA	Bicinchoninic acid assay
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BTA	3-bromoactonyltrimethylammonium bromide
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CE-ECL	CE coupled with electrochemiluminescent detection
CE-LIF	CE coupled with laser-induced fluorescence detection
CK	Cytokinin
CNT	Carbon nanotube
COI1	Coronatine insensitive protein 1
CZE	Capillary zone electrophoresis
DAD	Diode array detector
DCC	N,N'-dicyclohexylcarbodiimide
dCPE	Dual-cloud point extraction
DFMA	Difluoromethylarginine
DFMO	Difluoromethylornithine
Dicamba	3,6-dichloro-2-methoxybenzoic acid

DLLME	Dispersive liquid-liquid microextraction
DPV	Differential pulse voltammetry
DW	Dry weight
EDTA	Ethylenediamine-tetra-acetic acid
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
EOF	Electroosmotic flow
FLD	Fluorescence detector
FW	Fresh weight
GC	Gas chromatography
GC/MS	Gas chromatography coupled with mass spectrometry
GC/MS-CI	Gas chromatography coupled with mass spectrometry and chemical ionization
GC-ECD	GC coupled with electron capture detection
GC-FID	GC coupled with flame ionization detector
GH3	Gretchen Hagen3 auxin-responsive genes
H_2O_2	Hydrogen peroxide
HF-LLLME	Hollow fiber-based liquid-liquid-liquid microextraction
HOOBt	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HPLC-FLD	High performance liquid chromatography coupled to fluorescence detection
HPLC-UV	High performance liquid chromatography coupled to ultraviolet (UV) detection
HPTLC	High performance thin layer chromatography
HRP-IgGs	HRP-labeled immunoglobulins
IAA	Indole-3-acetic acid
IAA-Ala	Indole-3-acetyl-alanine
IAA-Asp	Indole-3-acetyl-aspartate
IAA-Glu	Indole-3-acetyl-glutamate
IAA-Gluc	Indole-3-acetyl-glucose
IAA-Gly	Indole-3-acetyl-glycine
IAA-HRP	IAA labeled with horseradish peroxidase
IAA-Inos	Auxin-myo-inositol conjugates
IAA-Leu	Indole-3-acetyl-leucine
IAAox	IAA oxidase
IAA-Phe	Indole-3-acetyl-phenylalanine
IAA-Trp	Indole-3-acetyl-tryptophan
IAA-Val	Indole-3-acetyl-valine
IBA	Indole-3-butyric acid
ICA	Indole-3-carboxylic acid

IEC	Ion exchange chromatography
IPA	Indole-3-propionic acid
IT	Ion trap
JA	Jasmonic acid
LAX3	Class of Like AUX1 (LAX) proteins
LC	Liquid chromatography
LC x LC	Comprehensive 2D-HPLC
LC/MS	Liquid chromatography coupled with mass spectrometry
LLE	Liquid-liquid extraction
LPD	Liquid phase desorption
MAE	Microwave-assisted extraction
Mag-MIPs	Magnetic MIPs
MBTH	3-methyl-2-benzothiazolinone-hydrazone-hydrochloride
MeIAA	IAA methyl ester
MEKC	Micellar electrokinetic chromatography
MIM	Molecularly imprinted monolayer
MIMs	Molecularly imprinted microspheres
MIPs	Molecularly imprinted polymers
MISPE	Molecularly imprinted SPE
MPA-CdS/RGO	3-mercaptopropionic acid stabilized CdS/reduced graphene oxide nanocomposites
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSI	MS imaging
NAA	1-naphtaleneacetic acid
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NGS	Next Generation Sequencing
OM	Olive medium
PAA	Phenylacetic acid
PAR	Photosynthetically active radiation
pCEC	Pressurized capillary electrochromatography
PDA	Photodiode array detector
PDAB	p-(dimethylamino)benzaldehyde
PEC	Photoelectrochemical immunosensor
PIN proteins	PIN-FORMED proteins
PMSF	Phenylmethylsulfonyl fluoride
POX	Peroxidases
PPO	Polyphenol oxidases
Put	Putrescine

PVP	Polyvinylpirrolidone
Q ICR FT-MS	Quadrupole ion cyclotron resonance Fourier transform MS
QC	Quiescent center
QCM	Quartz crystal microbalance
qMS/MS	Tandem quadrupole MS
QTOF	Quadrupole time-of-flight
Q-Trap	Triple quadrupole linear ion trap
QuEChERS	Acronym for quick, easy, cheap, effective, rugged and safe
RI	Refractive index
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RP	Reversed phase
SACE	Sol-gel-alginate-carbon composite electrode
SCF^{TIR1}	Skp, Cullin, F-box containing complex (Transport Inhibitor Response 1)
SEC	Size exclusion chromatography
SHAM	Salicylhydroxamic acid
SIM	Selected ion monitoring
Spd	Spermidine
SPE	Solid-phase extraction
Spm	Spermine
SPME	Solid-phase microextraction
SPR	Surface plasmon resonance
SRM	Selected reaction monitoring
TIBA	3,4,5-triiodobenzoic acid
TMB	3,3',5,5'- tetramethylbenzidine
TMOS	Tetramethoxysilane
TOF-MS	Time-of-flight mass spectrometry
Trp	Tryptophan
VMAE	Vacuum microwave-assisted extraction
VPE	Vapor phase extraction
WOX5	WUSCHEL-RELATED HOMEOBOX 5 transcription factors

Thesis publications

The present work is based on the following manuscripts:

Porfírio S., Da Silva M.D.R., Cabrita M.J., Azadi P., Peixe A. (2016), Reviewing current knowledge on olive (*Olea europaea* L.) adventitious root formation. *Scientia Horticulturae* **198**: 207-226. doi:10.1016/j.scienta.2015.11.034

Porfírio S., Gomes da Silva M., Peixe A., Cabrita M.J., Azadi P. (2016), Current analytical methods for plant auxin quantification – A review. *Analytica Chimica Acta* **902**: 8-21. doi:10.1016/j.aca.2015.10.035

Porfirio S., Sonon R., Gomes da Silva M.D.R., Peixe A., Cabrita M.J., Azadi P. (submitted), Development of a dispersive liquid-liquid microextraction microwave assisted derivatization method for the quantification of free auxins in olive (*Olea europaea* L.) cuttings and microshoots by gas chromatography / mass spectrometry analysis.

Porfirio S., Calado M.L., Noceda C., Cabrita M.J., Da Silva M.G., Azadi P., Peixe A. (2016), Tracking biochemical changes during adventitious root formation in olive (*Olea europaea* L.). *Scientia Horticulturae* **204**: 41-53. doi:10.1016/j.scienta.2016.03.029

Other publications

Macedo E., Vieira C., Carrizo D., **Porfírio S.**, Hegewald H., Arnholdt-Shmitt B., Calado M. L., Peixe A. V. Adventitious root formation in olive (*Olea europaea* L.) microshoots: anatomical evaluation and associated biochemical changes in peroxidases and polyphenol oxidases activities (2013). *Journal of Horticultural Science and Biotechnology* **88** (1): 53–59

Peixe A., Calado M. L., **Porfírio S.** (2013), Propagação da oliveira – metodologias e sua evolução. In: *O grande livro da oliveira e do azeite*, Dinalivro Editora, Lisboa, Portugal, 101-119. ISBN 978-972-576-620-0

Macedo E., Vieira C., Carrizo D., **Porfírio S.**, Hegewald H., Arnholdt-Schmitt B., Calado M. L., Peixe A. (2012), Formação de raízes adventícias em microestacas de oliveira (*Olea europaea* L.); avaliação anatómica e alterações bioquímicas associadas à atividade das peroxidases e polifenoloxidases, *VI Simpósio Nacional de Olivicultura*, Actas Portuguesas de Horticultura n. 21: 25-37

Results from this thesis have been presented in several scientific events:

Oral presentations

Peixe A., Noceda C., **Porfírio S.** (2013), Procurando compreender a dificuldade de enraizamento de algumas variedades de oliveira (*Olea europaea* L.), *Workshop Nacional "Investigação em Olivicultura e Azeite – Resultados e Aplicações*", June 27-28, Évora – Portugal.

Porfirio S., Sonon R., Peixe A., Cabrita M.J., Gomes da Silva M., Azadi P. (2015), "Development of a Dispersive Liquid-Liquid Microextraction Microwave Derivatization Method for the Quantification of Free Auxins from Olive (*Olea europaea* L.) Cuttings by GC/MS", *Pittcon 2015 Conference and Expo*, March 8-12 New Orleans, LA, USA

Poster publications

Porfírio S., Hegewald H., Carrizo D., Vieira C., Peixe A., Calado M. L. (2012), *New data on the activity of oxidative enzymes during rooting of semi-hardwood olive (Olea europaea L.) cuttings*, VII Simpósio Internacional de Olivicultura, San Juan, Argentina

Porfírio S., Sonon R., Gomes da Silva M., Peixe A., Cabrita M.J., Azadi P. (2015), *Dispersive liquid-liquid microextraction and microwave derivatization applied to the quantification of free auxins from olive (Olea europaea L.) cuttings by GC/MS*, 9th Annual Glycoscience Symposium, Complex Carbohydrate Research Center, The University of Georgia, Athens, GA, USA

Preface

Introduction and context

This dissertation is submitted as partial fulfillment of the requirements for the Doctoral Degree in Agronomical Sciences and includes the results of my Ph.D. carried out from September 2011 to August 2015 in the Escola de Ciências e Tecnologia from the Universidade de Évora in cooperation with LAQV – REQUIMTE, Departamento de Química da Faculdade de Ciências e Tecnologia – Universidade Nova de Lisboa. Part of this work was performed abroad, as visiting research scholar at the Complex Carbohydrate Research Center from The University of Georgia, Athens, GA, USA.

Problem statement

Adventitious root formation in Olea europaea (L.)

Olive is one of the most important crop fruit species in the Mediterranean basin, where 95% of the world's olive orchards are planted, and is mainly propagated by traditional methods using semi-hardwood cuttings. However, while some cultivars root very easily, others present very low rooting rates and can even be considered recalcitrant in many cases. Thus, improving rooting ability in cuttings from recalcitrant olive cultivars has become a critical topic, which implies fundamental research on the anatomy, physiology, biochemistry and genetics of the adventitious root formation process. Research on olive adventitious root formation has been mostly based on trial and error approaches, contributing to a high dispersion of information and a lack of systematic studies. For this reason, this thesis is focused on understanding the biochemical mechanisms governing the process of adventitious root formation in olive (*Olea europaea* L.) cuttings.

Auxins and oxidative enzymes

Among other factors, the involvement of auxins and oxidative enzymes in the regulation of this complex physiological process has been suggested by many authors. However, we are still very far from fully understanding the underlying mechanisms that control adventitious rooting in *O. europaea* (L.).

Auxins are a class of phytohormones widely used in plant propagation to induce root formation in cuttings. The main natural auxin – indole-3-acetic acid (IAA) – is the most studied plant hormone and many of its metabolic pathways are described. However, the auxin most commonly applied in rooting treatments is actually indole-3-butyric acid

(IBA), another natural auxin that exists endogenously in concentrations lower than those of IAA. Auxins act at very low concentrations, and their metabolism is therefore highly regulated. Auxins can be irreversibly degraded by oxidative enzymes, or they can be reversibly conjugated with sugar moieties or with aminoacids, peptides and proteins. They can also be interconverted into each other, and the conversion of IBA into IAA has been described in *O. europaea* (L.). Changes in auxin levels have been related to the different stages of adventitious root formation (induction, initiation and expression) and differences in the rate of auxin conjugation and of IBA-IAA conversion have been associated with different rooting ability. Hence, monitoring the levels of free and conjugated auxins throughout adventitious root formation is highly desirable and recommended during research studies.

Oxidative enzymes such as polyphenol oxidases (PPO) and peroxidases (POX) have also been associated with the regulation of adventitious rooting for a long time. Both classes of enzymes are described to participate in a broad range of biological processes, from defense against pathogens to stress responses. POX are also involved in the metabolism of IAA, through the isoform IAA oxidase (IAAox), a group of enzymes which has been largely associated with adventitious rooting. The activity of oxidative enzymes, especially POX, has been suggested as a biochemical marker for the different stages of adventitious rooting and has been related with the rooting ability of cuttings. However, although a lot of information is available on the literature regarding the involvement of oxidative enzymes in adventitious rooting, the results are frequently contradictory and appear to be species- or even cultivar-dependent.

Analytical methods for auxin quantification

While it is highly desirable to monitor the levels of plant hormones during adventitious root formation, the naturally low levels of these compounds in plant samples present a major obstacle to the development of high-throughput methods of analysis. Furthermore, because levels of plant hormones vary with species, it is important to adapt the analytical methodology to the sample under study. Plant hormones, and particularly auxins, have been studied for a very long time and, over the years, large efforts have been put into the development of more sensitive and precise methods of analysis and quantification of plant hormone levels in plant tissues. Hence, auxin analysis has mirrored the evolution of analytical chemistry. However, most of the methods developed for this purpose use herbaceous tissues, frequently from model

plants such as *Arabidopsis*, and there are very few reports of auxin quantification in olive tissues and even fewer in olive semi-hardwood tissues.

Auxin quantification in samples submitted to root-inducing treatments is even more challenging for two main reasons: (1) the *low endogenous* levels of IAA in plant tissues, which are embedded in a very complex sample matrix and (2) the very *high exogenous* levels of IBA introduced in the sample by the root-promoting treatment. This precludes the use of a shared internal standard for the two auxins, which is common when quantifying endogenous levels, and requires a more robust method, capable of extracting both auxins with equal yields and high recoveries.

Aims and motivation

The main goal of this thesis is to determine if auxin levels and the activities of oxidative enzymes are associated with the different rooting ability of olive cultivars. Can differences in these two parameters explain the different rooting behaviors?

To answer this question, we compared two cultivars with contrasting rooting behavior: 'Cobrançosa' (easy-to-root) and 'Galega vulgar' (difficult-to-root). Semi-hardwood cuttings of both cultivars were used in rooting studies, as well as microshoots of 'Galega vulgar'. To mimic the behavior of a difficult-to-root cultivar *in vitro*, the microshoots were treated with an inhibitor of adventitious root formation - salicylhydroxamic acid (SHAM). By analyzing the levels of free auxins and the activities of oxidative enzymes in both cultivars and treatments, we looked for biochemical clues which could help understand the different behavior of the chosen cultivars. Thus, the specific aims of this work are:

- to perform rooting trials using semi-hardwood cuttings of 'Cobrançosa' and 'Galega vulgar' in different seasons, as well as using 'Galega vulgar' microshoots treated with IBA and with SHAM+IBA;

- to develop an analytical method for the quantification of free IAA and IBA levels in the samples collected during the rooting trials;

- to determine the levels of free IAA and IBA, as well as the activities of oxidative enzymes (PPO, POX and IAAox), in both semi-hardwood cuttings and microshoots over the rooting period;

- to potentially establish a relationship between free auxin levels, activities of oxidative enzymes and rooting ability of cuttings.

Thesis outline and content

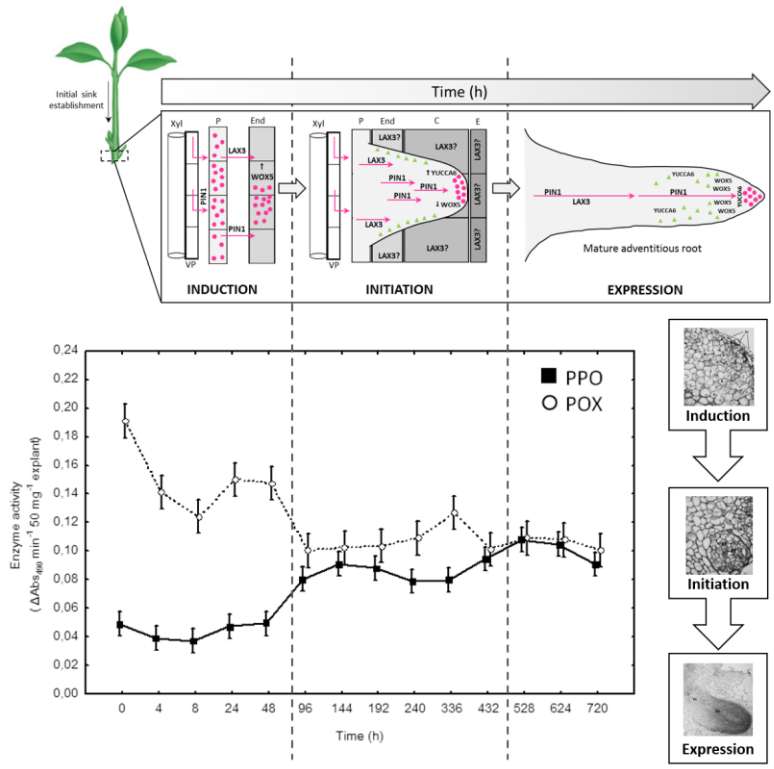
This thesis contains four chapters, each of which is a scientific paper presented in the form it was submitted for publication in a peer-reviewed journal. After the chapters, the main conclusions of this work and the areas of future work are summarized and discussed in a separate section.

This work is organized as follows:

Chapter I

Review and discussion of the current knowledge on adventitious root formation in *O. europaea* (L.). In this chapter, adventitious rooting in olive is critically discussed from an anatomical, biochemical and biological point of view. The available information on this subject concerning olive is also compared with other species. The most recent models for adventitious root formation in dicots are presented and the proposed biochemical network controlling the process is described. The role of plant hormones, oxidative enzymes, polyamines and hydrogen peroxide (among other factors) is debated, as well as the contribution of biological associations with other organisms. This manuscript was published in *Scientia Horticulturae* and is available online since December 17th, 2015.

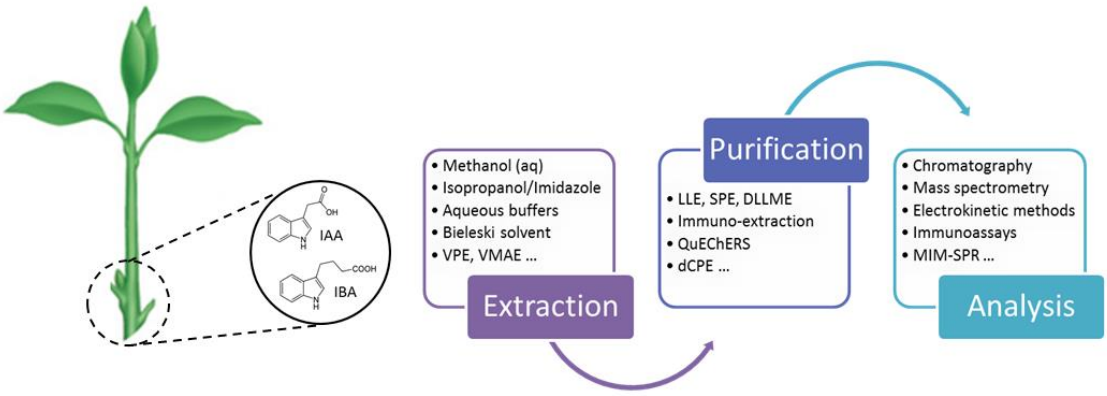
Graphical abstract of Chapter I



Chapter II

Review and discussion of the most recent advances in analytical methods for auxin quantification. While this is still a tricky procedure, phytohormone quantification is a major component of physiological and agronomical studies. Therefore, the analytical methods used to identify and quantify free and conjugated auxins (as well as other phytohormones) are continuously being improved and the number of references describing such methods is constantly increasing. In this chapter, procedures frequently used for extraction, purification and analysis of auxins are described and compared, focusing on references published in the past 15 years. This manuscript was published in *Analytica Chimica Acta* and is available online since November 6th, 2015.

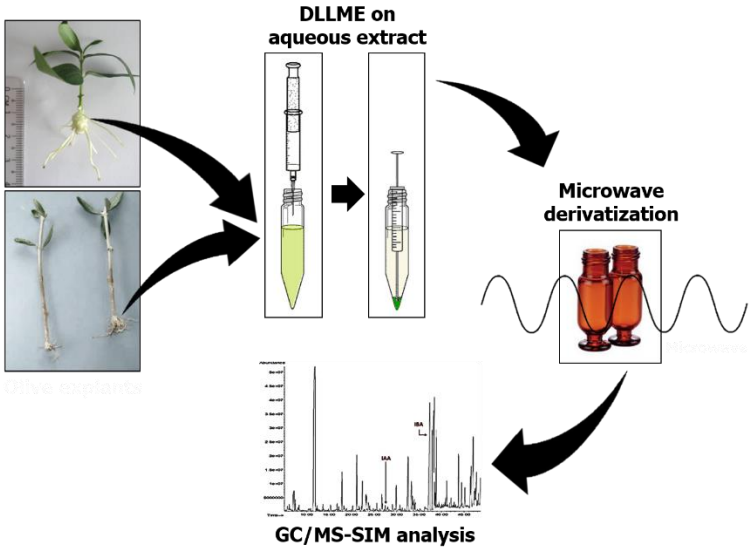
Graphical abstract of Chapter II



Chapter III

Development of an analytical method for the identification and quantification of free auxins in olive cuttings. The optimization of dispersive liquid liquid microextraction (DLLME) conditions, as well as microwave derivatization (MAD) conditions, is described. Method validation, including determination of linear ranges, limit of detection (LOD) and limit of quantification (LOQ) is also presented. Samples of *O. europaea* (L.) semi-hardwood cuttings and microcuttings were subjected to the developed method and the results are shown and compared with the literature. This manuscript was submitted for publication in *Analytical Methods* on May 2nd 2016.

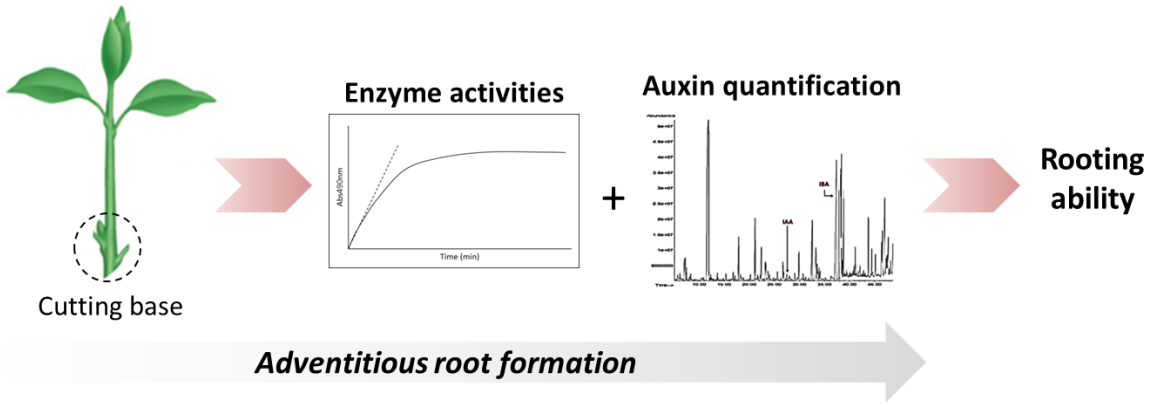
Graphical abstract of Chapter III



Chapter IV

Comparison of the biochemical changes occurring during adventitious root formation of olive microshoots and semi-hardwood cuttings. Rooting trials were performed with semi-hardwood cuttings of the two chosen cultivars and with microshoots of 'Galega vulgar', treated both with IBA and with IBA + SHAM. The activities of oxidative enzymes and the levels of free auxins were evaluated over time and the results of such analyses are described as a comparative study. A putative model for the molecular and biochemical interactions occurring during adventitious rooting is presented. This manuscript was published in *Scientia Horticulturae* and is available online since April 6th, 2016.

Graphical abstract of Chapter IV



It is worth mentioning that not all results produced during the development of this thesis are organized in chapters. Thus, negative or inconclusive results, as well as smaller contributions in other publications are presented as appendices of this dissertation.

Appendix I – Method development towards analytical separation of auxins by GC/MS. Description of alternative analytical procedures evaluated prior to the development of the method presented in Chapter III.

Appendix II – Changes in oxidative enzyme activities and auxin levels during adventitious root formation of olive semi-hardwood cuttings. Description of biochemical changes occurring during adventitious rooting of 'Cobrançosa' and 'Galega vulgar' semi-hardwood cuttings in two different seasons.

Appendix III – Macedo E., Vieira C., Carrizo D., Porfírio S., Hegewald H., Arnholdt-Schmitt B., Calado M. L., Peixe A. (2013), Adventitious root formation in olive (*Olea europaea* L.) microshoots: anatomical evaluation and associated biochemical changes in peroxidase and polyphenol oxidase activities, *Journal of Horticultural Science & Biotechnology*, 88 (1): 53–59.

Chapter I

REVIEWING CURRENT KNOWLEDGE ON OLIVE (*Olea europaea*) ADVENTITIOUS ROOT FORMATION

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Reviewing current knowledge on olive (*Olea europaea* L.) adventitious root formation

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Abstract

Olive (*Olea europaea*) is one of the most important fruit species in the Mediterranean basin, where 95% of the world's olive orchards are planted, and it has become an economically valuable crop worldwide, due to an increasing interest in olive oil for human consumption. New olive orchards are being planted outside the Mediterranean, calling for an effort to identify the genotypes best adapted to the new conditions. However, some olive cultivars remain difficult to propagate, which significantly reduces the capacity to use the full genetic diversity of the species. Improving rooting ability in cuttings from recalcitrant olive cultivars has become a critical topic, which implies fundamental research on the anatomy, physiology, biochemistry and genetics of the adventitious root formation process. Besides, the existence of different rooting behaviors among olive cultivars also makes the species a candidate model plant for these studies. Olive propagation techniques evolved through time from field- or nursery-planted hardwood cuttings, to semi-hardwood cuttings in greenhouses under mist, and, more recently, to *in vitro* culture techniques. Nevertheless, research about adventitious root formation carried on each propagation method was mostly based on trial and error approaches. Researchers have mainly investigated different factors involved in the process of adventitious rooting by testing their effect in the rooting capacity of different cultivars, leading to a high dispersion and fragmentation of the available information. The goal of this review is to present the most relevant results achieved on adventitious root formation in olive cuttings, aiming to provide an integrated perspective of the current knowledge.

Keywords: adventitious rooting, auxins, polyamines, propagation, *Olea europaea*, oxidative enzymes

1. General overview

1.1. Conceptual basis of adventitious root formation

Given its simplicity in relation to other techniques, multiplication by cuttings is one of the most relevant vegetative propagation methods, and, its success, depends on the cuttings' ability to form adventitious roots. While species like chrysanthemum (*Chrysanthemum indicum*) root easily and display a uniform rooting capacity (Sagee et al., 1992), others, like olive (*Olea europaea*), show different rooting responses among cultivars (Fouad et al., 1990), and, despite all the research done on the subject, a scientific answer able to explain this contrasting behavior is still unavailable. In fact, most of the current knowledge on adventitious root formation is based on trials developed with model plant species, like *Arabidopsis* sp. or *Tobacco* sp. (Kevers et al., 2009; Pijut et al., 2011). In woody perennials, like *Olea europaea*, the anatomy, biochemical background, genetic control of the process, and the action of exogenous factors able to affect it, remains largely unknown.

Adventitious root formation can occur naturally as part of the normal development of the plant. This happens in most monocotyledonous, where they constitute the main root system (Geiss et al., 2009), as well as in many dicotyledonous, such as strawberries (*Fragaria* spp.), hops *(Humulus lupulus*), African violets (*Saintpaulia* spp.), or blackberries (*Rubus* spp.) (Bellini et al., 2014), that naturally propagate by vegetative structures. It also represents a plant's response to a stress situation, which can be naturally caused (*i.e.* environmental stress), or mechanically induced, as a result from wounding following tissue culture or cutting severance (Bellini et al., 2014; Li et al., 2009a).

Two pathways may give rise to adventitious roots; i) **direct** organogenesis from established cell types, like the cambium, cortex, pericycle or vascular bundles, which involves cell re-differentiation; ii) **indirect** formation from callus tissue, formed upon mechanical damage induced by explant preparation. Although the two pathways can occur in the same species, generally, the direct pathway is displayed by easy-to-root species (e.g. *Hedera helix*), while difficult-to-root ones (e.g. *Pinus radiata*) are associated with the indirect pathway (Altamura, 1996; Hartmann et al., 1990).

Adventitious rooting was initially considered a one-step process, but, histological and physiological studies (De Klerk et al., 1999; Friedman et al., 1985; Jasik and De Klerk, 1997; Sircar and Chatterjee, 1973), reclassified it as a developmental mechanism, organized in a sequence of interdependent stages, each having its own requirements

and features (De Klerk, 1996; Gaspar et al., 1992, 1997; Jarvis, 1986; Kevers et al., 1997b; Rout et al., 2000).

According to Berthon et al. (1990), Heloir et al. (1996), Li et al. (2009a) and Pacurar et al. (2014), three phases can be distinguished; i) **induction**, corresponding to the period preceding any visible histological event, comprising molecular and biochemical events, ii) **initiation**, which starts when the first histological events take place, like root primordia organization, and is characterized by the occurrence of small cells with large nuclei and dense cytoplasm, iii) **expression**, that involves the development of the typical dome shape structures, intra-stem growth and emergence of root primordia.

Such description of the adventitious rooting phases is perfectly adapted to a large number of genuses (e.g. *Salix*, *Populus*, *Jasminum*, *Citrus*), where preformed adventitious root primordia are present in the stem, but remain dormant until the cuttings are prepared and submitted to conditions favorable to rooting (Altamura, 1996; Blakesley et al., 1991; Geiss et al., 2009; Pacurar et al., 2014). Nevertheless, in cases where these pre-specified cells are not present, an additional dedifferentiation phase exists before induction, where cells reacquire their competence for cell proliferation and organ regeneration (Bellini et al., 2014; Pacurar et al., 2014).

The length of the different phases differs among species (Blakesley et al., 1991; Nag et al., 2001; Naija et al., 2008). In apple (*Malus domestica*) microcuttings, where no preformed root primordia exist, the dedifferentiation phase occurs during the first 24h, when cells become competent to respond to auxin. De Klerk et al. (1995) showed that after recognizing auxin signals, and until 96h after cutting severance, certain cells become determined to form roots (induction phase). From 96h onwards, these determined cells produce a root primordium from which the adventitious root is developed (differentiation phase). These observations were coincident with histological changes; i) formation of starch grains, during dedifferentiation; ii) first cell divisions and meristem formation, during induction; iii) formation and development of root primordia, during differentiation (De Klerk et al., 1995).

Regulation of the formerly described rooting phases is influenced by a large number of factors, whose interaction remains poorly understood and the underlying molecular mechanisms governing the process remain unknown (Geiss et al., 2009; Legué et al., 2014). Among others, phytohormones (especially auxins and ethylene), polyamines, and oxidative enzymes, are some of the factors that seem to influence and regulate the process of adventitious root formation (reviewed in Li et al., 2009a; Geiss et al., 2009; Pijut et al., 2011).

1.2. Molecular basis of adventitious root formation

1.2.1. Auxins

Auxins are a class of phytohormones widely used in plant propagation to induce root formation in cuttings (Preece, 2013; Rademacher, 2015). The main endogenous auxin is indole-3-acetic acid (IAA), but there are other natural [indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA), phenylacetic acid (PAA)] and synthetic compounds [1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-2-methoxybenzoic acid (dicamba), and 4-amino-3,5,6-trichloropicolinic acid (picloram)] with auxin activity. IBA was originally chemically synthesized, being only later discovered its endogenous presence in plants (Epstein et al., 1989; Ludwig-Müller and Epstein, 1991), and is, in fact, the second most relevant natural auxin.

Endogenous IAA is synthesized in meristems and young tissues, such as the shoot apex and young leaves (Ljung et al., 2005; reviewed in Di et al., 2015). From these source tissues, IAA is transported basipetally through the stem to sink tissues. It can move passively in the bulk flow, or be actively transported through the vascular cambium in a polar manner (Teale et al., 2006). This type of polar transport, is gravity independent (Peer and Murphy, 2007) and mediated by carrier proteins (reviewed in Peer et al., 2011). Auxin can enter the cell by diffusion in the protonated form (IAAH) (Ljung, 2013), or through carrier-mediated co-transport, by the action of **AUX1/LAX** proteins (Blakeslee et al., 2005; Peer et al., 2011). Once inside the cell, because of the neutral cytosolic pH, IAA is in its unprotonated form (IAA^-) and can only exit the cell through efflux carriers – **PIN** and **ABCB proteins** (Zažímalová et al., 2010). When applied exogenously, auxins can also be transported acropetally in the xylem (Blythe et al., 2007). Both IAA and IBA can be transported directionally in different tissues and evidence suggests that specific active IBA transporters may exist, as many IAA transporters don't transport IBA (reviewed in Strader and Bartel, 2011). Appropriate auxin transport from the site of application to the site of root initiation can influence the effectiveness of the treatment (Blythe et al., 2007) and several studies have demonstrated the importance of auxin transport in adventitious rooting (Pacurar et al., 2014).

As plant hormones, auxins act at very low levels and their cellular concentration must be highly regulated. This can be achieved by regulation of *de novo* synthesis (Zhao, 2010) or auxin metabolism. Auxin levels can be irreversibly decreased through oxidative degradation (as discussed in Section 2.2.2.), or reversibly by conjugation with sugar moieties (**ester conjugates**), amino acids, peptides, or proteins (**amide**

conjugates) (Ludwig-Müller, 2011). Conjugation occurs ubiquitously in higher and lower plants. Interestingly the type of conjugates produced differs between plants (**Table 1**): while amide conjugates prevail over ester conjugates in dicotyledonous, the reverse happens in monocots (Bajguz and Piotrowska, 2009).

Table 1: Auxin amide conjugates identified by GC/MS, LC/MS and HPLC-FLD.

Conjugate	Species	Reference
IAA-Asp	Pea (*Pisum sativum*) Transgenic tobacco (*Nicotiana tabacum*) Walnut (*Juglans nigra* x *Juglans regia*) Rice (*Oryza sativa*) Cherry (*P. cerasus* x *P. canescens*) Chestnut (*Castanea sativa* × *Castanea crenata*) *Helleborus niger*	Nordström et al. (1991) Sitbon et al. (1993) Gatineau et al. (1997) Matsuda et al. (2005) Štefančič et al. (2007) Gonçalves et al. (2008) Pěnčík et al. (2009)
IAA-Glu	Transgenic tobacco (*Nicotiana tabacum*) Rice *(Oryza sativa*) *Helleborus niger*	Sitbon et al. (1993) Matsuda et al. (2005) Pěnčík et al. (2009)
IAA-Ala	*Arabidopsis thaliana* *Helleborus niger*	Kowalczyk and Sandberg (2001) Pěnčík et al. (2009)
IAA-Leu	*Arabidopsis thaliana* *Helleborus niger*	Kowalczyk and Sandberg (2001) Pěnčík et al. (2009)
IAA-Gly	*Helleborus niger*	Pěnčík et al. (2009)
IAA-Phe	*Helleborus niger*	Pěnčík et al. (2009)
IAA-Val	*Helleborus niger*	Pěnčík et al. (2009)
IAA-Trp	*Arabidopsis thaliana*	Staswick (2009)

Conjugation is one way of maintaining a constant pool of free IAA in tissues where auxin homeostasis has been disturbed. Another way of doing so is through IAA storage in the form of IBA (Korasick et al., 2013). As well as IAA, IBA can also be conjugated with other moieties via amide- or ester-linkages (Bajguz and Piotrowska, 2009; Korasick et al., 2013). IBA conjugates are more easily hydrolyzed and more slowly transported (Bajguz and Piotrowska, 2009), which can influence the amount of free auxin at the base of a cutting.

IBA can also be shortened into active IAA through a peroxisomal enzymatic process, similar to fatty acid β-oxidation (Zolman et al., 2000). Several enzyme candidates for IBA β-oxidation have been identified in *Arabidopsis* (Strader et al., 2011; Zolman et al., 2007; Zolman et al., 2008) and previous doubt regarding IBA having auxin activity by itself (Woodward and Bartel, 2005; Normanly et al., 2010) no longer seems to make sense, as some authors are confident that the conversion IBA-IAA is essential to IBA auxin activity (Strader and Bartel, 2011; Korasick et al., 2013).

1.2.2. Polyamines

Polyamines are low molecular weight cations, ubiquitous to all living organisms (Cohen, 1998), that act as growth regulators and have been described to interact with plant hormones (Alabadi et al., 1996; Altman, 1989; Tonon et al., 2001), although their classification is still controversial (Rademacher, 2015). Given their role in DNA replication, they have been associated with a large number of developmental processes in plants, including cell division and organogenesis, embryogenesis, floral initiation and development, fruit development, root growth, senescence and abiotic stress (Alcázar et al., 2010; Bais and Ravishankar, 2002; Galston and Sawhney, 1990; Kaur-Sawhney et al., 2003; Martin-Tanguy, 2001; Takahashi and Kakehi, 2010).

The major polyamines in plants are putrescine (Put), spermidine (Spd) and spermine (Spm), and their role in adventitious root formation has been suggested by several authors working with woody species (*Phaseolus*, Jarvis et al., (1985); *Vigna*, Friedman et al., (1985); apple, Wang and Faust, (1986); tobacco, Tiburcio et al., (1987, 1989); pear (*Pyrus* sp.), Baraldi et al., (1995); *Prunus avium*, Biondi et al., (1990); poplar (*Populus tremula* x *P. tremuloides*), Hausman et al., (1995a, 1995b); cork oak (*Quercus suber*), Neves et al., (2002); hazelnut (*Corylus avellana*), Cristofori et al., (2010)), including olive (Rugini and Wang, 1986).

1.2.3. Oxidative enzymes

Oxidative enzymes have long been related to adventitious root formation. The first reports on this subject suggested that phenolic compounds stimulated root formation as IAA synergists, possibly through inhibition of IAA-oxidase (IAAox) (Gorter, 1969). Later, a protein complex corresponding to polyphenol oxidase (PPO), POX, and IAAox, was reported to emerge in the early stages of rooting of *Phaseolus aureus* (Frenkel and Hess, 1974). Treatment of cuttings with a PPO inhibitor promoted root formation in

Phaseolus vulgaris and *Vigna radiata,* and its effect was more pronounced during the initiation phase (Gad and Ben-Efraim, 1988), suggesting that PPO had a main role in the regulation of adventitious root formation. However, in contrast, Upadhyaya et al. (1986) claimed that POX and PPO were not involved in the initiation of roots, but rather in their development. In fact, although the involvement of oxidative enzymes in adventitious rooting is abundantly described in the literature, results are frequently contradictory and thus appear to be species- or cultivar-dependent.

Plant peroxidases (class III POX EC1.11.1.7) are hemic proteins involved in a broad spectrum of physiological processes (for a review see Passardi et al., (2005)), including auxin metabolism (Galston et al., 1953). They catalyze the oxidation of diverse electron donors, including phenolic compounds, as well as auxin (Bandurski et al., 1995; Hiraga et al., 2001), using hydrogen peroxide (H_2O_2) as oxidative agent (Dawson, 1988). Although under most conditions non-decarboxylative oxidation is the main pathway for IAA degradation *in vivo* (Normanly, 2010), enzymatic oxidative decarboxylation of IAA can also occur, being catalyzed by a group of POX isoforms named IAAox (Ljung et al., 2002), a group of enzymes which has been largely associated with adventitious rooting (Bansal and Nanda, 1981; Güneş, 2000) (see Section 2.2.2.).

Polyphenol oxidases are a group of copper-containing oxidative enzymes that catalyze two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity (tyrosinase) EC 1.14.18.1) (Espín et al., 1997; Mayer, 2006) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity (catechol oxidase) EC 1.10.3.2) (Constabel and Barbehenn, 2008; Escribano et al., 2002; Mayer, 2006). The quinones produced in this reaction, can further polymerize then react non-enzymatically with other compounds, resulting in the formation of products that are believed to protect damaged tissues against herbivores and pathogens (Hind et al., 1995; Mayer, 2006; Robards et al., 1999). In addition to the mono- and di-phenols mentioned above, PPO are also capable of degrading other phenolic compounds that are structurally more complex, such as anthocyanins and other polyphenols (Jiménez and García-Carmona, 1999). PPO display a wide functional diversity, from protection against environmental stress (Thipyapong et al., 1995) to browning reactions (Ciou et al., 2011; Sciancalepore and Longone, 1984; Spagna et al., 2005; Subramanian et al., 1999; Waliszewski et al., 2009). The involvement of PPO activity in adventitious rooting has also been suggested by several authors (see Section 2.2.2.).

1.3. Suggested models for adventitious rooting in dicots

In the past decades, using *Arabidopsis* as plant model, significant progress has been achieved in understanding the physiological and molecular mechanisms behind primary and lateral root development (Casimiro et al., 2003; Muraro et al., 2013; Petricka et al., 2012; Ubeda-Tomás et al., 2012). In contrast, work on adventitious root formation has been more challenging and despite remarkable progresses already made in monocotyledonous species like rice and maize (Hochholdinger et al., 2004; Hochholdinger and Zimmermann, 2008; Zhi-Guo et al., 2012), the knowledge on the mechanisms controlling the process, is not as advanced in dicotyledonous species. Nevertheless, an initial model of adventitious rooting regulation has been proposed based on studies in *Arabidopsis* (Della Rovere et al., 2013; Gutierrez et al., 2012; Sorin et al., 2006). According to the model proposed by Della Rovere et al. (2013), PIN1 (proteins associated with auxin efflux) transporters initially divert IAA from the basipetal flow along the vascular parenchyma cells towards pericycle cells, activating LAX3 (auxin-inducible protein active in auxin cellular uptake) and promoting subsequent auxin accumulation. PIN1 and LAX3 retain IAA in the recently formed inner and outer layers of the adventitious root and WOX5 (auxin-induced protein associated with the positioning of the quiescent center (QC)) is expressed. At this point cytokinin downregulates PIN1 and LAX3 in the periphery and base of the newly formed adventitious root primordium, driving the auxin flow towards the primordium tip through the middle cell rows. This results in an apical auxin maximum which is also a result from IAA biosynthesis by YUCCA6 (auxin biosynthesis-related gene). This auxin maximum limits WOX5 expression at the distal tip therefore positioning the QC. Protrusion is possibly favored by LAX3 present in the hypocotyl epidermis, cortex and endodermis around the root primordium. In the mature adventitious root, the auxin maximum maintained by WOX5 expression and YUCCA6-derived biosynthesis also incorporates the QC. At the root tip, auxin homeostasis is partially maintained by cytokinin through downregulation of PIN1 and LAX3.

Gutierrez et al. (2012) also propose a model where auxin regulates adventitious root formation through the regulation of jasmonic acid (JA) homeostasis. In this model auxin-induced activation of ARF (Auxin Response Factor) proteins indirectly regulates the rooting-inhibiting COI1 (protein equivalent to auxin regulatory protein SCF^{TIR1}) pathway.

Although the molecular mechanisms behind adventitious root formation in model species like *Arabidopsis* are starting to be revealed, it is still unknown whether it will be

possible to translate the current knowledge on adventitious root development in herbaceous species to practical use in woody species (Bellini et al., 2014). Recently, Legué et al. (2014) summarized the recent progress made in the identification of transcription factors associated with the regulation of adventitious rooting in woody species, using *Populus* sp. as model organism. The present work aims to summarize the current knowledge on adventitious root formation in *Olea europaea*, another woody species and an important Mediterranean crop, focusing on the anatomical events and biochemical control of the process.

2. Adventitious root formation in olive stems

2.1. Stem anatomy and associated histological changes

Studies on stem comparative anatomy are fundamental to better understand the histological events leading to adventitious root formation. They allow; i) to identify cells/tissues giving rise to adventitious roots, and which are the target for auxin and other inducing factors; ii) to determine the presence or absence of pre-formed root primordia; iii) to establish a relationship between stem anatomical features and rooting capacity; iv) to create a relationship between physiological and biochemical data and the anatomical phases of root formation (Altamura, 1996).

In semi-hardwood olive cuttings, stem cross sections of easy and difficult-to-root cultivars were already compared and no anatomical differences were found between the studied genotypes. A continuous sclerenchyma ring between the phloem and the cortex was observed (Ayoub and Qrunfleh, 2008; Peixe et al., 2007b). This is considered to be a characteristic feature of the *Olea* genus (Ayoub and Qrunfleh, 2006) and has been previously pointed out as a possible mechanical barrier to root emergence in recalcitrant cultivars (Ciampi and Gellini, 1963; Qrunfleh and Rushdi, 1994; Salama et al., 1987). However, the most recent data on this subject show that such a ring, even with 3-6 cell layers, can't be a restricting factor for rooting as it crumbles during the rooting process, even in cultivars where only callus formation occurs and root formation isn't achieved (Ayoub and Qrunfleh, 2006, 2008).

Altamura (1996) already suggested that this putative relation between the sclerenchyma ring and the adventitious rooting capacity of a species be ruled out as a cause for recalcitrance in two ways; i) cell expansion and proliferation induced by auxin treatments can cause breaks in the sclerenchyma ring; ii) the developing root

primordium instead of pushing through the ring, can go around it, turning downward and emerging from the cutting base.

In *Grevillea* spp., as in olive, cultivars with differing rooting ability also presented stem cuttings that were anatomically similar, with a continuous sclerenchyma ring separating the cortex and the phloem. However, unlike olive, different anatomical changes were observed during rooting of *Grevillea* cuttings. In the easy-to-root cultivars, cell division was observed only in a localized area of vascular tissue, displacing the sclerenchyma fibers, leaving unaffected other regions of these tissues. In contrast, the difficult-to-root cultivars showed rapid cell division in all tissues (except the pith) and total disaggregation of the sclerenchyma ring, yet these events did not result in organization of new cells to form root primordia. Authors suggest that the lower rooting ability of those cultivars might be related to the loss of competence at the cellular level (Krisantini et al., 2006).

Using *in vitro* microcuttings from the olive cultivar 'Galega vulgar', Macedo et al. (2013), showed that the events corresponding to the induction phase take place in the first 96h after auxin treatment, when cells regain meristematic features. From 96h until 336h, the first meristemoids and morphogenetic root zones were observed, events corresponding to the initiation phase. These events are followed by high mitotic activity that eventually leads to the expression phase, which starts at 528h after the root-inducing treatment (**Figure 1**).

Despite some differences in timing, the cytological events observed in olive by Macedo et al. (2013), are in accordance with observations made in other temperate fruit species like *Malus pumila* 'KSC-3' (Hicks, 1987), *Prunus avium* x *Prunus pseudocerasus* (Ranjit et al., 1988), or *Castanea sativa* (Gonçalves et al., 1998). Timing differences were expected as the time required for root initiation varies among species from two to eight days (Auderset et al., 1994; Bressan et al., 1982; Gonçalves et al., 1998; Harbage et al., 1993; Ranjit et al., 1988; Samartin et al., 1986; San-José et al., 1992)

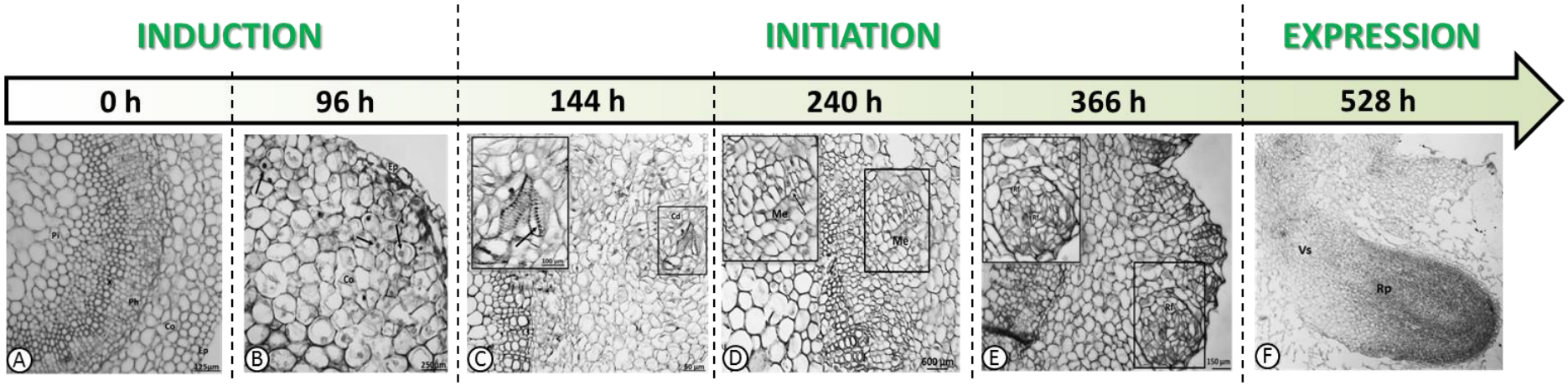

Figure 1. Histological events happening during adventitious root formation in olive cuttings after root-inductive treatment with 14.7 mM IBA (adapted from Macedo et al., 2013). (A) Anatomical structure of the stem before IBA treatment, showing a vascular bundle (Pi, pith; Co, cortex; Ep, epidermis; Ph, phloem; X, xylem); (B) Cells in the cortex re-acquire meristematic features, with dense cytoplasm, large nuclei, and visible nucleoli (arrows) (Ep, epidermis; Co, cortex); (C) First cell divisions (Cd) leading to callus formation; (D) Stem section showing two meristemoid structures (Me) in the upper phloem; (E) Morphogenic root zones (Rf) developing from subepidermal cells; (F) Root primordium (Rp) and differentiated vascular system (Vs). Magnifications of selected areas are shown in figures insets.

Studies performed in apple (Hicks, 1987; Naija et al., 2008; Zhou et al., 1992), chestnut (Gonçalves et al., 1998; Vieitez et al., 1981), oak (*Quercus robur*) (San-José et al., 1992), *Rosa multiflora* (Collet, 1985), camellia (*Camellia japonica*) (Samartin et al., 1986) or artichoke (C*ynara scolymus*) (Dridi, 2003), all describe similar changes in the stem tissues, with the appearance of larger nuclei (nucleus swelling) and dense cytoplasm in cambial cells and adjacent phloem being the first histological sign of adventitious root formation (Naija et al., 2008). The following step is the occurrence of cell division in or near the cambium zone. In the apple rootstocks MM 106, first cell divisions took place in the phloem region near the cambium (Naija et al., 2008). In other species adventitious roots originate near the vascular cambium (Ahkami et al., 2009, 2013; Hicks, 1987; Park et al., 2002; Rigal et al., 2012; San-José et al., 1992; Syros et al., 2004). The final stage of root formation involves the development of primordia into organized roots, where root primordia protrude among other tissues and roots emerge from the cutting surface (Naija et al., 2008). Generally, in woody perennials, the origin site is located close to the central core of vascular tissues (Geiss et al., 2009). However, this generalization may comprise many sites of origin (Blakesley et al., 1991; De Oliveira et al., 2013; Jasik and De Klerk, 1997) as the region of the stem tissues where cells become activated seems to depend in part on physiological gradients of substances entering the shoot from the medium and on the presence of competent cells to respond to stimuli (Naija et al., 2008).

In *Olea europaea*, most authors have observed adventitious roots arising from the cambial region of the stem [Bakr et al. (1977) on cultivar 'Wetaken', Salama et al. (1987) on 'Manzanillo', 'Mission', 'Kalamata', or 'Hamed' and Ayoub and Qrunfleh (2006) on 'Nabali' and 'Raseei']. Studies recently published by Macedo et al. (2013) showed that in cuttings of the easy-to-root cv. 'Cobrançosa' the first morphogenic root fields were also found to be formed in cambial cells, confirming previously acquired data. Nevertheless, the same authors reported, in the case of the difficult-to-root cv. 'Galega vulgar', that morphogenic fields were always found in cells from the callus tissue around the base of the cutting. This is in accordance with Therios (2009), who stated that callus formation is a prerequisite for adventitious root formation but, depending on the species, that callus formation may be independent of rooting.

This emphasizes once again the diversity of rooting behaviors among olive cultivars (**Table 2**), confirming *Olea europaea* as a candidate model plant to study adventitious rooting. As stated by Blakesley et al. (1991), rooting differences occurring within the same species arguably provide a better system for investigation as this system removes the chance of genetic causes on evaluation of results.

Table 2. Rooting capacity of the most commonly cultivated olive cultivars (from Fabbri et al., 2004)

Cultivar (country)		
High (100 – 66%) **"Easy-to-root"**	**Medium (66 – 33%)** **"Average Rooting"**	**Low (33 – 0%)** **"Difficult-to-root"**
Aglandau (France)	Aggezi Shami (Egypt)	Azéradj (Algeria)
Arbequina (Spain)	Azapa (Chile)	Bella di Spagna (Italy)
Ascolana tenera (Italy)	Bardhe i Tirane (Albania)	Bianchera (Italy)
Barnea (Israel)	Bella di Cerignola (Italy)	Biancolilla (Italy)
Bouteillan (France)	Bical Castelo Branco (Portugal)	Büyük Topak Ulak (Turkey)
Coratina (Italy)	Bidh el Hammam (Tunisia)	Carrasquenha (Portugal)
Cordovil Castelo Branco (Portugal)	Cailletier (France)	Chemlal (Algeria)
Frantoio (Italy)	Çakir (Turkey)	Chemlali de Sfax (Tunisia)
Gordal de Granada (Spain)	Carrasqueño (Spain)	Domat (Turkey)
Leccino (Italy)	Chalkidiki (Greece)	Empeltre (Spain)
Lechin de Sevilla (Spain)	Chemchali (Tunisia)	Farga (Spain)
Lucques (France)	Erkence (Turkey)	Gordal Sevillana (Spain)
Manzanilla de Sevilla (Spain)	Galega Vulgar (Portugal)	Leccio del Corno (Italy)
Mission (USA)	Kalamata (Greece)	Lianolia Kerkyras (Greece)
Mixan (Albania)	Picholine (France)	Nabali Baladi (Israel)
Moraiolo (Italy)	Picholine Marocaine (Morocco)	Nocellara Etnea (Italy)
Nocellara Messinese (Italy)	Picual (Spain)	Ogliarola Messinese (Italy)
Oblica (Croatia)	Sigoise (Algeria)	Oueslati (Tunisia)
Pendolino (Italy)	Taggiasca (Italy)	Salonenque (France)
Verdal (Spain)	Verdale de L'hérault (Spain)	Verdeal Alentejana (Portugal)

2.2. The biochemical regulatory network

In Section 1 the biological function of the major molecules involved in adventitious rooting has been described. Herein the interaction between these factors and how they participate in a complex regulatory network, which culminates in adventitious root formation, will be discussed.

2.2.1. Role of plant growth regulators

Auxins play a major role in the control of root development and the current knowledge on this subject is far more developed than it is in adventitious rooting (reviewed in Overvoorde et al., 2010). Nevertheless, the involvement of auxins in adventitious root formation has been proven by several authors (Bellamine et al., 1998; Cooper, 1935; Haissig and Davis, 1994; Zeev Wiesman et al., 1989). Evidence suggests that IAA potentially promotes adventitious rooting through a signaling network similar to that in lateral roots. This network involves auxin response factors (ARF) that regulate the synthesis of auxin-inducible genes (GH3) by modulating jasmonic acid (JA) homeostasis (Gutierrez et al., 2009, 2012). Several lines of evidence show that auxin can stimulate the production of ethylene (Abel et al., 1995; Peck and Kende, 1995; Wilmowicz et al., 2013; Yun et al., 2009), which in turn may promote adventitious rooting (Pan et al., 2002). However, the precise mechanism of auxin action remains poorly understood (Pop et al., 2011).

Auxin and ethylene regulate each other's metabolism (Robles et al., 2013), in a cross-talk that was also proposed to have a putative regulatory role on adventitious root development. In *Arabidopsis* hypocotyls, an increased number of adventitious roots was obtained after treatment with ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) or in ethylene over-producing mutants (*eto1*), which also display reduced auxin transport. On the other hand, ethylene insensitive mutants (*ein2-5* and *etr1-1*) with enhanced auxin transport showed an increased number of roots. Taken together, these results indicate a negative regulatory role of ethylene on auxin transport and accumulation (by modulating levels of ABCB19 carrier proteins) and ultimately adventitious rooting (Sukumar, 2010). Contradictory results, however, were found in flooded tomato plants, where ethylene stimulated auxin transport through a positive feedback loop (Vidoz et al., 2010). Ethylene also stimulated IAA biosynthesis in roots of *Arabidopsis* (Růzicka et al., 2007; Swarup et al., 2007). Therefore, the role of ethylene in the regulation of adventitious root formation is still unclear as results differ greatly

from species to species. Ethylene can promote adventitious rooting in many species (De Klerk et al., 1999; Druege et al., 2014; Negi et al., 2010; Vidoz et al., 2010), inhibit the process in others (Nordström and Eliasson, 1984; Sukumar, 2010), or even have no effect at all (Batten and Mullins, 1978). Conversely, auxin treatments induce the production of ACC and ethylene, resulting in enhanced adventitious rooting (Riov and Yang, 1989; Visser et al., 1996).

Even though it is assumed that ethylene, unlike auxin, stimulates root expression (Druege et al., 2014), a current hypothesis suggests that ethylene acts indirectly by stimulating auxin biosynthesis and transport to the elongation zone, consequently inhibiting root elongation (Muday et al., 2012; Rahman et al., 2001; Růzicka et al., 2007; Stepanova et al., 2005, 2007). Moreover, unlike auxin-related genes which show a phase-specific pattern, the expression of ethylene-related genes indicates that ethylene is important to stimulate adventitious rooting but not to regulate the process (Druege et al., 2014).

A model of the hormone interactions involved in the different phases of adventitious root formation is proposed by Da Costa et al. (2013) and a more specific model of the regulation of adventitious rooting by auxin and ethylene is suggested by Druege et al. (2014). This information was compiled with the model of auxin flow, gene expression and cytokinin localization during adventitious rooting proposed by Della Rovere et al. (2013) (see Section 1.3.) and is presented in **Figure 2**.

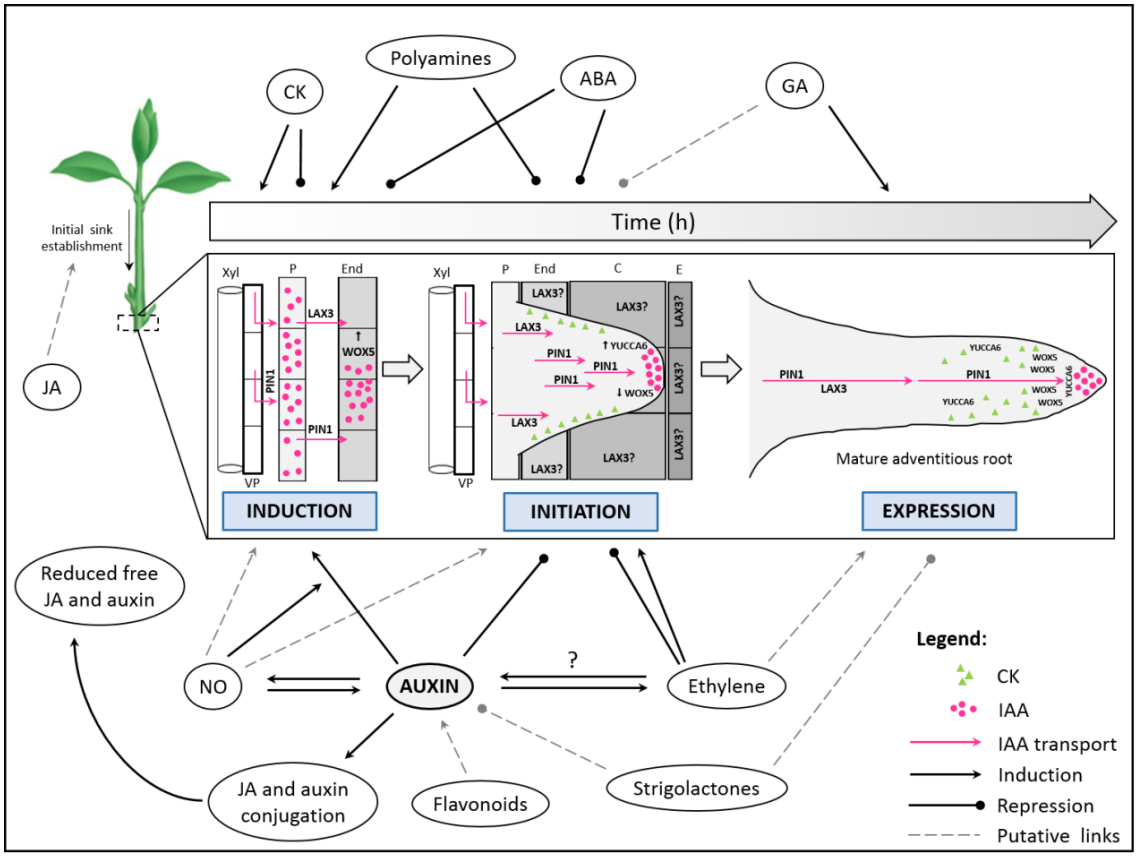

Figure 2. Possible histological and physiological events and hormone interactions involved in adventitious root formation (adapted from Da Costa et al., (2013), Della Rovere et al., (2013) and Druege et al., (2014)). Initially, JA may promote carbohydrate sink establishment before induction. During induction phase, auxin (IAA) is diverted from the basipetal flow in the xylem by PIN1 transporters, through the pericycle activating LAX3 proteins, and accumulates in the endodermis, increasing the expression of WOX5. This phase is positively regulated by auxin, polyamines, CK and ethylene. CK and ethylene seem to have a dual role, by also negatively regulating induction. ABA inhibits this stage. Levels of JA and auxin are decreased by conjugation with aminoacids, allowing the progress of initiation phase. Strigolactones may suppress auxin action or may directly inhibit adventitious rooting. On the contrary, NO is considered to stimulate both induction and initiation phases. During initiation, PIN1 transporters drive the auxin flow towards the root primordium tip through the middle cell rows because CK down-regulates PIN1 and LAX3 in the peripheral cell layers. This results in an auxin maximum at the distal tip of the adventitious root primordium (ARP), decreasing WOX5 expression and establishing the quiescent center (QC). This auxin maximum is maintained through increased auxin biosynthesis by YUCCA6. Protrusion is possibly favored by active LAX3 in the endodermis, cortex and epidermis around the ARP tip. Initiation phase is negatively regulated by polyamines, auxin, ABA and possibly GA and strigolactones. During expression phase the QC is incorporated in the auxin maximum where WOX5 and YUCCA6 expression are maintained, creating a constant apical auxin accumulation. CK is also present in the ARP and contributes to auxin homeostasis by down-regulating PIN1 and LAX3. Ethylene and GA stimulate expression, while ABA acts as a repressor. Xyl, xylem; P, vascular parenchyma; P, pericycle; End, endodermis; C, cortex; E, epidermis; JA, jasmonic acid; CK, cytokinin; ABA, abscisic acid; GA, gibberellin; NO, nitric oxide; aa, aminoacids.

Although the genotype appears to have a stronger influence, 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; Heloir et al., 1996; Krisantini et al., 2006; Nag et al., 2001; Sagee et al., 1992). According to De Klerk et al. (1995), the high auxin levels needed for the success of induction phase, become inhibitory during root expression, meaning that IAA catabolism is mandatory to avoid the inhibition of root development, since high auxin concentrations inhibit root elongation and promote cellular differentiation (Li et al., 2009a). Indeed, auxin influx carrier genes are downregulated during induction phase (Druege et al., 2014) and, for woody species in particular, the development of functional roots throughout the acclimatization of induced microcuttings demands an auxin-free culture medium (Kevers et al., 2009). This way, IAA levels could be used as a marker for the stages of adventitious rooting or to distinguish recalcitrant genotypes from non-recalcitrant.

When auxin reaches the base of a cutting, it could be expected to accumulate in those tissues, even if only transiently. Therefore, differences in auxin transport and accumulation in cuttings could explain the different rooting capacity of easy- and difficult-to-root plants. For example, while easy-to-root cuttings of flametip (*Leucadendron discolor*) transported more ^{3}H-IBA to the leaves, more free IBA was accumulated at the base of difficult-to-root cuttings (Epstein and Ackerman, 1993). However, many possible scenarios could explain the behavior of difficult-to-root cuttings: they may metabolize IAA faster than easy-to-root cuttings, leading to lower basal free IAA levels, or the rate of basipetal IAA transport may be lower in this case; there may be a higher concentration of rooting inhibitors at the base of these cuttings; the cells that give rise to root primordia could be less sensitive to auxin or less competent for re-differentiation (Ford et al., 2001).

Differences between easy- and difficult-to-root genotypes have been associated with their capacity to inactivate auxins through conjugation, as suggested by Epstein et al. (1993) working with *Prunus avium*, where rooting of a difficult-to-root cultivar was enhanced by inhibitors of IBA conjugation. Authors also reported that IBA conjugation was faster in the difficult-to-root cultivar, suggesting that recalcitrant cultivars may lack the capability of hydrolyzing IBA conjugates during adventitious root formation. Actually, different cultivars of the same species may differ in endogenous amounts of IBA (Ludwig-Müller, 2000).

In olive, to the best of our knowledge, no auxin conjugates have been identified so far. In fact, there is no research done in this area using olive cultivars and there are very few reports of quantification of free auxin levels. Contradicting results found in other species (Krisantini et al., 2006; Sagee et al., 1992), in olive, free IAA levels were found

to be higher in the difficult-to-root cv. 'Nabali' than in the easy-to-root cv. 'Raseei' (Ayoub and Qrunfleh, 2008). It is, however, worth mentioning that in this study the levels of growth regulators were measured in buds and leaves and not in the base of the cuttings where root formation occurs.

Differences in IBA-IAA conversion could also explain differences in rooting ability. This conversion was reported in cuttings of *Pinus sylvestris* (Dunberg et al., 1981), *Malus pumila* (Alvarez et al., 1989), *Populus tremula* (Merckelbach et al., 1991), *Pyrus communis* (Baraldi et al., 1995), *Vigna radicata* (Chang and Chan, 1976) and *Persea americana* (García-Gómez et al., 1994). In IBA-treated avocado microcuttings a 2-fold increase in free IAA levels was observed when compared to control cuttings, as well as an increase in IAA-Asp levels before differentiation (García-Gómez et al., 1994). Some authors also have suggested that IBA treatment can increase the internal free IBA concentration or synergistically modify the action or synthesis of endogenous IAA (Van der Krieken et al., 1993).

IBA-IAA conversion has been described in olive by Epstein and Lavee (1984). After treating 'Kalamata' (difficult-to-root) and 'Koroneiki' (easy-to-root) cuttings with radioactive IBA-^{14}C, most of the recovered radioactivity was found in the form of IAA-^{14}C at the base of the cuttings, and higher conversion rates were found in 'Koroneiki' cuttings. Interestingly, the process was faster in difficult-to-root cultivars (Epstein and Lavee, 1984). This is a very important result considering the inhibitory effect of high auxin levels during initiation phase (De Klerk et al., 1995). If a difficult-to-root cultivar converts IBA into IAA very fast (before and during the induction phase) and doesn't metabolize the resulting free IAA, the initiation phase could be suppressed by the high amounts of auxin and no further root development would be observed. Hence this could explain the recalcitrant behavior of some cultivars. Additionally, exogenous IBA promoted rooting of *Arabidopsis* stem segments which was inhibited by the auxin polar transport inhibitor 3,4,5-triiodobenzoic acid (TIBA) suggesting a conversion of IBA into IAA (Ludwig-Müller et al., 2005).

2.2.2. Role of oxidative enzymes – activity and isoforms

As previously mentioned (see Section 1.2.3.), IAA levels can also be regulated by irreversible enzymatic degradation through the action of the POX isoform IAAox. Indeed, POX activity is one of the most studied factors involved in adventitious root formation and it has also been suggested as a marker for root formation. In this section

the involvement of POX and other oxidative enzymes in adventitious rooting will be described.

So far there is very few information regarding this subject in olive (*Olea europaea*). Several studies have partially purified, characterized and identified the cellular location of PPO and POX in olive fruits (Ben-Shalom et al., 1977; Lopez-Huertas and Del Rio, 2014; Saraiva et al., 2007; Shomer et al., 1979; Tzika et al., 2009) and both POX and PPO activities have been investigated during unrelated processes such as browning (Goupy et al., 1991; Sciancalepore and Longone, 1984; Segovia-Bravo et al., 2007) and ripening (Ebrahimzadeh et al., 2003; Ortega-García et al., 2008; Ortega-García and Peragón, 2009).

Differences in PPO activity among *Vitis* rootstocks have been found during rooting (Satisha et al., 2008). Increased PPO activity was observed in response to caffeic acid during rooting of mung bean (Batish et al., 2008) and associated with improved rooting in *Excoecaria agallocha*, *Cynometra iripa* and *Heritiera fomes* (Basak et al., 2000). A positive relationship between PPO activity and rooting ability was also found in walnut by Cheniany et al. (2010) and the use of PPO as a marker of the onset and duration of the different phases of rooting was suggested.

Serra et al. (2007) compared the levels of PPO activity in tissues of two olive cultivars with different rooting behavior while trying to establish a relationship between PPO activity and rooting capacity. In both cultivars, higher activity was detected in auxin producing tissues, such as leaves and buds, as described in other species (Szecskó et al., 2004). In agreement with results from eucalyptus (*Eucalyptus urophylla*) (Li and Huang, 2002) higher activity was found in the easy-to-root 'Cobrançosa' than in the difficult-to-root 'Galega vulgar'. In other species, like the case of *Vitis vinifera*, however, no relationship was found between PPO activity and rooting capacity (Kose et al., 2011; Yilmaz et al., 2003). The opposite was even observed in *Rhododendron* sp. where enzyme activity was higher in difficult-to-root cultivars (Foong and Barnes, 1981).

However, it is believed that PPO doesn't influence root formation directly but its effects should rather occur through a disturbance in POX activity, and an inverse relationship between the activities of these two enzymes probably exists (Cheniany et al., 2010). Such a relationship was found in olive by Macedo et al. (2013) while studying the evolution of POX and PPO activities during IBA-induced adventitious root formation of microcuttings of the difficult-to-root 'Galega vulgar'. The inverse trend of POX and PPO activities had been previously described by Qaddoury and Amssa (2003) and by

Cheniany et al. (2010) who attributed the decrease in PPO activity to an accumulation of monophenolic compounds, which in turn stimulated POX activity. The results found in olive are in agreement with those described for grapevine (*Vitis vinifera*, Kose et al., (2011); Yilmaz et al., (2003), *Camellia sinensis* Rout (2006)) and walnut (*Juglans regia*, Cheniany et al., (2010)). In *Vitis vinifera* cultivars, a decrease in PPO activity was found to happen at the same time root formation was observed, leading to the conclusion that a decrease in PPO activity is necessary for root formation (Yilmaz et al., 2003). The magnitude of changes in PPO activity appears to be larger in easy-to-root cultivars (Cheniany et al., 2010) and, despite some speculation about a relationship between phenolic content and rooting performance (Nag et al., 2001), a clear relation between PPO activity and rooting ability hasn't been found (Rout, 2006; Yilmaz et al., 2003).

POX activity was first associated with rooting capacity by Quoirin et al. (1974) who found higher POX activity in easy-to-root plants, an observation also described by Van Hoof and Gaspar (1976), although it is disputed by some authors (Faivre-Rampant et al., 1998; Foong and Barnes, 1981). Low POX and IAAox activities also favored rooting in *Bruguiera parviflora*, *Thespesia populnea* (Basak et al., 2000) and eucalyptus (*Eucalyptus urophylla*) (Li et al., 2000). Van Hoof and Gaspar (1976) also linked rhizogenesis with a decrease in POX activity and several authors subsequently described an increase in POX activity before root emergence followed by a decrease thereafter (*Phaseolus mungo* (Bhattacharya and Kumar, 1980); *Sequoiadendron giganteum* (Berthon et al., 1987), *Vitis vinifera* (Mato et al., 1988), *Populus tremula* × *P. tremuloïdes* (Hausman, 1993); *Casuarina equisetifolia* (Rout et al., 1996); *Phaseolus radiatus* (Pan and Gui, 1997); *Prunus dulcis* (Caboni et al., 1997); *Elaeis guineensis* (Rival et al., 1997); *Populus nigra*, *Populus alba* and *Populus tremula* (Güneş, 2000)). IAAox activity was also reported to decrease during rooting of *Castanea sativa* (Mato and Vieitez, 1986) and *Glycine max* and this decrease was accompanied by an increase in endogenous levels of IAA (Liu et al., 1996). On the contrary, rooting success in poplar cuttings was attributed to IAA catabolism mediated by an increase in IAAox activity (Güneş, 2000).

The initial reports suggested that a peak of POX activity would mark the end of the induction phase, which was not confirmed by Gaspar et al. (1992) who established that the peak of POX activity determines the end of the initiation phase instead and suggested POX activity as a marker for adventitious root formation. However, its reliability as a biochemical marker can be contested (De Klerk, 1996) in part because in some species the opposite trend was observed: POX activity decreased in the first stages of rooting and increased subsequently [*Castanea sativa* x *C. crenata*

(Gonçalves et al., 1998); *Cucurbita moschata* (Xiaoman et al., 1998)]. Unlike the case of most auxin-related studies, where *Arabidopsis* is used as a model organism (Bellini et al., 2014; Della Rovere et al., 2013), research involving POX activity is based in a wide range of species which prevents us from drawing definitive conclusions.

Using microcuttings of the difficult-to-root 'Galega vulgar', Macedo et al. (2013) studied the evolution of POX and PPO activities during IBA-induced adventitious root formation (**Figure 3**). By comparing the changes in enzymatic activities with histological data authors were able to identify the putative physiological stages of the rooting process in this species for the first time. POX activity decreased to a minimum in the first 96h after treatment, which corresponded to the end of the inductive phase, in agreement with findings from Gaspar et al. (1992, 1994). POX activity increased subsequently reaching a peak at 336h. The period between 96h and 336h could correspond to the initiation phase, as proposed by Gaspar et al. (1992, 1994), however in the case of olive a clear relationship between POX activity and root initiation is not observed as it is in other species (Gaspar et al., 1992; Rival et al., 1997; Rout et al., 2000). In the period 336 – 528h, which coincided with intense mitotic activity and development of newly formed root meristems, POX activity decreased significantly. The expression phase was observed from 528 – 720h, when both POX and PPO activities decreased, in agreement with Gaspar et al. (1992).

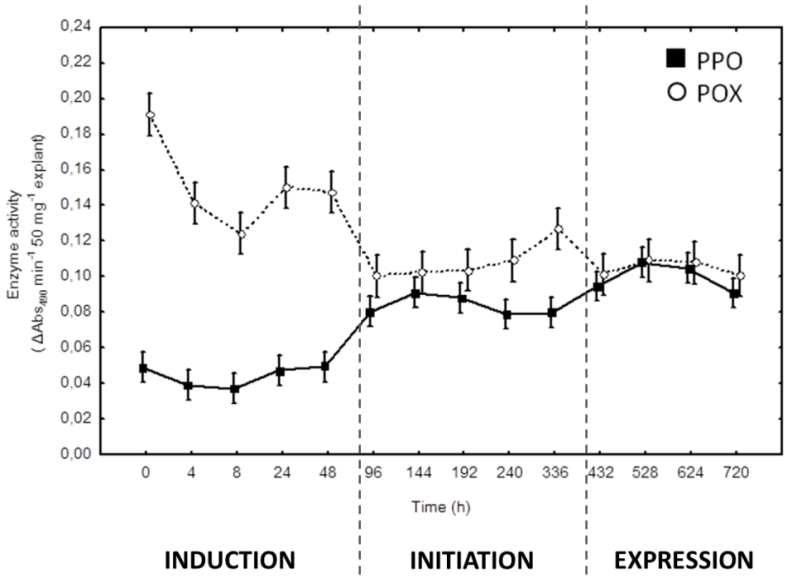

Figure 3. Changes in peroxidase (POX) and polyphenol oxidase (PPO) activities at different time-points during the development of adventitious roots in *in vitro*-cultured 'Galega vulgar' olive microshoots. Vertical bars denote ± standard errors. Rooting phases are highlighted for clarity of presentation (adapted from Macedo et al., (2013)).

The available information on this subject in *Olea europaea* is still very sparse. The evidence described in the literature regarding other species is extremely inconsistent, and contradictory results can be found among different cultivars of the same species, as the case of *Vitis* rootstocks (Kose et al., 2011; Satisha et al., 2008). Therefore further studies involving other cultivars are necessary either to confirm or at least clarify the existing results.

The pattern for POX activity found in olive was also found in several other woody species such as grapevine (*Vitis vinifera*, Yilmaz et al., (2003), *Elaeocarpus sylvestris*, Gu et al., 2004), walnut (*Juglans regia*, Cheniany et al., (2010) and mung bean (*Vigna radiata*, Nag et al., (2001, 2013)). In these species the inductive phase was marked by a minimum of POX activity, followed by a peak that established the end of initiation phase and a subsequent decrease during expression phase. The characteristic peak in POX activity at the end of initiation phase is most likely due to an increase in specific isoforms of POX, as was demonstrated in mung bean where new isoforms were detected at the same time the activity peak was observed (Nag et al., 2013). The isoforms involved in this process are probably the so-called IAAox, as the activity of this group of enzymes was inversely related with IAA levels during rooting of *Vigna radiata* hypocotyls (Nag et al., 2001). An increased expression of POX was also observed in the first 24 h after cutting severance in *Petunia hybrida*, which was also associated with IAAox activity (Druege et al., 2014). Interestingly the POX isoform profile (number and relative activity of isoforms) varies throughout the different phases of adventitious rooting (Pastur et al., 2001; Syros et al., 2004) and also differs with rooting ability (Ludwig-Müller, 2003) making it a potential indicator of the underlying processes happening during root formation (Pastur et al., 2001). In olive, Bartolini et al. (2008) reported the emergence of a “new polypeptide” that could be related to root formation. However, the results are not clear, the putative protein was not further purified and the conclusions drawn are merely speculative. On the contrary, in narrow-leafed ash (*Fraxinus angustifolia*, (Tonon et al., 2001)) another species of the *Oleaceae* family, POX activity increased in the expression phase, as described also in other species like date palm (*Phoenix dactylifera*, (Qaddoury and Amssa, 2003)), *Camellia sinensis* (Rout, 2006) and apple (rootstock MM106, (Naija et al., 2009)). This is supported by findings of Tartoura et al. (2004) who suggested that POX (IAAox) is only involved in initiation and expression of adventitious roots, as during root induction IAA conjugation is the most likely cause of downregulation of IAA levels. These findings reinforce the need for more studies in olive as they show that changes in POX activity during adventitious root formation may vary within species. So far no data is available

regarding POX activity in olive cuttings with different rooting ability. This is definitely an important area of future investigation, as it has been described that the POX activity peak can shift in time when the rooting success rate is low (Rival et al., 1997) and that the POX peak can be higher in easy-to-root cultivars (Cheniany et al., 2010; Kose et al., 2011), therefore establishing a relation between POX and rooting capacity.

2.2.3. Role of hydrogen peroxide

Most studies on oxidative enzymes consider that POX affects adventitious root formation through regulation of IAA levels accomplished by IAAox. However, some authors have suggested that POX may act indirectly through H_2O_2, given the rooting-stimulatory effect of H_2O_2 (Sebastiani et al., 2002; Sebastiani and Tognetti, 2004) and the inhibitory effect of its scavengers (Li et al., 2009b). Li et al. (2009b) reported an increase in endogenous H_2O_2 levels in mung bean seedlings after IBA treatment and removal of the primary root, suggesting that IBA may induce rooting indirectly through a pathway involving polyamines and H_2O_2. Moreover, treatment of mung bean seedlings with H_2O_2 resulted in a decrease of POX activity (Li et al., 2009c). In olive, exogenous application of Put induced a POX peak (Özkaya and Celik, 1994; Rugini et al., 1990, 1997) which could be a result of Put degradation through the Δ'-pyrroline pathway, where H_2O_2 is the main by-product (Tiburcio et al., 1997). H_2O_2 accumulation could then stimulate POX activity (Gaspar et al., 1997; Rugini et al., 1992) and ultimately root formation. Furthermore, H_2O_2 seems to interact with alternative oxidase (AOX) (Macedo et al., 2009, 2012) and nitric oxide (NO) (Da Costa et al., 2013).

2.2.4. Role of polyamines – depletion/accumulation

Treatments with inhibitors of polyamine synthesis, like difluoromethylornithine (DFMO) and α- difluoromethylarginine (DFMA), tend to inhibit adventitious rooting (Hausman et al., 1994; Martin-Tanguy and Carré, 1993; Naija et al., 2009) and this effect can be partially reversed by exogenous polyamine treatment (Biondi et al., 1990). Additionally, depletion of polyamine pools has been linked to root growth inhibition (Couée et al., 2004) and polyamine accumulation was related to rooting (Altamura et al., 1991, 1993; Friedman et al., 1982, 1985; Jarvis et al., 1983).

In olive, polyamines (especially Put) have been reported to stimulate rooting in several cultivars. Combined treatments with IBA + Put increased rooting percentage and promoted early rooting of 'Frangivento', 'Pendolino', 'Frantoio', 'Moraiolo', 'Dolce

Agogia' and 'Chondrolia Chalkidikis' cuttings, while the stimulatory effect of pure Put was dependent on season (Grigoriadou et al., 2002; Rugini, 1992; Rugini et al., 1990). Interestingly, the positive effect of polyamines was also observed when rooting was induced by *Agrobacterium rhizogenes* in the absence of exogenous auxin (Rugini, 1992). The endogenous polyamine content of the cuttings also seems to influence their rooting capacity. Easy-to-root olive cuttings show higher polyamine content and the peak of free polyamine content is coincident with the highest rooting percentage (Denaxa et al., 2014). This supports the role of free polyamines in rooting previously suggested by other authors (Faivre-Rampant et al., 2000; Neves et al., 2002; Rugini et al., 1993, 1997; Tiburcio et al., 1989). Thus, the recalcitrance of genotypes like 'Kalamata' has been attributed to a low content of free polyamines (Denaxa et al., 2014) and, interestingly, the predominant polyamine found in cuttings appears to be dependent on genotype. For example, in the case of 'Arbequina' and 'Kalamata', even though Spd was found to be the predominant polyamine, Put was the most effective in promoting rooting (Denaxa et al., 2014).

Nevertheless the effect of polyamines in adventitious rooting is still controversial and seems to be dependent on species. Polyamine treatment had little to no effect on cuttings of *Malus pumila*, *Prunus dulcis*, *Pistacia vera* (Rugini, 1992), chestnut, jojoba (*Simmondsia chinensis*) and apricot (*Prunus armeniaca*) (Rugini et al., 1993), but it decreased rooting in walnut and increased rooting in apple and olive (Naija et al., 2009; Rugini et al., 1993). Other factors such as basal shoot darkening, type of explant and endogenous level of polyamines also seem to influence the response to polyamine treatments. In fact, the content of total free polyamines in cuttings (which in olive is low) seems to be inversely related with the response to exogenous Put treatments (Rugini et al., 1993). There seems to be a relationship between the endogenous polyamine content and the early stages of rooting (Jarvis et al., 1985; Biondi et al., 1990; Heloir et al., 1996; Neves et al., 2002) and therefore, polyamines have been suggested as markers for adventitious rooting. Much like POX, changes in Put levels have been related to rooting phases: an early peak followed by a decrease was attributed to induction phase (Denaxa et al., 2014; Gaspar et al., 1997; Kevers et al., 1997a) and a second peak marked the expression phase (Denaxa et al., 2014; Nag et al., 2001). In fact, explants treated with auxin had increased levels of polyamines before root emergence (Desai and Mehta, 1985; Friedman et al., 1982; Geneve and Kester, 1991).

Some authors suggest that polyamines stimulate rooting through an increase in POX activity since treatment with H_2O_2 (a product of Put catabolism and POX substrate) increased rooting percentage and promoted early rooting (Rugini et al., 1992). On the

other hand, evidence suggests a combined role of polyamines and auxins in the regulation of the early events of adventitious rooting. Put and IAA levels varied in parallel during root induction and initiation of *Vigna* cuttings (Nag et al., 2001).

2.3. Factors affecting rooting performance by interfering with the availability of biochemical compounds

In the previous sections the most crucial factors controlling adventitious rooting were presented. However, their availability can be affected by other numerous endogenous and exogenous factors. For instance, auxins are produced in young leaves and buds (source tissues) and transported to the base of the cutting (sink tissues), therefore, the age of the cutting, as well as the presence of leaves and buds, can affect auxin availability.

Most research aiming to improve rooting of difficult-to-root olive cultivars, is based on the effect of such factors. Studies are not systematic and were mostly performed on trial and error basis. The main results of such studies are compiled in **Supplementary Material** and briefly discussed below. However, this section doesn't aim to be an exhaustive review on all the endogenous and exogenous factors affecting adventitious rooting in olive, but instead to present the major results achieved with experiments involving factors able to affect the availability of biochemical compounds.

2.3.1. Cutting size and age

Several authors have studied the effect of cutting size on rooting as it can affect the amount of available auxins. The results achieved for olive are not consistent, being highly dependent on the cultivars and cutting type (**Supplementary Material**).

In hardwood cuttings, the increase in cutting size negatively affects rooting being accompanied by a decrease in the number of roots per cutting (Awan et al., 2012). A reverse trend is observed in semi-hardwood cuttings and microcuttings (De Oliveira et al., 2003; Haq et al., 2009). On such propagation materials, rooting tends to improve with increasing cutting size, something that can potentially be attributed to greater carbohydrate reserves, higher amounts of accumulated endogenous auxins, and higher number of competent cells (Haq et al., 2009).

Furthermore, the maturity of the shoot used for cutting preparation seems to strongly affect rooting performance (Therios, 2009). In fact, it is now broadly accepted that the

origin of the propagation explant in relation to its position on the tree, can, independently of its nature (hardwood, semi-hardwood, or softwood), affect the capacity for adventitious root formation. Explants taken from the juvenile cone of the plant (basal part of the leader trunk) show higher rooting capacity than those taken from the upper part of the tree (Porlingis and Therios, 1976), which is probably related with the higher cell differentiation of the latter ones.

2.3.2. Type and concentration of auxin

The type and concentration of auxin used in root-inducing treatments is one of the most studied topics. When using semi-hardwood cuttings, IBA is the most commonly used auxin as it frequently promotes rooting more efficiently than NAA (Das et al., 2006; İsfendiyaroğlu and Özeker, 2008), with a few exceptions (Serrano et al., 2002). In other cases, however, the combination of both auxins results in better rooting rates, but genotype seems to play a major role (Denaxa et al., 2010; Khabou, 2002). IBA also produces better results than NAA in microcuttings (Bati et al., 1999). Although many concentrations of IBA have been tested (**Supplementary Material**), there isn't a universal concentration that will induce rooting in all cultivars. Actually, different rooting parameters can be improved by different IBA concentrations (Kurd et al., 2010; Pio et al., 2005). Nevertheless, optimal IBA concentrations for semi-hardwood cuttings are in the range 500 – 6000 ppm (mg L^{-1}) (**Supplementary Material**) and for microcuttings optimal concentrations of 1.25 and 1.5 mg L^{-1} have been reported (Ali et al., 2009; Haq et al., 2009).

2.3.3. Presence of buds and leaves

Leaves and buds are sites of photosynthesis and auxin production (Ljung et al., 2001) and therefore can potentially influence the rooting performance of cuttings by altering auxin levels and carbohydrate reserves.

In olive, the presence of leaves in the cuttings seems to play a minor role in adventitious root formation for some cultivars (De Oliveira et al., 2003), but an inhibitory effect was described in other cases when leaves and buds are removed (Avidan and Lavee, 1978; Caballero and Nahlawi, 1979; Fontanazza and Rugini, 1977). The presence of leaves has been reported to significantly improve callus and root formation, and to decrease outgrowth of buds to shoots (Suárez et al., 1999). However,

auxin treatments still seem to exert a stronger influence on rooting success (Pio et al., 2005).

2.3.4. Seasonality

The time of the year when cuttings are taken also seems to influence rooting performance and thus is important to choose the ideal season to get a maximum rooting response. Despite some discordant results (Mousa, 2003; Talaie and Ghassemi, 1996), there is a general seasonal trend for rooting percentage, with a maximum in summer and minimum during autumn and winter (Da Silva et al., 2012; De Oliveira et al., 2003; Fouad et al., 1990; Gellini, 1965; Mancuso, 1998; Therios, 2009; Usta, 1999). Differences in rooting response are attributed to the alternate-bearing behavior displayed by stock plants which in turn can be related to changes in carbohydrates over seasons (Denaxa et al., 2012; De Oliveira et al., 2003; Özkaya and Çelik, 1999). The proximity to harvest was also pointed as an explanation for a better rooting response at a certain time of year (De Oliveira et al., 2009). However, juvenility also needs to be taken into account: while juvenile cuttings have optimum rooting performances regardless of season, mature cuttings will root better if collected in late spring and/or summer (Therios, 2009).

The poor rooting response observed in 'Nabali' cuttings was attributed not only to IAA levels *per se* but also to their seasonal variation. Periods of higher rooting percentages were coincident with higher levels of IAA and ABA and lower levels of rooting inhibitors such as GAs and cytokinins (Ayoub and Qrunfleh, 2008).

Interestingly, *in vitro* explants carry over this seasonal behavior, which can be postponed by the darkening of the rooting medium (Mencuccini, 2003).

2.3.5. Light and darkening

Light and darkening can affect adventitious root formation in several ways. Light intensity, light quality or light exposure time are reported to influence rooting in many species (Fett-Neto et al., 2001; Jarvis and Shaheed, 1987; Sorin et al., 2005), including olive (Morini et al., 1990; Therios, 2009). Low light intensity can increase rooting percentage; long photoperiods can increase carbohydrate accumulation and induce rooting; light color can affect rooting, in a species-dependent manner (Therios, 2009). In addition, a dark environment at the base of cuttings can enhance the accumulation

of photosensitive auxins therefore improving rooting, as observed in many species (Pan and Van Staden, 1998).

In olive, it was found that darkening the bases of *in vitro* cultured explants enhances rooting response (Rugini et al., 1988, 1993). Initially, darkening was achieved by painting the outside of the vessels black or by placing black sterile polycarbonate granules in the surface of the rooting medium. However, this cumbersome approach was not practical and other alternatives were found. Black dye (Mencuccini, 2003) or activated charcoal (Peixe et al., 2007a) are frequently added to the rooting medium. In other cases, a 5 day-long dark pre-treatment before subculture is used (Rugini and Fedeli, 1990; Zacchini and De Agazio, 2004) to improve rooting performance, which indicates an inhibitory effect of light on rooting. In the absence of the dark pre-treatment, no rooting was obtained (Sghir et al., 2005) which could be attributed to a rooting inhibition associated with continuous exposure to auxin-like regulators, as shown for other woody species (Druart, 1997). Furthermore, darkening eliminates the differences in rooting ability observed *in vivo* among cultivars (Mencuccini, 2003).

2.3.6. Wounding

Wounding at the base of the cutting can enhance rooting, particularly when mature stock plants are used, by promoting cell division through auxin and carbohydrate accumulation at the wounding site. It also facilitates the absorption of exogenously applied auxin (Therios, 2009). Studies regarding wounding of semi-hardwood cuttings of olive are not abundant, and the few existing studies are quite contradictory and seem to depend on the cultivar (see **Supplementary Material**).

While in some cultivars basal cuts improved rooting (Talaie and Malakroodi, 1995), in other cases no differences in rooting response were observed between wounded and unwounded cuttings (Talaie and Ghassemi, 1996). In the case of girdling, results were affected by the type of cutting and growing season (Usta, 1999).

2.4. Other factors known to affect adventitious rooting

Much of the available information in the literature refers to the biochemical control of adventitious rooting, although other variables can affect the process, even if indirectly. Research in adventitious rooting of olive described other factors which may also influence the final outcome of cutting propagation (see **Supplementary Material**).

An **intact seed** in the cutting can prevent rooting through competition for assimilates (Del Rio, 1989). The **nutritional status of stock plants** is determinant to the rooting capacity of the cuttings produced from them. The type of **rooting substrate** can greatly affect the success of root formation as a result of specific properties such as substrate porosity, water retention capacity, pH and level of nutrients (Therios, 2009). In the case of microcuttings, **culture media** composition is detrimental to the efficiency of *in vitro* multiplication, as mineral composition of the medium (Cozza et al., 1997; Rugini and Pannelli, 1993), growth regulators (Chaari-Rkhis et al., 1997) and carbon source (Garcia et al., 2002; Leva et al., 1994) can determine the success of a micropropagation protocol. Application of **growth retardants** in combination with auxin can improve rooting ability, (Davis et al., 1985; Wiesman et al., 1989) possibly through inhibition of gibberellin biosynthesis (Rademacher et al., 1984).

Similarly, **fertilizers** containing essential nutrients such as *boron* and *zinc* can also enhance rooting response (Ali and Jarvis, 1988; Schwambach et al., 2005). Boron has been associated with the maintenance of cell division and cell enlargement (Josten and Kutschera, 1999) and lignin biosynthesis (George and De Klerk, 2008). It also has been suggested as a structural component of primary cell walls (Hu et al., 1996) and to have a role in the control of endogenous auxin levels (Jarvis and Booth, 1981) by promoting IAA destruction and translocation (Goldbach and Amberger, 1986; Jarvis, 1984). Zinc is required for the synthesis of the auxin precursor tryptophan (Trp) (Blazich, 1988; Marschner, 1995), and is also a structural component of auxin receptor ABP1 (Auxin-Binding Protein 1; Tromas et al., (2010)). *Manganese* and *iron* are co-factor and structural components of POX, respectively, and can therefore affect rooting rates by modulating this class of enzymes (Campa, 1991; Fang and Kao, 2000). Similarly, the inorganic composition of culture media can affect rooting performance, depending on species, cultivar and growth conditions (Geiss et al., 2009).

Carbohydrate reserves are the main source of energy to drive the formation of root primordia in cuttings (Calamar and De Klerk, 2002; Li and Leung, 2000) and have been related with rooting ability of cultivars (Aslmoshtaghi and Shahsavar, 2010; Yoo and Kim, 1996). Seasonal variations in carbohydrate levels have been suggested as an explanation for the seasonal rooting ability of olive cuttings (Del Rio et al., 1991). Carbohydrates also modulate gene expression (reviewed in Gibson, 2005) and interact with plant hormone signaling (reviewed in Eveland and Jackson, 2011; Gibson, 2004; León and Sheen, 2003). Daily fluctuations in sugar content have been related with changes in auxin levels (Sairanen et al., 2012) and it has been suggested that sugars affect auxin conjugation and/or transport (Ljung et al., 2015). However, the exact role of

carbohydrates in adventitious rooting is still controversial (Ragonezi et al., 2010) and apparently their allocation and distribution within the cutting has a greater influence than the content itself (Druege, 2009; Druege et al., 2000; Ruedell et al., 2013). Nevertheless, the positive effects of exogenous application of carbohydrates on rooting performance are often related with low reserves of the cuttings. Loach and Whalley (1978) obtained a 33% increase of *in vitro* rooting percentage of several woody species with the external application of 2% (w/v) sucrose, but only when the cuttings were subjected to low levels of light intensity. Likewise, Del Rio et al. (1986) were able to increase considerably the rooting capacity of 'Picual' olive cuttings, by immersing the base of the cuttings in a 15% (w/v) sucrose solution, although the rooting increase was only observed in stems containing floral buds.

The effect of other factors, such as **electrical impedance** and **cold storage**, has also been studied in different olive cultivars, being the main achieved results summarized in **Supplementary Material**.

3. Microbial symbiosis and adventitious rooting in olive

Although most research is focused on the chemical factors governing adventitious rooting, biological interactions with fungi and bacteria have also been described to enhance root formation and growth. Arbuscular mycorrhizae (AM) are broadly used in micropropagation for improving the performance of the propagated plantlets and reducing the acclimatization time (Kapoor et al., 2008). Inoculation of micropropagated plants with arbuscular mycorrhizal fungi (AMF) results in a highly branched root system, containing adventitious roots with higher diameter (Kapoor et al., 2008). It also increases photosynthetic efficiency, water conducting capacity, protects the plant from soil pathogens and environmental stress (Kapoor et al., 2008), and increases the survival rates of difficult-to-root plants (Azcón-Aguilar and Barea, 1997). Application of ectomycorrhizal fungi to the vegetative (micro)propagation of valuable plant species is a well-known and studied subject in woody plants like conifers (reviewed in Niemi et al., 2004).

Olive is known to form AM (Roldán-Fajardo and Barea, 1985) with obligate plant symbionts of the order Glomales (Calvente et al., 2004) (**Figure 4**). There is a wealth of information concerning the beneficial effects of these symbiotic associations in contexts such as drought and salinity tolerance (Porras-Soriano et al., 2009; Mekahlia et al.,

2013). AM fungi are reported to have a positive effect in the development and survival of micropropagated plants by improving plant establishment, increasing rhizosphere volume, improving nutrient uptake, protecting the plant against biotic and abiotic stress and improving soil structure. Their influence is particularly important during acclimatization of micropropagated plants by reducing the stress of transplantation (Azcón-Aguilar and Barea, 1997; Kapoor et al., 2008; Mechri et al., 2014; Smith and Read, 2010), as described for olive (Meddad-Hamza et al., 2010). However, studies involving the effect of mycorrhizae in adventitious rooting are less common.

Inoculation of olive cultivars with the AM fungus *Glomus mosseae* resulted in higher survival rates, shoot height and node number (Binet et al., 2007). This eventually resulted in a successful acclimatization and faster development of the economically valuable ecotype 'Laragne', which is able to grow beyond the limit of the Mediterranean climate (Chas, 1994). Thus, AM fungi can be included in micropropagation programs to promote the culture of important olive genotypes.

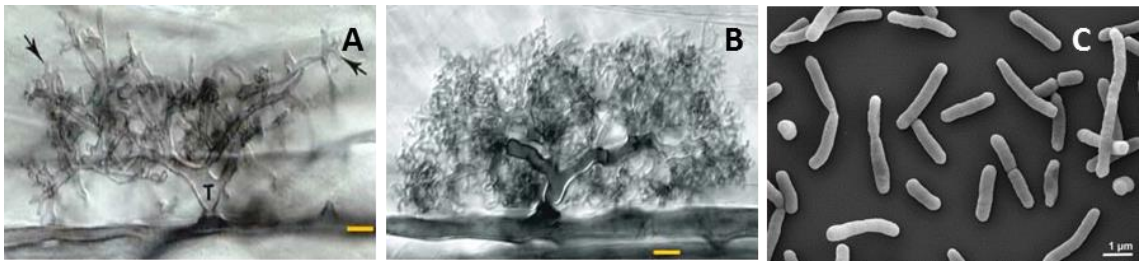

Figure 4. Microbial associations found in *Olea europaea***.** (A) Developing arbuscule of *Glomus mosseae* in a root cell; (B) Mature arbuscule of *Glomus mosseae* with branched hyphae; (A) and (B) Bar = 10 µm (from "Arbuscular Mycorrhizas," http://www.sft66.com/fungi/html/vam.html); (C) Scanning electron micrograph showing cells of *Chryseobacterium oleae* strain CT348T (from Montero-Calasanz et al., 2014).

Inoculation with selected AM fungi has been reported for 'Arbequina', 'Leccino', 'Moraiolo', 'Frantoio', 'Misión', 'Picual' and 'Cornicabra' cultivars. However, as described for other species (Camprubi and Calvet, 1996; Linderman and Davis, 2001), the degree of responsiveness to mycorrhizae was shown to be dependent both on genotype and AM fungi species or strain used for inoculation (Calvente et al., 2004; Citernesi et al., 1998; Estaún et al., 2003; Ganz et al., 2002; Piedra et al., 2003). Moreover, the substrate used for acclimatization of inoculated plants can influence root colonization (Bustos, 2012). AM fungi have even been used in wild olive (*Olea europaea* ssp. *sylvestris*) plants, where inoculation not only increased shoot biomass,

but also enhanced the activity of antioxidant enzymes as well as other physiological parameters (Alguacil et al., 2003; Caravaca et al., 2003).

Mechri et al. (2014) found that olive tree root colonization with the AM fungi *Glomus intraradices* induced significant changes in the bacterial community structure of olive tree rhizosphere compared to non-mycorrhizal plants. This mycorrhizal effect on rhizosphere communities may be a consequence of changes in characteristics in the environment close to mycorrhizal roots, granting suitable conditions for other microorganisms and eventual synergic interactions.

Besides AM, a species of plant growth promoting bacteria has been identified in olive. Very recently, Montero-Calasanz et al. (2014) identified a novel species of bacteria, *Chryseobacterium oleae* (type strain CT348T), isolated from the ectorhizosphere of an organically farmed olive tree cv. 'Arbequina'. Among the evaluated bioproducts of the strain, several polyamines were found that are known to be involved in adventitious root formation (discussed in Section 2.2.4.). Sym-homospermidine is the major polyamine produced but Spd and Spm were also determined as minor components. Traces of Put and cadaverine were also detected. *Chryseobacterium* genus members have also been considered an important bacterial group associated with plants (Anderson and Habiger, 2012; Brown et al., 2012; Lee et al., 2006) and are thought to possess plant-growth promoting activities (Dardanelli et al., 2010; Montero-Calasanz et al., 2013).

4. Conclusions

Much of the available information on olive adventitious root formation has been published in specific symposia and, frequently, only the abstract of such communications is accessible. This problem, associated with the absence of a continued line of work on the fundamental aspects of adventitious rooting (anatomy, physiology and genetics), has considerably hindered the collection of information presented in this paper.

In woody perennials, the difficulty in establishing analytical methods that allow collecting this type of data has definitely contributed to the lack of fundamental studies on adventitious rooting. In such species, most rooting assays are not systematic and so far have been based on empirical knowledge, where the effect of factors known to affect the formation of adventitious roots is evaluated.

From the gathered information, some conclusions seem however to be consolidated:

- In olive, there is no evidence for the existence of preformed roots. However, the presence of latent meristematic structures in older branches, which can evolve to form new adventitious roots when subjected to suitable conditions, seems to be established. Such structures, together with high levels of nutritional reserves in the cuttings, have clearly enabled the use of propagation by hardwood cuttings, a method used in olive propagation since ancient history.

- Differences in the rooting ability of cultivars cannot be explained by anatomical features of the stem. The sclerenchyma ring typically found in *Olea europaea* is not significantly different among varieties and therefore doesn't explain the observed variability in rooting behaviors.

- Auxin is a limiting factor in the rooting of semi-hardwood cuttings since acceptable rooting rates can't be obtained in the absence of exogenous treatments. In cultivars considered easy-to-root, the exogenous application of high concentrations of IBA (2000 – 6000 ppm) for short time periods (10 – 20 sec) has shown to be effective and allows good rooting rates (70 – 90%) which are compatible with commercial propagation. However, in difficult-to-root cultivars it became evident that other factors influence the cuttings' response, besides endogenous auxin levels. Although many parameters have been tested, including methods of application (solution, powder), concentrations, length of the treatment, and even other auxins (IAA, NAA), low rooting rates of these cuttings are still a persistent result.

In view of the above, many assays were performed with the goal of testing the effect of factors that are known to affect the rooting capacity of cuttings. Among these, some should be highlighted: the external application of polyamines (Rugini (1992), obtained good rooting rates with Put), the exogenous application of carbohydrates (Del Rio et al., (1986), show that external application is only effective during periods of natural deficiency), the nutritional status of mother plants (high nitrogen levels decrease rooting rates), the season (it seems established that spring and fall are the best seasons for rooting of semi-hardwood cuttings), the age of the starting plant material and that of the cutting (maintenance of mother plants with vigorous pruning is important and branches collected from the base of the stem are the best plant material for rooting).

Very few articles report the quantification of endogenous levels of compounds that affect the formation of adventitious roots in olive. In such cases, it's shown that difficult-

to-root cultivars struggle to control free auxin levels after the induction phase. The first results from genomic studies are now starting to emerge, with emphasis on the differential expression of AOX genes in easy- and difficult-to-root plants (Hedayati et al., 2015).

Developing a base of knowledge about adventitious rooting in olive to a level similar to what has been achieved in model species is certainly the main goal of future research studies. However, that doesn't necessarily require following the same steps. Sometimes, taking a step back allows us to learn from our mistakes and have a different view of what path to take.

For instance, the use of hardwood or semi-hardwood cuttings in fundamental studies on adventitious rooting is ill-advised, considering the extremely random response of this type of material, which is also dependent on uncontrollable factors (light, temperature, and humidity). *In vitro* culture techniques allow a much more effective control of such factors and therefore a much more homogenous response from the plant material.

It is impractical to keep using the base of the cuttings as analytical sample since it is known that only a small portion of cells participate in the process of adventitious root formation. The recent progress in techniques such as confocal microscopy and scanning electron microscopy, together with laser microdissection, opens up new possibilities for the study of pre-primordial and primordial cells.

Furthermore, it is also inefficient to continue quantifying biochemical compounds associated with adventitious root formation in the absence of a complementary genetic analysis of the process. Next Generation Sequencing (NSG) techniques (Tsai, 2013) are currently an extremely valuable tool in the search for genetic variability between individuals with different rooting behavior and can be a vital tool in studies on adventitious root formation.

As mentioned by Haissig and Davis (1994), "many researchers have collected numeric data, based on the hypothesis that enough numbers and properly compared will yield repeatable, interrelated sequences that can be used to decipher the most basic process underlying rooting. However, that hypothesis remains unproven. We have not been able to identify any underlying law(s) that our data and, therefore, rooting obeys."

A complex process demands an integrated approach. Only this way we can aspire to, sometime, be able to root any olive cultivar, where we want and how we want.

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Supplementary material

Supplementary table 1 – Endogenous and exogenous factors affecting adventitious root formation of olive cuttings.

Hardwood cuttings					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Cutting size	Azarbaijan	----	- IBA 3000 ppm - Plastic tunnels covered with nylon shade cloth	The increase in cutting size was accompanied by a decrease in number of roots per cutting and root length	Awan et al. (2012)
	Uslu	Low			
	Improved Nabali	----			
	Manzanillo	----			
	Leccino	High			
Electrical impedance	Minerva (Leccino clone)	----	- KIBA 3600 ppm - Perlite	Highest rooting percentages were related with conditions characteristic of high metabolic activity (low intracellular resistance and high relaxation times in the shoot)	Mancuso (1998)
Semi-hardwood cuttings					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Type and concentration of auxin	Galega vulgar	Low	(A) IBA 5000 ppm in 30% ethanol (B) Powder formulation of 0.8% IBA (Seradix) (C) Powder formulation of 0.2% NAA (Rhizopon B)	- Rhizopon B: highest average rooting percentage - Seradix: lowest average rooting percentage - Treatment with IBA 5000 ppm was ineffective	Serrano et al. (2002)
Type and concentration of auxin	Chemlali	----	- Several combinations of IBA (1000 - 4000 ppm) + NAA (0 - 2000 ppm) + IAA (0 - 2000 ppm) - Perlite under mist	Highest rooting rate (60%) was obtained with IBA 2000 ppm	Khabou (2002)
	Meski	----		Highest rooting rate (82%) achieved with IBA 2000 + NAA 500 ppm	
	Chemchali	----		Highest rooting rate (75%) obtained with IBA 2000 ppm	
	Oueslati	----		Best rooting percentage (73%) obtained with IBA 4000 ppm, or IBA 2000 + NAA 1000 + IAA 1000 ppm, or IBA 2000 + NAA 500 + IAA 500 ppm	

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Type and concentration of auxin	Leccino	High	(A) IBA 3000, 5000 ppm (B) NAA 500, 1000 ppm	- Highest rooting rate (90%) obtained with IBA 5000 ppm - Rooting percentage increased with increasing concentration of auxin - IBA produces higher rooting rates than NAA	Das et al. (2006)
Type and concentration of auxin	Domat	Low	(A) IBA 5000 ppm (B) NAA 1000, 3000, 5000, 7000 ppm (C) SA 2500, 5000, 7500, 10 000 ppm (D) SA in different combinations with IBA	- Highest rooting (63%) obtained with IBA 5000 ppm followed by NAA 3000 ppm (37%) - All SA treatments inhibited rooting	İsfendiyaroğlu and Özeker (2008)
Type and concentration of auxin	Arbequina	High	(A) IBA 500, 1000, 2000, 4000 ppm (B) NAA 500, 1000, 2000, 4000 ppm (C) Combinations of both auxins	Highest rooting percentage obtained with IBA 2000 ppm in summer and IBA+NAA 1000 ppm in autumn	Denaxa et al. (2010)
	Mastoidis	Medium		Best rooting performance achieved with NAA 1000 ppm regardless of season	
	Kalamata	Low		Highest rooting percentage (5%) obtained in summer with IBA 500 ppm	
Type and concentration of auxin	Clonavis Sevillana Manzanilla	---- ---- ----	IBA 2000, 3000, 4000 ppm	3000 ppm induced the highest rooting percentage in all cultivars.	Talaie and Malakroodi (1995)
Type and concentration of auxin	Domat N.D. Belice Biancolilla Pendolino Coratina	Low ---- ---- ---- ----	- IBA 2000, 4000, 6000 ppm - Cuttings planted in sand : silt : clay (1:1:1) in plastic tunnels	IBA 4000 ppm promoted the highest: - Sprouting percentage - Survival percentage - Shoot and root length - Root number	Khattak et al. (2001)
Type and concentration of auxin	Nabali Improved Nabali	Low Medium	- IBA 2000, 4000, 6000, 8000 ppm - Perlite	Highest rooting percentage (25.3% Nabali, 35.2% Improved Nabali) with 6000 ppm	Mousa (2003)

Semi-hardwood cuttings (cont.)

Tested factor	Cultivar	Rooting ability	Rooting conditions	Results	Reference
Type and concentration of auxin	Ascolano 315	Low	- IBA 1000, 3000, 5000 ppm - Rooting media compared: I. Sand II. Soil III. Vermiculite IV. Sand : soil (1:1 v/v)	IBA 3000 and 5000 ppm: - Higher rooting percentage - Higher number of roots per cutting - Higher and root length	De Oliveira et al. (2003a)
Type and concentration of auxin	Grapollo	----	- IBA 1000, 2000, 3000 ppm - Plantmax®	- IBA 2000 ppm: highest rooting percentage - 3000 ppm : Longer roots and higher number of roots per cutting	Pio et al. (2005)
Type and concentration of auxin	Ascolano 315	Low	- IBA 1000, 2000, 3000 ppm - Rooting media compared: I. Perlite II. Perlite : vermiculite (1:1, v/v)	IBA 3000 ppm: - Higher rooting percentage - Higher number of roots per cutting	De Oliveira et al. (2009)
Type and concentration of auxin	Coratina	----	IBA 3000, 4000, 5000 ppm	- 4000 ppm: Highest root number and root length - 3000 ppm: Highest rooting percentage	Kurd et al. (2010)
Type and concentration of auxin	*Olea europaea* L. subsp. Cuspidata	Low	IBA 0, 10, 20, 25, 30, 35 and 40 µg/cutting	Optimal IBA dosage in the range of 20–40 µg/cutting	Negash (2003)
Cutting size	Ayvalık	High	- IBA 4000 ppm - Rooting media compared: I. Control (Sand) II. Perlite: Peat: Sand: Silt (1:1:1:1) III. Perlite: Peat: Sand: Silt (1:2:1:2) IV. Perlite: Peat: Sand: Silt (1:1:2:2) V. Perlite: Peat: Sand: Silt (0:0:1:1) VI. Perlite: Peat: Sand: Silt (1:0:1:1)	- All tested sizes can be used for successful root formation - Root formation depends on the combination of cutting size and rooting medium used	Gerakakis and Özkaya (2005)
	Domat	Low		None of the cutting sizes and/or rooting media induced rooting of this cultivar	

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Cutting size	Picual Arbequina	Medium High	- IBA 3000 ppm - Perlite, bottom temperature 23 – 25°C	- Rooting percentage increased with the increase of cutting size - No differences were found between cuttings with the same number of nodes	De Oliveira et al. (2003b)
Type of cutting	Domat	Low	IBA 4000 ppm	Medial cuttings: - Higher number of rooted cuttings - Reduced callus formation	Usta (1999)
Presence of leaves and buds	Gordal	Low	- IBA 8000 ppm - Perlite at 25°C basal heating	- Presence of lateral buds: no effect - Presence of leaves: improved callus and root formation, decreased outgrowth of buds - Cuttings without leaves: lower rooting rates and increased shooting of buds, even when lateral buds were detached	Suárez et al. (1999)
Presence of leaves and buds	Grapollo	Low	- IBA 1000, 2000 and 3000 ppm - Plantmax® substrate in nebulization chamber	IBA treatment was the prevalent factor, inducing rooting independently of the presence of leaves	Pio et al. (2005)
Presence of intact seed in the cutting	Picual	Medium	- IBA 3000 ppm - Perlite at 20 – 22°C	Inhibitory influence of the intact seed: callus and rooting percentage was higher in cuttings with killed-seed, but lower than in cuttings with fruit removed	Del Rio and Rallo (1991)
Juvenility	Chondrolia Chalkidikis	Medium	Not specified	- Rooting ability decreases with the transformation from juvenile to mature form - Cuttings taken from the crown of the trunk rooted much more readily than cuttings taken from the top of the tree	Porlingis and Therios (1976)
Type of rooting medium	Ayvalık	High	- IBA 3000 ppm - 25 different media either on their own or as mixtures	- 95% rooting with perlite : vermiculite (1:1 v/v) - ≥ 90% rooting with rockwool, peat : polystyrene (2:1 v/v) and sand : perlite (1:2 v/v) - 5-28% rooting with sand, peat and peat : sand mixtures	İsfendiyaroğlu et al. (2009)

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Type of rooting medium	Ayvalık Domat	High Low	- IBA 4000 ppm applied for 10 sec - Rooting media compared: I. Control (Sand) II. Perlite: Peat: Sand: Silt (1:1:1:1) III. Perlite: Peat: Sand: Silt (1:2:1:2) IV. Perlite: Peat: Sand: Silt (1:1:2:2) V. Perlite: Peat: Sand: Silt (0:0:1:1) VI. Perlite: Peat: Sand: Silt (1:0:1:1)	- Best results obtained with media containing silt and sand - A bigger ratio of sand to silt leads to a decrease in survival rates of the cuttings	Gerakakis and Özkaya (2005)
Type of rooting medium	Arbequina Koroneiki Picual	High Medium Medium	- IBA 4000 ppm - Sand, perlite and peat-moss	- Sand: Highest rooting percentages - Perlite: Highest root length and number - Peat-moss: Large swellings at the base of cuttings and occasional apical necrosis	Hechmi et al. (2013)
Type of rooting medium	Roghani Zard Zeitoun	---- ----	- IBA 4000 ppm - Rooting media compared: I. Peatmoss + perlite II. Sawdust + sand III. Peatmoss + sand IV. Perlite V. Sand VI. Perlite + sand	- Perlite: Highest rooting percentage (53%) - Peat-moss + perlite: Lowest rooting percentage (44%)	Talaie and Ghassemi (1996)
Type of rooting medium	Arbequina	High	- IBA 4000 ppm - Rooting media compared: I. Sand II. Perlite III. Peat-moss	- Better rooting response observed with perlite - More calli, less and shorter roots per cutting obtained with peat-moss - Highest rooting percentage (90%) and survival rate (99%) was achieved with sand	Mehri et al. (2013)
Type of rooting medium	Ascolano 315	Low	- IBA 1000, 3000 and 5000 ppm - Rooting media compared: I. Sand II. Soil III. Vermiculite IV. Sand : soil (1:1 v/v)	- Best rooting response (48% rooting) with sand : soil - Vermiculite was the worst rooting medium (10% rooting)	De Oliveira et al. (2003a)

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Type of rooting medium	Ascolano 315	Low	- IBA 3000 ppm - Rooting media compared: I. Perlite II. Perlite : vermiculite (1:1, v/v)	- Similar rooting percentages (9.6%) w/ both substrates - Addition of vermiculite only increased root length - No significant differences in root number or rooting percentage between the two substrates	De Oliveira et al. (2009)
Time of collection of cuttings (season)	Weteken Dermlali Khoderi Souri Picual Mission Frantoio Koroneiki	High Low Low Low Medium Medium Medium Medium	- IBA 4000 ppm - Peat moss : sand (2 : 1)	- Season can affect rooting performance - Maximum rooting percentage in summer - Minimum rooting percentage in winter	Fouad et al. (1990)
Time of collection of cuttings (season)	Domat	Low	IBA 4000 ppm	- In April, good rooting rates obtained with apical and medial cuttings - Best response was achieved with basal cuttings in September - Overall, rooting rates increased from spring to summer/early autumn	Usta (1999)
Time of collection of cuttings (season)	Roghani Zard Zeitoun	---- ----	- IBA 4000 ppm - Rooting media compared: I. Peatmoss + perlite II. Sawdust + sand III. Peatmoss + sand IV. Perlite V. Sand VI. Perlite + sand	- March: Highest rooting percentage - August: Lowest rooting response	Talaie and Ghassemi (1996)
Time of collection of cuttings (season)	Raseei Nabali	High Low	Not specified	Good rooting performance regardless of time of collection - Lowest rooting percentage in February - Highest rooting percentage in September	Ayoub and Qrunfleh (2008)

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Time of collection of cuttings (season)	Ascolano 315	Low	- IBA 3000 ppm - Rooting media compared: I. Perlite II. Perlite : vermiculite (1:1, v/v)	- April and June: highest rooting percentage and root length - No differences were found among other collection times	De Oliveira et al. (2009)
Time of collection of cuttings (season)	Ascolano 315	Low	- IBA 1000, 3000 and 5000 ppm - Rooting media compared: I. Sand II. Vermiculite III. Sand : soil (1:1, v/v) IV. Soil	Better rooting response in February	De Oliveira et al. (2003a)
Time of collection of cuttings (season)	35 cultivars	----	- IBA 3000 ppm - Sand	For some cultivars, cuttings collected in April showed better rooting response (rooting percentage and number of roots per cutting), although this was significantly dependent on the interaction cultivar x collection time.	Da Silva et al. (2012)
Time of collection of cuttings (season)	Nabali Improved Nabali	Low ----	- IBA 2000, 4000, 6000 and 8000 ppm - Perlite	December: Higher rooting percentage and root number	Mousa (2003)
Time of collection of cuttings (season)	Leccino	High	- IBA 0.3% (talc powder) - Coarse sand	- Spring: Highest rooting percentage and root number (February and April, respectively) - Winter: Highest survival rate and lowest number of roots	Ahmed et al. (2002)

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Wounding	Roghani Zard Zeitoun	---- ----	- IBA 4000 ppm - Rooting media compared: I. Peatmoss + perlite II. Sawdust + sand III. Peatmoss + sand IV. Perlite V. Sand VI. Perlite + sand	No differences in rooting response were observed between wounded and unwounded cuttings	Talaie and Ghassemi (1996)
Wounding	Clonavis Sevillana Manzanilla	---- ---- ----	IBA 2000, 3000, and 4000 ppm	- Basal cuts increased the number of roots and root percentage - No effect was observed in root length	Talaie and Malakroodi (1995)
Wounding	Domat	Low	IBA 4000 ppm	- Girdling in medial cuttings showed the best results - Growing season also had an effect on rooting response	Usta (1999)
Light quality	Leccino	High	- IBA 2500 ppm - Plastic basins containing wet perlite closed inside transparent polyethylene bags	- Yellow light has a positive effect on rooting percentage, root length and number of persisting leaves - Effect was considerably more noticeable in IBA-treated cuttings	Morini et al. (1990)
Cold storage	Nocellara del Belice Cassanese	High ----	IBA 2500 ppm	The highest rooting percentage was obtained with IBA treatment followed by cold storage of the cuttings Best results were achieved with cuttings stored at 4°C for 2 days and treated with IBA afterwards	Briccoli-Bati and Lombardo (1987)
Growth retardants	Arbequina Manzanillo	High ----	- Rooted cuttings were used - Rooting conditions not specified	Paclobutrazol is a weak growth retardant in olive	Navarro et al. (1989)

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Growth retardants	Kalamata Manzanillo	Medium ----	- Talc powder containing 0.8% IBA - Peat : crushed plastic foam (1:1, v:v) at 25°C	- Spray treatment of mother plants improved rooting and increased callus formation in both cultivars - Soil application of paclobutrazol led to very poor rooting, although it increased plant survival - Chlormequat: least efficient growth retardant - The effect of IBA treatment was significantly enhanced by the paclobutrazol in 'Manzanillo'	Wiesman and Lavee (1994)
Growth retardants	Barnea Manzanillo Souri Uovo de Piccione	High Medium Low Medium	- IBA (0.8% talc powder) combined with urea-phosphate (UP) and/or paclobutrazol (PB) - Peat and crushed plastic foam (1:1, v:v) maintained at 25°C	- When applied alone, neither UP nor PB improved rooting - When in combination with IBA, both increased rooting - PB was more efficient than UP - The triple treatment IBA + UP + PB significantly improved rooting and survival in all cultivars	Wiesman and Lavee (1995b)
Fertilizers	Ascolano 315 Arbequina	Low High	- IBA 3000 ppm - Perlite	- Only fertilizers containing Zn increased callus percentage - No increase in rooting percentage was observed (inhibitory effect of high Zn concentrations) - Highest rooting percentage: fertilizer containing Zn, an adequate concentration (0.27%) of organic C and B (0.18%)	De Oliveira et al. (2010a)
Fertilizers	Ascolano 315	Low	- IBA 3000 ppm - Perlite 20 ± 2°C	- Fertilizers slightly improved rooting performance - N may acidify the rooting substrate when in high concentrations - Fertilizers containing Zn can improve rooting by increasing tryptophan synthesis	De Oliveira et al. (2010b)
Nutritional status of stock plants	Barnea	High	- Rooted cuttings exposed to different concentrations of: ✓ N (0.4 to 14.1 mM) ✓ P (0.01 to 0.62 mM) ✓ K (0.25 to 5.33 mM) - Perlite	- K and P: minor role in propagation success - N negatively affected rooting rates and cutting survival: reduction in N concentration in irrigation water caused a threefold increase in propagation success	Dag et al. (2012)

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Hydrogen peroxide	Frantoio Gentile di Larino	High Low	A) 10 s in IBA 2000 ppm B) 10 s in IBA 4000 ppm C) 30 s in 3.5% (w/v) H_2O_2 + 10 s in IBA 2000 ppm D) 30 s in 3.5% (w/v) H_2O_2 + 10 s in IBA 4000 ppm Perlite	- Rooting response was improved by H_2O_2 treatment - Root formation occurred independently of IBA dosage - Root formation only occurred at high IBA concentration (4000 ppm) - Rooting response was improved by H_2O_2 treatment, counteracting the lack of root formation at lower IBA concentration	Sebastiani et al. (2002)
Hydrogen peroxide	Frantoio Gentile di Larino	High Low	(A) 10 s in IBA 4000 ppm (B) 30 s in 3.5% (w/v) H_2O_2 + 10 s in IBA 4000 ppm Perlite	H_2O_2 improved rooting in both cultivars at least in one of the years of the study	Sebastiani and Tognetti (2004)
Carbohydrates	Barnea Manzanillo Kalamata	High ---- Low	- IBA 6000 ppm - Peat and crushed plastic foam (1/1, v/v)	- Rooting of 'Manzanillo' cuttings was improved by sucrose applied together with IBA - Amyloplasts levels decreased during rooting, especially in IBA-treated cuttings	Wiesman and Lavee (1995a)
Carbohydrates	Gemlik Domat	High Low	(A) IBA 4000 ppm (B) Wounding (C) Wounding + IBA 4000 ppm Shaded Polyethylene Tunnels (SPT)	- Carbohydrate content decreases over time and is higher in cuttings from "on-years" - A relationship between total carbohydrates content and rooting couldn't be established	Özkaya and Çelik (1999)
Carbohydrates	Arbequina Kalamata	High Low	- IBA 2000 ppm - Plant plugs under an automatic mist unit	- Time of planting: 'Arbequina' cuttings had higher amounts of total soluble sugars and lower levels of starch - Rooting percentage in 'Arbequina' was correlated with the initial levels of fructose and glucose, which didn't seem to depend on auxin treatment - During rooting of 'Arbequina' cuttings, levels of glucose and mannitol decreased - Starch concentration also decreased during rooting, however at a lower rate than that of individual sugars	Denaxa et al. (2012)

Semi-hardwood cuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Carbohydrates	Leccino	High	IBA 4000 ppm	- Mannitol was main soluble carbohydrate - After an initial increase, mannitol content decreased during rooting of both cultivars - The content of total soluble carbohydrates increased during root differentiation	Bartolini et al. (2008)
	Leccio del Corno	Low			

Microcuttings					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Type and concentration of auxin	Nocellara etnea	Low	(A) IBA 0.5, 1.5, 2.5 ppm (B) NAA 0.5, 1.5, 2.5 ppm	- IBA produced better results than NAA - Rooting percentage increased with auxin concentration	Bati et al. (1999)
Type and concentration of auxin	Dolce Agogia	Medium	- IBA at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75 and 2.0 mg L^{-1} - Modified OM (half macro & micro elements) medium supplemented with 100 mg L^{-1} Brilliant Black dye - Acclimatization in soil: sand (1:1) in glass house	Highest rooting percentage (95.3%) achieved with tetranodal cuttings and IBA 1.25 mg L^{-1}	Haq et al. (2009)
Type and concentration of auxin	Moraiolo	High	(A) IBA 0.5, 1, 1.5, 2, 2.5, 3 mg L^{-1} (B) NAA 0.5, 1, 1.5, 2, 2.5, 3 mg L^{-1}	- IBA produced better results than NAA - Highest rooting percentage (86.7%) IBA 1.5 mg L^{-1}	Ali et al. (2009)
Microcutting size	Dolce Agogia	Medium	- Modified OM (half macro & micro elements) medium supplemented with 100 mg L^{-1} Brilliant Black dye - IBA at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75 and 2.0 mg L^{-1} - Acclimatization in soil: sand (1:1) in glass house	- Rooting percentage increased with the increase of microcutting size - Tetranodal cuttings achieved maximum rooting percentages at lower IBA concentrations than other cutting sizes	Haq et al. (2009)

Microcuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Time of collection of cuttings (season)	Frantoio Dolce Agogia Moraiolo	High Medium High	MS medium with 5 µM NAA	Explants not submitted to darkening: - Maintained their *in vivo* seasonal rooting response - Low rooting rates in January - High rooting rates in May and September	Mencuccini (2003)
Light and darkening	Moraiolo	High	Bourgin and Nitsch (1967) medium with macro elements reduced to half, 2% sucrose, with or without 5 µM NAA and 0.7% agar	Darkening improved rooting and promoted earlier root emergence	Rugini et al. (1993)
Light and darkening	Frantoio Dolce Agogia Moraiolo	High Medium High	MS medium with 5 µM NAA, 2% sucrose, 0.7 % agar, pH 5.5 Black dye (Brilliant Black) added to the medium in 4 concentrations: 0, 10, 100, 200 mg L^{-1}	- Darkening (100-200 mg/L) enhanced root formation by 100% regardless rooting period or cultivar - Darkening eliminates the differences in rooting ability observed *in vivo* among cultivars	Mencuccini (2003)
Light and darkening	Nebbiara	----	OM medium with half-dose macronutrients, 20 g L^{-1} sucrose, 3.22 µM NAA	- The darkening treatment promoted rooting in all tested explants - Root number was decreased in the treated cuttings - Dark exposure didn't affect root length	Zacchini and De Agazio (2004)
Light and darkening	ZDH4 Lucques Haouzia Dahbia Amellau Salonenque Picholine du Languedoc Picholine marocaine	High High Medium Medium Medium Medium Low Low	OM medium with 5.37 µM NAA or 24.6 µM IBA (single-phase); or two-phase protocol with a 5 day induction phase in liquid 24.6 µM IBA solution in the dark with further cultivation on regulator-free OM medium	- In the absence of the dark pre-treatment, no rooting was obtained for either of the cultivars tested - All cultivars rooted to varying degrees when exposed to darkness	Sghir et al. (2005)

Microcuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Culture media composition	ZDH4	High	OM medium with 5.37 µM NAA or 24.6 µM IBA (single-phase); or two-phase protocol with a 5 day induction phase in liquid 24.6 µM IBA solution in the dark with further cultivation on regulator-free OM medium	- NAA-containing medium induced rooting only in 'Picholine marocaine' - Shoots planted in IBA-containing medium developed calli without root formation - 5-day induction step stimulated rooting in all cultivars, particularly 'Picholine marocaine' (65% rooting)	Sghir et al. (2005)
	Lucques	High			
	Haouzia	Medium			
	Dahbia	Medium			
	Amellau	Medium			
	Salonenque	Medium			
	Picholine du Languedoc	Low			
	Picholine marocaine	Low			
Culture media composition	Galega vulgar	Low	- OM basal medium, supplemented with 4.9 µM IBA, or 14,700 µM IBA for 10 sec (pulse technique) - All media contained 2 g L^{-1} activated charcoal - Jiffy-Pots filled with a vermiculite : perlite (3:1, v/v) mixture subsequently wetted with the OM mineral solution	- Highest rooting rates were obtained using the pulse technique, followed by inoculation in OM regulator-free medium - Multiplication was improved using coconut water and BAP as zeatin substitutes, and sucrose as D-mannitol substitute	Peixe et al. (2007)
Culture media composition	Wild olive (*Olea europaea* ssp. *maderensis*)	Low	Half-strength DKW medium with: 5.4 µM NAA 26.8 µM NAA 4.1 µM IBA 20.7 µM IBA 2 mM IBA Peat : perlite (3:2) treated with fungicide	- Rooting was only achieved with IBA-containing media - This approach gave better results than short exposure to high-concentration IBA solutions - Further acclimatization allowed 70% survival of the micropropagated plants	Santos et al. (2003)
Inorganic compounds	Nocellara etnea	Low	IBA (2.5 ppm) or NAA (1.5 ppm) combined with several concentrations of H_3PO_4 (50 – 400 ppm)	- Interaction between auxins (IBA and NAA) and H_3PO_4 - IBA treatment promoted rooting regardless of the presence of H_3PO_4 - Rooting response was enhanced in the combined treatment	Bati et al. (1999)
	Carolea	High			

Microcuttings (cont.)					
Tested factor	**Cultivar**	**Rooting ability**	**Rooting conditions**	**Results**	**Reference**
Inorganic compounds	Chondrolia Chalkidikis	Medium	IBA 12 µM + NAA 3 µM IBA 12 µM + NAA 3 µM + 30 µM putrescine IBA 12 µM + NAA 3 µM + 1 mM H_3PO_4 IBA 12 µM + NAA 3 µM +1 mM H_3PO_4 + 30 µM putrescine Peat : perlite (4:1, v/v)	Phosphoric acid showed no apparent influence on the rooting of this cultivar	Grigoriadou et al. (2002)

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Chapter II

CURRENT ANALYTICAL METHODS FOR PLANT AUXIN QUANTIFICATION – A REVIEW

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Current analytical methods for plant auxin quantification – A Review

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Abstract

Plant hormones, and especially auxins, are low molecular weight compounds highly involved in the control of plant growth and development. Auxins are also broadly used in horticulture, as part of vegetative plant propagation protocols, allowing the cloning of genotypes of interest. Over the years, large efforts have been put in the development of more sensitive and precise methods of analysis and quantification of plant hormone levels in plant tissues. Although analytical techniques have evolved, and new methods have been implemented, sample preparation is still the limiting step of auxin analysis. In this review, the current methods of auxin analysis are discussed. Sample preparation procedures, including extraction, purification and derivatization, are reviewed and compared. The different analytical techniques, ranging from chromatographic and mass spectrometry methods to immunoassays and electrokinetic methods, as well as other types of detection are also discussed. Considering that auxin analysis mirrors the evolution in analytical chemistry, the number of publications describing new and/or improved methods is always increasing and we considered appropriate to update the available information. For that reason, this article aims to review the current advances in auxin analysis, and thus only reports from the past 15 years will be covered.

Keywords: Plant hormones, Auxins quantification, Sample preparation, Chromatographic analysis, Mass spectrometry, Immunoassays

1. Introduction

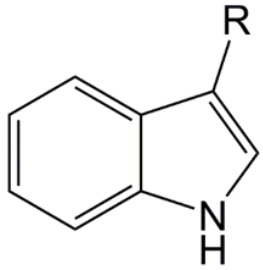
Plant hormones are a group of structurally diverse compounds which regulate most processes involved in plant growth and development [1,2]. Auxins are by far the most studied group of plant hormones mainly because they were the first to be discovered [3,4], and because they are widely used in plant propagation protocols [5–9], given their role in adventitious root formation in different species [10,11].

Although there are several compounds with auxin activity, indole-3-acetic acid (IAA) is by far the most physiologically important plant hormone. In fact, it is surprising how such a small molecule can influence so many different processes. IAA has been shown to be involved in many aspects of plant growth and development: cell elongation, regulation of apical dominance, vascular differentiation, fruit development, lateral and adventitious root formation [2]. Indeed, IAA has long been considered “the growth hormone” [4,12].

The widespread use of auxins in plant propagation protocols and physiological studies [9,13], has led to many efforts towards the development of analytical methods for the quantification of the very low auxin levels in plants. The goal of this review is to summarize the recent advances (since 2000) in analytical methods for the quantification of two naturally occurring auxins, IAA and indole-3-butyric acid (IBA) in plant tissues.

2. Analytical methods for auxin quantification

Auxins are indolic acids distinguishable by a variable side chain (see **Figure.1**).



Auxin	**R**
ICA – indole-3-carboxylic acid	COOH
IAA – indole-3-acetic acid	CH_2COOH
IPA – indole-3-propionic acid	CH_2CH_2COOH
IBA – indole-3-butyric acid	$CH_2CH_2CH_2COOH$
MeIAA – IAA methyl ester	CH_2COOCH_3

Figure. 1 Chemical structure of auxins (adapted from [16])

One of the main obstacles to auxin quantification is the low endogenous concentration of analyte present in plant samples. Like any plant hormone, auxins are typically found

in trace amounts in plant tissues, usually at the ppb level – 0.1 – 50 ng g^{-1} FW [14,15], making the qualitative and quantitative analysis of these compounds very difficult [16]. The analysis is further hindered by the high amount of interfering substances contained in crude plant extracts [17]. However, the main difficulty associated with auxin quantification may be the low yield frequently obtained as a result of oxidation processes and the tendency of indolic compounds to bind irreversibly to glass [18]. Nevertheless, these effects can be compensated by the use of **isotope of dilution techniques** (described in detail in [18]). Stable isotope-labeled compounds are very good internal standards on account of their physical and chemical similarities with the original analytes, providing correction for analyte loss and ion suppression by co-eluting substances [16,17]. Structural similarities between analytes and internal standards entail an identical or nearly identical behavior during extraction and chromatographic separation, yet the difference in mass allows them to be distinguished by mass spectrometry (MS) [17]. Nevertheless, it should be noted that the mass difference between analyte and internal standard must be enough to avoid isotopic interference [19], which is why [$^{13}C_6$]IAA is the best internal standard for IAA quantification: the incorporation of six ^{13}C atoms in the benzene ring of the indole group provides a mass difference of 6 units between analyte and internal standard. In the case of IAA, different types of isotopically-labeled standards are commercially available, but this is not so for other auxins. To quantify IBA, for example, proper internal standards (such as [$^{13}C_8$,$^{15}N_1$]IBA) have to be synthesized, as reported by some authors [20], which brings an extra workload. Alternatively, other compounds can be used as internal standards provided they are closely related to the target compounds in terms of physicochemical properties and stability, and are not naturally produced by the plant or are produced in undetectable amounts [17].

Considering the above, the development of extremely sensitive and selective analytical methods is crucial for the accurate quantification of plant hormones, considering that most current studies require increasingly smaller amounts of plant material and faster analyses. Many methods have been developed for the simultaneous quantification of several plant hormones (**Figure. 2**) [21–25], however, until recently, a rapid, sensitive, accurate and efficient standard method was still needed for faster progress in botany research [14].

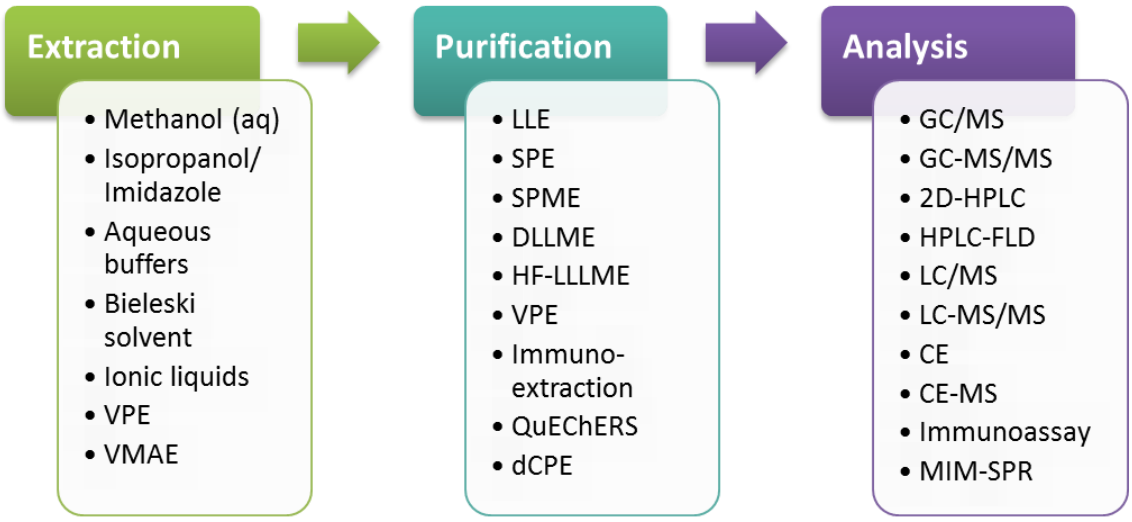


Figure. 2 Examples of analytical methods used in auxin analysis.

2.1. Sample preparation

Despite the advances in analytical methods in the past decades, and with the exception of microtechniques [20,26], sample preparation is still the major step in auxin quantification, taking up to 80% of the total time of analysis [27]. Depending on the type of plant material and the method used, the complete process of sample preparation can involve sample homogenization, extraction of analytes from the matrix and purification of the extract to remove co-extracted interfering substances (extract enrichment) [16].

Sample collection is the first of a series of key steps in the preparation of samples prior to analysis. It is very important to work fast and collect the samples in a way that avoids changes in hormone levels induced by wounding [28]. One way of doing so involves flash-freezing the samples in liquid nitrogen when they are collected from the plant, a step particularly important when dealing with large sample amounts (≥ 50 mg). In this case, the next crucial step involves grinding the frozen samples, which can also be done in liquid nitrogen to prevent defrosting of the sample and chemical degradation of auxins [14]. However, if a small amount of sample is used (few mg) grinding should be bypassed to avoid sample loss. Instead, tissues may be disrupted by ceramic beads in a tissue homogenizer [22] or homogenized directly with extraction buffer in vibrating-ball micromills [21].

Another option involves freeze-drying the samples before grinding, which eliminates time constraints related to the possibility of defrosting and minimizes chemical degradation of analytes. Actually it has been shown that freshly frozen and freeze-dried plant tissues do not differ in plant hormone contents [29]. However, it should be mentioned that freeze-drying is not suitable for all types of plant tissues, so the method used in sample preparation should be chosen based on the type of plant material.

2.1.1. Extraction

Because plant samples are in solid form, the first step of any analytical protocol is a classical solid-liquid extraction that will extract the analytes into a liquid phase, which can be used for further purification and concentration steps.

Extraction yield is highly dependent on the choice of the right extraction solvent, which frequently is a mixture rather than an individual solvent. An ideal solvent would extract the maximum amount of auxins and the minimum amount of matrix components, but since the interfering matrix is in large excess over auxins, it is very difficult to find such a solvent.

Auxins are only slightly soluble in water, and highly soluble in organic solvents (e.g. methanol, ethanol, acetone, diethyl ether and dimethyl sulfoxide) or in aqueous alkaline solutions such as basic buffers [30].

Many different solvents have been applied in auxin extraction: methanol [21,31,32], methanol : water [33–35], acetone : water [36], methanol : KH_2PO_4 buffer [37], isopropanol : H_2O : HCl [28], isopropanol : imidazole buffer [18,38]. There are also some references to the use of aqueous buffers (phosphate buffer pH 6.5) [39] and, in an attempt to use more environment-friendly extraction solvents, several ionic liquids were tested as extraction solvents of IBA from pea samples [40]. Although good results were obtained with 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4mim][PF_6]), a previous extraction step using phosphate buffer is still required [40]. Among these different mixtures, methanol has become the most popular solvent for extraction of plant hormones possibly because it easily penetrates plant cells during extraction due to its low molecular weight and high polarity ([15] and references therein). Nevertheless, auxin extraction with primary alcohols can possibly result in the esterification of IAA [18], which should be taken into account when choosing an analytical protocol. To avoid this type of artifacts, secondary alcohols such as

isopropanol or solvents with similar polarity, such as acetonitrile can be used instead [18,41].

The choice of extraction solvent also should be influenced by the analytical technique to be used. Recently, Novák et al. [42] showed that organic solvents may be unsuitable for LC/MRM/MS analysis. When comparing the performance of 80% methanol, 70% acetone and 2-isopropanol/Na-phosphate buffer pH 7.0 (2:3), unbuffered organic solvents extracted a much higher concentration of interfering compounds such as lipids and pigments. However, phosphate buffers have been suggested to cause enzymatic degradation of auxins during extraction, and acetone is reported to produce lower recoveries than methanol and acetonitrile [43].

Auxins are easily oxidized and degraded by exposure to light, oxygen and high temperatures [30]. Although this is less of a problem when working at the microscale, if the sample preparation procedure is long, which is usually associated with large sample sizes and bulk extractions, an antioxidant can be added to the extraction solvent to prevent auxin degradation. The most widely used antioxidants are butylated hydroxytoluene (BHT) [33–35,39] and diethyl dithiocarbamate [36,44]. In such cases, considering the reasons above, extraction is normally carried out for several hours at low temperature. It should be mentioned, however, that such additives interfere with subsequent analysis and their use can and should be avoided if rapid analysis methods are to be used. Extraction efficiency can be improved using microwave energy (microwave-assisted extraction (MAE)), which also speeds up the whole procedure. However, the high temperatures produced by microwaves can destroy some plant compounds [45]. To overcome this problem, extraction can be performed under vacuum conditions. This procedure not only prevents oxidation of analytes, but also allows extraction to be performed at low temperatures preventing thermal degradation. An example of this procedure was described by Hu et al. [46] who used vacuum microwave-assisted extraction (VMAE) to extract IAA and IBA from pea and rice seeds.

As previously mentioned, depending on the type of plant material and technique used, further sample clean-up may be still necessary between extraction and analysis. While this type of procedures is losing significance in most modern protocols [26], sample purification is still very important to remove interferents and increase sensitivity of the analytical methods when working with bulk extractions.

2.1.2. Purification and clean-up

Sample purification can be crucial for a successful analysis because it isolates the analytes of interest from their matrix constituents, while cleaning the sample. This procedure not only improves separation and detection by the analytical methods used, but also reduces the cost of analysis by increasing the instrument's maintenance interval [27]. However, the type of plant tissue and the available instrumentation will greatly influence the need for purification methods. When working with small amounts (a few mg or even less) of herbaceous tissues and having access to powerful instrumentation such as high-resolution MS, sample clean-up becomes less important and can even be detrimental. Nevertheless, auxin quantification is frequently performed in more ligneous tissues using less powerful instrumentation. In these situations, purification of crude extracts still is a fundamental step of sample preparation.

2.1.2.1. Adaptations of liquid-liquid extraction (LLE) and solid-phase extraction (SPE)

Classical techniques such as LLE and SPE are by far the most used methods of purification in auxin analysis (see **Tables S1-S4**). Given their simplicity and the possibility of customization and automation, they became the preferred purification techniques for most analytes [47], although SPE has been associated with higher recoveries than LLE [48]. Particularly, the purification of IAA by C18-SPE has been optimized in detail as part of analytical protocols starting from samples extracted with 80% methanol [49]. Ion exchange chromatography (IEC) has also been applied as a purification step in combination with SPE and/or LLE. For example, DEAE columns have been combined with C18 SPE cartridges [50,51], or with LLE [52] or even with other IEC columns [53] (**Tables S1 and S2**). In other cases, a dual-mode SPE purification step including ion exchange columns (Oasis MCX) in combination with C18 cartridges was used to isolate IAA from other plant hormones [54,55]. Mixed-mode cation-exchange cartridges such as Oasis MCX can improve detection by LC/ESI-MS/MS by reducing the matrix effect through the selective retention of interferents, like pigments and lipids [56]. Further improvements in analyte recovery can be achieved by combining SPE with LLE in the same protocol, as described by Cui et al. [48], who performed a comparative study on the performance of different SPE cartridges (Oasis HLB, HyperSep C18, Oasis MAX and Oasis MCX) and LLE solvents (ethyl acetate, hexane and dichloromethane). The authors concluded that Oasis MCX cartridges

combined with ethyl acetate LLE was the best combination to extract auxins (among other plant hormones) from two-month-old leaves of oilseed rape.

Nevertheless, the relatively large amount of sample needed (frequently hundreds of mg), the high solvent waste produced, as well as the length of operation time associated with both LLE and SPE methods, have stimulated the development of microextraction techniques, such as solid phase microextraction (SPME) and dispersive liquid-liquid microextraction (DLLME), which consume minimal volumes of toxic solvents and can even be performed in a solvent-less, faster manner [57]. Still, it is worth mentioning the work of Liu et al. [20] who developed a miniaturized SPE protocol for auxin isolation from plant tissues. These authors developed a high-throughput purification protocol based on SPE TopTips for the quantification of IBA, IAA and IAA precursors by GC/MS/MS using less than 20 mg of tissue. The protocol, successfully applied to *Arabidopsis* and tomato tissues, not only minimizes the volume of solvents used (overcoming the main disadvantage of SPE) but also can be customized based on the choice of SPE resin. A similar approach had previously been developed by Müller et al. [21], but in this case the protocol was designed for the isolation of multiple classes of plant hormones, including IAA, from *Arabidopsis* tissues (20 – 200 mg FW). Other approaches used SPME to extract IAA and IBA from xylem fluids and foliage material of *Musa basjoo* and *Viola baoshanensis*, respectively [37], and carbowax-coated fibers were more efficient than polyacrylate fibers. Although the method was successfully applied to both types of samples, it was more efficient when applied to the xylem fluid as no matrix effect was found in this case, which narrows the application field of the method. Indeed, SPME is only seldom used in auxin extraction. Other constraints for this technique include the limited number of commercially available fiber coatings [37] and the requirement for volatile or semi-volatile analytes [58]. For instance, polydimethylsiloxane fibers have been used for SPME extraction of methyl jasmonate [58,59], however they were not useful in the extraction of its non-volatile form, jasmonic acid [60]. Nevertheless, a polyaniline nanofiber was recently developed for *in vivo* SPME detection of three acidic plant hormones, which did not include auxins [61].

Further adaptations of SPE include the application of molecularly imprinted polymers (MIPs) as SPE sorbents [62] in a process called molecularly imprinted SPE (MISPE) (for reviews see [63–65]). MIPs are tailor-made polymeric materials designed for the selective extraction of a particular analyte. This technology is gaining more and more attention due to the evolution on the way these materials are being synthetized, allowing to increase molecular recognition [66,67]. A particular example of this process

includes molecularly imprinted microspheres (MIMs) used as sorbent [68]. In this work MIMs were prepared by aqueous suspension polymerization using 3-hydroxy-2-naphthoic acid and 1-methylpiperazine as mimic templates of the analytes and used as selective sorbents for IAA and IBA purification from banana samples. Because the template used for MIM synthesis was not one of the target analytes, the prepared MIMs, with a diameter distribution of 30 – 60 µm, were able to overcome the common problem of template leakage. Moreover, the MISPE procedure showed higher extraction efficiency and better selectivity than conventional C18-SPE [68]. An alternative variation of the MISPE method uses magnetic MIP (mag-MIP) beads as sorbent. Auxin-complementary mag-MIPs can be synthesized by microwave heating-induced polymerization of 4-vinylpiridine and β-cyclodextrin and, after adsorption, can easily be collected with a magnetic bar, simplifying the isolation step [46,69]. Mag-MISPE has been applied to the extraction of IAA and IBA from wheat, pea and rice seeds [46,69] but IBA was never successfully extracted from any of the tested samples. This probably happened because IAA was used as a template to prepare the mag-MIPs and the selectivity obtained for IBA is not enough to extract the very low endogenous amounts normally present in plants [20,28]. Although MISPE can be advantageous in terms of increased specificity and faster purification than conventional SPE, the main disadvantage of this technique is the high amount of sample needed. At least in these initial reports, several grams of sample were used to produce a crude extract. It is likely that the required sample size will decrease with the development of the technology, but currently MISPE applied to auxin analysis still needs improvements.

Another adaptation of SPE based on magnetic properties of the sorbent was described by Liu et al. [70] for the quantification of IAA and other plant hormones from rice leaves. Instead of being packed into a cartridge, a magnetic sorbent made of TiO_2/magnetic hollow mesoporous silica spheres was dispersed into the sample by vortex, and could be easily separated from the sample by an external magnet. The adsorbed analyte was then derivatized *in situ* with 3-bromoactonyltrimethylammonium bromide (BTA) in preparation for UPLC/MS/MS analysis. More recently, Cai et al. [71] used Fe_3O_4@TiO_2 magnetic nanoparticles, synthesized by liquid-phase desorption (LPD), as sorbent for the purification of IAA and other plant hormones from rice seedlings. The purified analytes were further analyzed by UPLC/MS/MS. Because they are dispersed in solution and don't need to be packed into an SPE cartridge, magnetic adsorbents allow a faster sample preparation by dramatically increasing the contact surface area between sample and sorbent and by avoiding the column blocking step commonly

used in conventional SPE [71]. However, despite the advantages named here and the potential of these techniques, several constraints impede their broad application in auxin analysis. A major disadvantage is the limited commercial availability of this type of sorbent, a lack which frequently implies in-house modification. Correct functionalization of magnetic nanoparticles may take several months and not all labs are equipped with the necessary tools for this kind of procedure. It may also lead to high variability between batches. Therefore, despite the future potential and elegance of these techniques, the inherent drawbacks that method development with magnetic particles may arise cannot be disregarded.

Adaptations of the classical LLE technique have also been described in the literature. Wu and Hu [24] introduced the hollow fiber-based liquid-liquid-liquid microextraction (HF-LLLME), where the analytes are transferred from an aqueous solution (donor phase) to another aqueous solution (acceptor phase), through an organic solvent (organic phase). The protocol is performed with inexpensive equipment and low solvent consumption; however, it was only applied to the quantification of IAA from coconut water samples. Although a good enrichment factor was obtained (215-fold), the applicability of the method to solid samples was not tested.

Microtechniques such as DLLME have been used in the extraction of auxins from the green algae *Chlorella vulgaris* [35]. This approach greatly reduced the extraction time (< 1 min) and allowed good enrichment factors (10-fold for IAA and 60-fold for IBA). However, the same method could not be used for auxin quantification in the shrub *Duranta repens* due to “severe background interference” which represents a main disadvantage, as the method can’t be applied to plant samples. Nevertheless, an analogous DLLME method was developed for the quantification of IAA and IBA from olive (*Olea europaea*) samples (Porfirio et al., unpublished). Actually this method was efficient in extracting auxins from two very different types of tissues (semi-hardwood cuttings and microcuttings) proving the reliability of DLLME as extraction/purification method for auxin analysis in plant samples.

2.1.2.2. Purification by immunoaffinity columns

Several authors have used immunoaffinity columns for the purification of plant extracts. Immunoaffinity purification is based on the highly selective antibody-antigen interaction and therefore significantly reduces common SPE problems such as co-extraction and matrix interferences [72]. Immunoaffinity columns are packed with sorbents that contain immobilized antibodies against a specific analyte, also called immunosorbents,

allowing sample concentration [73]. Because low molecular mass compounds are unable to induce immune responses, the development of antibodies against these analytes includes their binding to a large carrier molecule, typically bovine serum albumin (BSA) [72], allowing protein recognition by the antibody. This was the case of the protocol developed by Pěnčik et al. [74], who generated IAA-BSA conjugates that were used to produce polyclonal antibodies in rabbit. In this work, samples (30 mg) of *Helleborus niger* were firstly extracted with phosphate buffer and pre-purified by SPE. The resulting eluate was further purified in an immunoaffinity column containing immobilized polyspecific rabbit polyclonal antibodies against the IAA-BSA conjugate. Because IAA is attached to BSA through its carboxylic group, these antibodies are also able to interact with other indolic compounds such as indole-3-acetamide and indole-3-acetonitrile (IAA precursors) [74]. Although some cross-reaction can happen with IBA or IAA-Aspartate (IAA-Asp), this issue is circumvented by methylation of the analytes with diazomethane before immunoaffinity purification. Indeed, this method allowed identification and quantification of several IAA conjugates including IAA-Glycine (IAA-Gly), IAA-Phenylalanine (IAA-Phe) and IAA-Valine (IAA-Val) in the pg g^{-1} FW range. Although IAA-Gly and IAA-Val had been previously described in crown gall cell cultures [75], this was the first report on these conjugates in higher plants.

Similar procedures were used by other authors to purify IAA and in some cases its conjugates from seaweed concentrates [76], roots of *Ricinus communis* infected with *Agrobacterium tumefaciens* [77] and tobacco BY-2 cells [55].

As previously mentioned, immunosorbents present major advantages in comparison with traditional sorbents. In fact, home-made immunosorbents can retain consistent analyte binding capabilities even after hundreds of utilizations over a period up to 1 year [72]. However, despite its superior behavior, immunoaffinity purification is most definitely not the main purification method used in auxin analysis, mainly because of the high costs associated with its operation, the difficulties in producing antibodies, or the high cost of commercially available antibodies, and the necessity of synthesizing analyte-protein conjugates capable of generating an immune response. Furthermore, the fact that reproducible immunosorbents can only be obtained with monoclonal antibodies [72] greatly increases the difficulty and cost of the entire procedure.

2.1.2.3. Other purification methods

Aside from the methods described above, less common purification strategies can also be found in the literature.

Schmelz et al. [22,78] used Super Q filters and open-top capped vials to perform what they called vapor phase extraction (VPE) after a conventional sample pretreatment including tissue homogenization with an extraction solvent. In this protocol, pre-derivatized plant samples were heated at 200°C, so that methylated IAA was volatilized and retained in the Super Q filters which were eluted for further GC/MS-CI analysis.

Another example of a particular extraction procedure was described by Yin et al. [79], who used dual-cloud point extraction (dCPE) for quantification of IAA and IBA in acacia leaves, buds, and bean sprout. The procedure consists on the formation of a cloud point, mediated by a thermostatic bath, between an acidic aqueous solution and a surfactant resulting in the formation of two phases. The two phases are separated by centrifugation and, after increasing the viscosity of the surfactant phase with an ice bath, the aqueous phase is removed. Then the surfactant phase containing the analytes is mixed with an alkaline solution, into which the analytes will be extracted. A new cloud point is formed by incubation in a thermostatic bath and the resulting aqueous phase is collected after centrifugation.

Many references also use HPLC fractionation as a purification step before analysis [50,55,80–87], however, this procedure is very cumbersome and incompatible with high-throughput analysis, and protocols most recently developed focused in eliminating this step [18,20].

Finally, among other plant hormones, IAA has been extracted from zucchini samples by the QuEChERS (acronym for quick, easy, cheap, effective, rugged and safe) methodology using 1% acetic acid in acetonitrile, anhydrous magnesium sulphate, sodium chloride, sodium citrate dehydrate and disodium citrate. However, the method was only able to extract IAA from one out of seven tested samples [88].

2.1.3. Derivatization or labeling

Derivatization refers to a group of modifications intended to make analytes more compatible with the detection method, ultimately increasing sensitivity and selectivity [47,89]. For instance, ionization in ESI-MS is frequently improved by derivatization [90–92], and IAA response in ESI-MS/MS can increase up to 200-fold after methylation [93].

Several factors determine the choice of a derivatization procedure, including the analyte's chemical structure, separation method and type of detector. Incorporation of UV-absorbing or fluorescent groups is commonly used in LC and CE, and a large

variety of reagents is available [89]. A decrease in polarity and increase in hydrophobicity, desirable both in GC and MEKC, is achieved by addition of alkyl, acyl or silyl groups [94]. In GC, an increase in volatility is also desirable for many compounds, including auxins, and this is often achieved through the addition of non-polar groups using silylation [34] and methylation [13] reactions. In fact, these are the derivatization procedures most commonly used in preparation of auxins for GC/MS analysis (**Table S1**), although examples of other reactions can also be found in the literature [50,52,77].

Methylation is frequently accomplished using diazomethane, a reagent that specifically modifies the carboxylic group of auxins in a short reaction time [18]. Diazomethane is normally used in preparation of samples for GC/MS analysis, where, in the case of auxins, derivatization is mandatory, but it can also be applied to LC/MS analysis as a way of increasing the hydrophobicity of the analytes and improve separation [44,51,95]. It has also been applied before ELISA detection of IAA [84]. Besides diazomethane, other reagents have been used in derivatization reactions preceding LC/MS/MS analysis. One example is bromocholine, which contains a quaternary amine moiety and converts carboxyl groups in positively charged groups improving the detection of some plant hormones. Although auxins don't require this kind of modification, as they can be analyzed in positive ion mode, given their structure they are still modified in the reaction with bromocholine [53].

Like GC-FID and GC/MS, CE often requires derivatization. The reaction can occur in pre-, on- or post-capillary modes, or even on-line (reviewed in [89]). Several examples of auxin modification can be found in the literature. In preparation for CE-electrochemiluminescent detection (CE-ECL), IAA has been derivatized through AEMP labeling with 2-(2-aminoethyl)-1-methylpyrrolidine (AEMP) using N,N'-dicyclohexylcarbodiimide (DCC) and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt) as coupling agents [79]. When using CE with laser-induced fluorescence detection (CE-LIF) several auxins were derivatized *in situ* with 6-Oxy-(acetyl piperazine) fluorescein (APF) [96], a derivatizing reagent for carboxyl compounds that has also been applied to HPLC-FLD detection of auxins [97]. Recently, a new mass probe was developed for the detection of IAA and IBA by CE-ESI-TOF-MS. BTA contains a permanent positive charge that improves the ionization of acidic plant hormones, like auxins, allowing a better signal response in TOF-MS and multiple reaction monitoring (MRM) [70,98]. In fact, BTA has also been applied as *in situ* derivatization reagent to improve sensitivity in UPLC/MS/MS [70].

Derivatization can tremendously improve sensitivity, as demonstrated by Prinsen et al. [93]. This is especially important when dealing with low concentration analytes, such as auxins. While in the case of GC analysis derivatization is not optional, it can also improve auxins' response when using other techniques. Considering the low price of derivatizing reagents and the resulting analytical improvements, derivatization is an extremely important and advantageous step of sample preparation.

2.2. Analysis

The last step in the analytical process is, of course, analysis of the purified sample in its natural or derivatized form. Chromatographic techniques have long been the preferred methods for analysis of plant hormones. GC/MS and LC/MS provide the separation and sensitivity required for accurate quantification of compounds present in trace amounts in complex matrices, such as auxins [16]. Immunoassays also have been an important tool in plant hormone analysis, since early 1980's [99], and ELISA is still commonly applied to auxin quantification (**Table S4**). Nevertheless, other methods such as MEKC [100], pressurized capillary electrochromatography (pCEC) [101,102], CZE [103], CE [79,96,98,104] and surface plasmon resonance (SPR) [105] have also been applied.

2.2.1. Separation and detection

2.2.1.1. Chromatographic methods

Chromatography is the prevalent analytical technique for plant hormones, and because several reviews on this subject have been published in the past [14–16], only the most relevant approaches will be discussed here.

2.2.1.1.1. GC and GC/MS

GC/MS is the most classical method of auxin quantification. Although a few reports used GC-ECD for IAA quantification, compound identification was still performed by GC/MS in such cases [77]. More sensitive than LC/MS [41], GC/MS has been widely applied to auxin analysis, especially after [$^{13}C_6$]IAA was chemically synthesized and proposed as internal standard for IAA quantification [106]. Although other standards, such as deuterated IAA ([$^{2}H_2$]IAA and [$^{2}H_5$]IAA) (**Table S1**) and methylated IAA (MeIAA) [49], are used sometimes, [$^{13}C_6$]IAA offers several advantages over the

deuterium labeled standards, namely, nonexchangeability of the isotope label, high isotopic enrichment, and chromatographic properties identical to that of the analyte [106].

Initially performed using SIM [107] or high-resolution SIM [108,109], the sensitivity of this technique was highly improved with the development of multisector instruments that allow MRM. Hence, when accessible, this is currently the preferred mode of analysis when using GC/MS, allowing very good sensitivity and low detection limits (**Table S1**). One of the first examples of the use of GC/MS/MS on IAA quantification is the work of Müller et al. [21], who used a multiplex technique to quantify multiple acidic plant hormones in a single run. This method allowed them to generate whole-plant organ-distribution maps of IAA (among other plant hormones) in *Arabidopsis thaliana*. In the following years, other authors used GC/MS/MS to study auxin transport and synthesis in *Arabidopsis* and pea [33,34]. More recently, a high-throughput assay, which uses typically 2 – 10 mg FW of tissue, was developed for the quantification of IBA, IAA and IAA precursors in *Arabidopsis* and tomato [20].

2.2.1.1.2. LC and LC/MS

LC, coupled to various types of detectors, has also been broadly applied to auxin analysis (**Table S2**), and it is a more suitable technique than GC since no derivatization step is required. Given its sensitivity and selectivity, MS detection is most commonly used, and different mass analyzers are described in the literature: IT, quadrupole time-of-flight (QTOF), tandem quadrupole (qMS/MS), and triple quadrupole linear ion trap (Q-Trap) (see **Table S2**). Currently, IAA and IBA can be separated from other auxins within 7 min by LC/ESI-ITMS [110]. With the development of LC/MS instruments, MRM mode became a reality and the technique surpassed GC/MS due to its simplicity. Currently, it is the most commonly used method of auxin quantification [16] (**Table S2**).

Recently LC/MRM-MS was used to analyze the *Arabidopsis* IAA metabolome from amounts of tissue as small as 20 mg. In the same protocol, most IAA precursors and degradation products were analyzed simultaneously, demonstrating the analytical power of this technique. However, in all studied tissues, IBA levels were below the detection limit of the method [42]. Sensitivity and detection limits can be highly improved by the use of nanoflow-LC/MRM-MS. Izumi et al. [111] reported detection limits in the fmol range and a 5-10 fold increase in sensitivity when using nanoflow-LC/ESI-IT-MS/MS with MRM for plant hormone profiling.

Some authors have also performed two-dimensional HPLC (2D-HPLC) for auxin quantification. Dobrev et al. [112] firstly described a “heart-cutting” 2D-HPLC method to separate and purify IAA and abscisic acid (ABA) from several plant species. In the first dimension, a silicacyanopropyl column was used and the elution was performed in reverse-phase mode at a low concentration of organic solvent, allowing a close elution of the two analytes. This was beneficial for the separation in the second dimension, which was done in a hydrophobic C18 column, because it concentrated IAA and ABA in narrow peaks. The full injection-to-injection cycle was smaller than 30 min and the analytes were detected by DAD and fluorescence (FLD) detectors connected in series. The method was subsequently used by other authors [113,114].

In an attempt to improve the speed of analysis, Stoll et al. [115] developed a fast and comprehensive (LC x LC) 2D-HPLC/DAD method for metabolomics studies. The speed of the second dimension separation was improved by using an ultra-fast and high temperature gradient elution, which reduced cycle time. For that purpose, a high-pressure mixing configuration was used to generate each second dimension gradient, instead of two separate binary pump systems. This design eliminated the differential in retention time between sequential separations, allowing a reduction in dwell volume higher than an order of magnitude. The two columns used (Discovery HS-F5 and ZirChrom-CARB, first and second dimension, respectively) allowed a high degree of orthogonality and thus the method was used to separate 26 IAA derivatives from maize samples in a single injection cycle, in a practical analysis time of 25 min.

2D-LC has the potential to be an extremely powerful separation technique mainly due to its exceptional resolving power compared to conventional 1D-LC methods (see [116] for a thorough review). In theory, it has a very broad application range as it allows performing separations using a large combination of LC modes (SEC, RP, IEC, etc.), although in practice the combination of certain modes is very difficult, if not impossible [117]. Despite the tremendous potential of 2D-LC, several disadvantages prevent its widespread use. The main drawback is still the very long timescale of comprehensive analysis (several hours). Unlike 2D-GC, where high speed separation is easier to implement, high speed separation in LC is more difficult because of pressure and instrumentation limitations (discussed in detail in [116]). As mentioned above, this problem was partially addressed by Stoll et al. [115], who were able to reduce the overall separation time to about 30 min by increasing the temperature and linear velocity of the second dimension column. An alternative to high temperatures in speeding up the second dimension analysis can be the use of monolithic columns, which can accommodate high flow rates without loss of resolution [117].

Furthermore, the number of parameters that need to be chosen, combined and optimized (column, flow rates, mobile phases, gradients and temperatures) for a 2D-LC method is considerably higher than for a 1D-LC method [116], which significantly increases the complexity of the technique. Combining 2D-LC separation with an MS detector is also a challenge considering that the flow rate has to be significantly reduced in order to be compatible with ESI [116]. The speed of the detector is also a problem, especially in the case of MS detectors, which can be slower than the LC separation [116].

Another major drawback of 2D-LC is data analysis. The amount of data resulting from a comprehensive 2D-LC analysis can be overwhelming, especially when using an MS or PDA detector, and currently there are no commercially available softwares that allow efficient and semi-automated analysis of 2D-LC data [117], although this reality may change in the near future.

Unlike 2D-GC, which was invented over two decades ago [118,119], has been continuously developed ever since, and is currently automated and commercially available, 2D-LC is still far from routine. To this date, 2D-LC remains still a promising technique.

Nevertheless, LC/MS accuracy and sensitivity is increasing even in 1D separations. Recently, high-resolution and accurate mass instruments have been used for the identification of a wide range of indolic compounds from crude plant extracts. A minimalistic sample purification protocol involving only centrifugation and dilution of the organic extract followed by quadrupole ion cyclotron resonance Fourier transform MS (Q ICR FT-MS) analysis allowed the identification of multiple indolic compounds, including free IAA, IAA amide-conjugates, tryptophan conjugates and other tryptophan derivatives from soybean, tomato and *Ginkgo biloba* [26,120]. Additionally, separation and quantification of four isomers of auxin-*myo*-inositol conjugates (IAA-Inos), as well as IAA and MeIAA, from *Zea mays* and *Arabidopsis thaliana* was also reported using QTOFMS [121].

2.2.1.1.3. Electrokinetic methods

Although chromatographic methods are the prevalent analytical strategy to study plant hormones, separation is hampered by the complex sample matrix and the high cost of analysis resulting from the need for isotopically-labeled internal standards [96]. CE has a higher separation power than LC and GC, which are based on the interaction

between analytes and the stationary phase, because analytes are separated according to their electrokinetic properties (i.e., mass *and* charge) [122]. Thus, separations with several hundred thousand theoretical plates can be achieved with CE [123]. Furthermore, CE displays several advantages over chromatography such as low sample (sub-nL) and reagent (sub-µL) consumption, short separation time and low instrumentation cost [123,124]. Several examples of the application of CE to auxin quantification can be found in the literature (**Table S3**).

A method based on CE-LIF was developed for auxin quantification from crude banana extracts, using APF as derivatizing reagent [96]. CZE was firstly tested as separation mode, but all analytes were flushed together using these conditions. Therefore MEKC was chosen as separation mode and all parameters (pH, SDS, ethanol concentration, water content) were optimized. Under optimized conditions, and without any sample clean-up, IAA and IBA were separated from other plant hormones and quantified within 20 min, with detection limits in the nM (µg mL^{-1}) range [96].

In other cases, CE-electrochemiluminescence (ECL) was used to analyze IAA and IBA from acacia tender leaves, buds, and bean sprout [79]. ECL detection is based on the formation of photons resulting from the decay of species that easily form excited states at the surface of electrodes, via an applied voltage. Among ECL systems, tris (2,2'-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) is one of the most commonly used, especially when in combination with CE [123]. Oxidation of $Ru(bpy)_3^{2+}$ by analytes containing tertiary amines generates an excited-state $Ru(bpy)_3^{2+*}$, whose decay to the steady state leads to the release of a photon. The amount of light energy released is therefore proportional to the analyte's concentration. The method is a powerful analytical tool with high sensitivity and wide linear ranges [123], but its application requires the presence of tertiary amine groups in the analytes. Analytes lacking a tertiary amine group in their structure, such as auxins, can be derivatized with AEMP in order to increase detection sensitivity [79]. Although this is a feasible solution, it also introduces an extra step in sample preparation. Good detection limits were obtained (nM), and the method was validated by HPLC-UV detection. $Ru(bpy)_3^{2+}$-$KMnO_4$ ECL had been used previously for the detection of IAA and IBA in mung bean sprouts [125]. In this work, $Ru(bpy)_3^{2+}$ was immobilized on an anion-exchange resin and the ECL reaction happened by contact with a diluted acidic $KMnO_4$ solution. This design reduces reagent consumption and does not require a mixing chamber or a pump. Furthermore, the reagent-containing resin could be used for at least six months as the relative ECL intensity only decreased 3% during that period.

Hybrid techniques like CEC and pCEC combine the efficiency of CE and the selectivity of HPLC, overcoming the disadvantages of CE [122]. In the case of pCEC, both a pressurized flow and the EOF push the mobile phase through the capillary, thus solving the problems associated with column drying-out and bubble formation [101]. Wang et al. [101] used pCEC to quantify IAA extracted from corn. Separation was carried out in a monolithic silica-ODS column, and detection was accomplished in a UV-Vis detector. Although the authors pointed out some disadvantages of pCEC, such as low concentration sensitivity associated with low sample volume and limited optical path length for UV-Vis detection, pCEC provided a better separation than LC. Yin and Liu [122] developed a method for the preparation of polydopamine-coated open-tubular capillary columns to be used in the detection of IAA and IBA. The capillary is filled with an aqueous solution of dopamine, and polydopamine is formed in the inner wall of the capillary through oxygen-derived oxidation of the dopamine solution, forming a permanent coating. The use of repetitive coatings allowed the formation of a layer with 200 nm thickness, providing a column with controllable EOF (the coating inhibits EOF, possibly by masking silanol groups in the inner wall of the capillary). The developed coating was stable under both acidic and alkaline conditions, resistant to the presence of methanol in the sample, and it can be stored for up to 2 months. Even though the coating was developed to separate IAA and IBA, a decreased interaction of IBA and the coating was observed, likely due to its longer chain. Nevertheless, both auxin standards were separated within 11 min in the single layer polydopamine-coated capillary, which showed improved resolution in comparison to the bare capillary. Although the method was successfully applied to the determination of IAA in culture media of IAA-producing bacteria (*Arthrobacter* sp., *Bacillus* sp. and *Enterobacter* sp.), its applicability in plant samples was not evaluated.

2.2.1.1.4. Immunoassays

Immunoassays such as RIA and ELISA have long been applied to auxin quantification [99,126]. They are based on highly specific antibody-antigen interactions where the analyte is an auxin conjugate that can be recognized by the antibody. Although ELISA is much less sensitive than LC/MS for the detection of plant hormones [127], and presents several obstacles such as complex sample preparation and cross-reactivity [16], its high selectivity and ease of operation make it a valuable tool for auxin analysis. Furthermore, anti-auxin monoclonal antibodies and full ELISA kits are commercially

available, which is not true for other analytical techniques. ELISA has been used for IAA quantification using IAA-ovalbumin or IAA-BSA conjugates [77,128,129].

Aside from ELISA assays, biosensors are gaining popularity due to their unique properties. Different kinds of biosensors have been adapted to plant hormone determination [130] and, particularly, immunosensors provide high specificity and sensitivity due to the use of antibodies or antigens as the sensing element [16].

2.2.1.1.5. Immunosensors and other biosensors

Biosensors are analytical devices that combine a biological component with a physicochemical detector, and convert a biological response into a signal that can be captured and interrogated [130]. As stated by Sadanandom and Napier [130], the ideal biosensor is selective, sensitive, gives a calibrated dose-response curve over physiologically relevant concentrations of analyte, gives a spatially resolved reading *in vivo*, and is not invasive.

A specific type of biosensor is an immunosensor, where the immunochemical reaction is coupled to a transducer and converted into an electrical signal (reviewed in [131]). Immunosensors can be classified based on the type of detector: electrochemical, optical and piezoelectric [131].

An immunosensor with a piezoelectric detector was designed for IAA detection [132]. A piezoelectric detector consists of a quartz crystal microbalance (QCM) which detects mass differences between the analyte-bound an unbound states of the biosensor. Because IAA is too small to produce a sufficient mass difference, an IAA-BSA conjugate with higher molecular weight was synthesized and used as antigen (analyte), increasing the sensitivity of the assay. Anti-[IAA-BSA] antibodies were purified from white rabbits and immobilized on the golden surface of the quartz crystal. This configuration allowed creating a QCM immunosensor capable of detecting IAA in a linear range of 0.5 ng mL^{-1} – 5 µg mL^{-1}. The capacity of the immunosensor was evaluated by determining IAA in solution at different concentrations, in the range 1 ng mL^{-1} – 1 µg mL^{-1}, and the calculated recoveries varied from 96 to 116%. However, although a functional immunosensor was developed, at the time of the study regeneration of the biosensor was an unsolved problem, which creates a great disadvantage. This problem was addressed in later work by the same authors who developed a renewable amperometric immunosensor for IAA detection [133]. This immunosensor consists of a sol-gel-alginate-carbon composite electrode (SACE),

produced from the sol-gel precursor tetramethoxysilane (TMOS), alginate and graphite powder, and contains anti-IAA antibodies on its surface. The detection was based on the enzyme-linked competitive immunoreaction between IAA in the sample and IAA labeled with horseradish peroxidase (IAA-HRP) on the SACE surface. The enzymatic activity of HRP bound to anti-IAA antibodies is measured by amperometric detection using H_2O_2 and 3,3',5,5'-tetramethylbenzidine (TMB) as substrates. This biosensor was capable of detecting IAA in the range 5 – 500 μg mL^{-1} and was applied to the analysis of hybrid rice grain samples. After each immunoassay, the sensor was regenerated by immersing the SACE in saline solution at pH 12 in order to rinse out the antibody immobilized on the SACE surface. The method was validated by analyzing the rice samples by HPLC.

Other types of biosensors were further developed for IAA detection. Mancuso et al. [134] described a non-invasive carbon-nanotube modified and self-referencing microelectrode for the study of auxin fluxes in root apexes. It is desirable that the microelectrode can detect IAA levels at precise distances from the tissues with good spatial resolution. Carbon nanotubes have high electrical conductivity, chemical stability and mechanical strength [134], solving some of the problems observed in previously developed electrodes [135]. Modifying the electrode surface with multiwalled carbon nanotubes increases the surface area available for electron transfer and enhances catalysis [136]. In fact, the authors showed the enhancing effect of multiwalled nanotubes on the oxidation peak current of IAA in comparison with a bare platinum electrode, further confirming the results of Wu et al. [137], who developed a similar but invasive electrode. The microelectrode created by Mancuso et al. [134] was used to monitor IAA fluxes in growing roots of maize, *Arabidopsis* and walnut. The method was validated by analyzing the samples by HPLC, and in both methods the amounts found in samples were in the ng g^{-1} range.

Nevertheless, this method displayed some disadvantages such as lower than desired temporal resolution and signal-to-noise ratio, as well as the need for exogenous IAA addition. Further improvements of this approach were already reported by McLamore et al. [136], who optimized a non-invasive self-referencing electrochemical microsensor for the measurement of endogenous IAA fluxes in maize roots. The microsensor included platinum black and carbon nanotube (CNT) surface modifications and can be used for real-time transport monitoring in surface tissues. Furthermore, the method can be performed simultaneously with live imaging techniques.

Zhou et al. [138] developed an electrochemical immunosensor based on gold nanoparticles (AuNPs) functionalized with HRP-labeled immunoglobulins (HRP-IgGs) and rat monoclonal antibodies against IAA (anti-IAA). A glassy carbon electrode was coated with graphene for an increased electrode surface and to facilitate electron transfer. The AuNPs were deposited on the electrode surface to allow IAA recognition. The HRP-labeling was used as a signal amplification tool to increase the sensitivity of the immunosensor, while IAA recognition and capture was performed by the monoclonal anti-IAA antibodies attached to the AuNPs-HRP-IGs. Electrochemical measurements were performed by differential pulse voltammetry (DPV) using $Fe(CN)_6^{3-/4-}$ as redox probe, and IAA was indirectly measured by the variation of oxidation current response of $Fe(CN)_6^{3-/4-}$. The determined LOD was comparable with other techniques (CE, chemiluminescence), and the method was applied to IAA quantification in mung bean sprouts (12 – 32 ng g^{-1}). A very similar immunosensor was described in the same year [139]. The IAA detection mechanism is the same, however in this case 4-aminophenylboronic acid (4-APBA) was used instead of graphene as coating agent for the electrode. Furthermore, in this case the HRP-IgGs were attached to Fe_3O_4-COOH magnetic nanoparticles while the anti-IAA antibodies were attached to the AuNPs, allowing double signal amplification. Also in this case the results from IAA quantification in seeds (wheat, corn, mung bean, soy bean, millet and brown rice) were comparable with results obtained by CE [139]. The LODs of both immunosensors are comparable (nM range).

Another example of the use of graphene in electrodes is the work of Sun et al. [140] who reported a photoelectrochemical (PEC) immunosensor using 3-mercaptopropionic acid stabilized CdS/reduced graphene oxide (MPA-CdS/RGO) nanocomposites for IAA detection. In this case graphene was chosen for its properties as electron-transfer matrix. PEC sensing is a promising technique that allows high sensitivity and high-throughput while using inexpensive devices, although to the best of our knowledge this is the only report describing the use of the technique. The immunosensor was successfully applied to IAA quantification from wheat, corn and bean seeds, with results comparable to those obtained using CE.

Yang et al. [141] developed an amperometric sensor based on a $CeCl_3$-DHP film modified gold electrode for IAA determination. In comparison with the bare and DHP modified gold electrodes, this sensor greatly increased the linear response of detection while decreasing the noise of the amperometric response. Mung bean sprout leaves were analyzed by this method, and results were comparable with HPLC analysis. Previously, a carbon paste electrode had been developed for IAA quantification using

square wave voltammetric determination based on surfactant effects [142]. This method presented very high sensitivity and low detection limits (20 nM) and was successfully applied to gladiola and phoenix tree leaves.

Finally, SPR was also applied to IAA monitoring in plant tissues [105]. SPR is a surface-sensitive technique based on the measurement of changes in refractive index (RI), which allows performing real-time and label-free analyte detection in complex matrices even without sample pre-treatment. Sensitivity can be highly improved by coating the sensor chip surface with a thin film of MIPs, which will selectively recognize a template molecule. In the work of Wei et al. [105], high selectivity was achieved by adsorbing a molecularly imprinted monolayer (MIM) on the SPR sensor chip surface containing preadsorbed IAA. The MIM consisted of a 2D monolayer of alkanethiol self-assembled around the template (IAA) pre-adsorbed on the surface of the gold-coated sensor chip. Selectivity was evaluated by applying the MIM-coated chip to the detection of IBA, and a much lower response was observed in this case. Moreover, an IBA-imprinted MIM was prepared which further showed the high selectivity of MIMs. The method was applied to different samples with good recoveries (95 – 98%) and very good detection limits (0.20 – 0.32 pM). The biggest disadvantage of this approach is the high cost of the SPR sensor chip, and its further functionalization with MIMs.

2.2.1.1.6. Other detection methods

A **fluorimetric assay** based on the reaction between IAA and acetic anhydride in the presence of perchloric acid as catalyst was used to quantify IAA in mung bean cuttings [143,144]. The method is highly specific as IBA didn't form detectable amounts of fluorescent derivatives, which can also be a disadvantage as no other auxins can be detected.

A **colorimetric method** for the detection of both IAA and IBA was described by Guo et al. [145], based on the reaction between auxins and Ehrlich reagent (*p*-(dimethylamino)benzaldehyde (PDAB)) under acidic conditions. PDAB reacts with indolic compounds, and IAA and IBA respond differently to reaction temperature and incubation time. While at 25°C IBA's reaction with PDAB generates a blue compound, IAA barely reacts with PDAB at this temperature during the first 60 min, allowing selective determination of IBA absorbance. At 70°C, both auxins react intensively with PDAB, however forming products with different colors (IBA – blue; IAA – pink). Therefore, IAA concentration can be determined as the difference between the total absorbance and IBA absorbance. The specificity of the method was tested by

determining IAA and IBA concentration in the presence of other auxins (phenylacetic acid (PAA), naphtaleneacetic acid (NAA) and indole-3-carboxylic acid (ICA)) and tryptophan (Trp). Although no interference from PAA, NAA and Trp was observed, ICA is a powerful interferent, and IAA and IBA determination cannot be performed in its presence. The method had linear ranges of 0.28 – 56 μM (IAA) and 0.84 – 42 μM (IBA) with detection limits of 0.10 μM (IAA) and 0.28 μM (IBA). It was applied to the analysis of bean sprouts and results were comparable to results obtained by CE-ECL.

Although a wide variety of methods are available for auxin isolation and analysis, conventional (i.e. chromatographic) methods are still the predominant techniques used in this field, because they are well established within the laboratory setting as well as in literature. Techniques such as biosensors and immuno-based methods provide novel alternatives to the field of study, but the overall investment needed to implement and incorporate them into existing laboratories may be still prohibitive for many laboratories. Furthermore, issues such as high operation cost, limited commercial availability, low signal-to-noise ratios, standardization and low or non-selective specificity are some of the drawbacks to consider [72,146,147]. Therefore, although worth noting for this review, these alternative approaches still remain difficult to implement in a broad scale.

3. Conclusions

Analytical methods for auxin analysis have greatly evolved since they were first described (reviewed in [41]) and more accurate, sensitive, precise and high-throughput methods are available nowadays. Although sample preparation remains the bottleneck of the analytical process, currently there are several approaches that can process large numbers of samples per day. A broad range of sample preparation methods is available, including the classical SPE and LLE and their respective variations (SPME, MISPE, DLLME, HF-LLLME), as well as alternative techniques such as VPE, QuEChERS, dCPE and immunoextraction. Many options are also available for sample analysis including GC, GC/MS, GC/MS/MS, LC, LC/MS, LC/MS/MS, CE, CE/MS, immunoassays and other reported methods. Among these, LC/MS and especially LC/MS/MS are the most advantageous methods considering their excellent selectivity, superior sensitivity, high-throughput and high accuracy. It is also desirable that auxin analysis, as well as the simultaneous analysis of all plant hormones (hormone profiling, or hormonome [53]), becomes a routine practice. The development of 2D-LC has brought us closer to this goal, and with further improvements in software and instrumentation, comprehensive hormone profiling can become a reality. Moreover, the

comprehensive analysis of a plant's metabolome can also help understand, at the molecular level, the plant's response to different conditions such as stress, mutations, and hormone treatments. However, the chemical diversity of plant hormones poses a great challenge to the development of simple, efficient, fast and universal methods [16], a problem exponentially bigger in the case of metabolomics analysis. Another obstacle to this ideal analytical method is the current lack of automation for many sample preparation techniques, a problem that is being addressed in some cases [18,20], but still lacks a universal solution.

As stated by Du et al. [16], the development of techniques that allow highly sensitive, noninvasive, *in vivo*, *in situ* and real-time detection of auxins and other plant hormones is still an ambition. For example, while MS imaging (MSI) has been used with great success to map the spatial distribution of small metabolites in animal tissues [148], its application to the study of plant tissues has only recently begun [149,150]. Although MSI only allows a qualitative analysis, it will definitely be a valuable tool that can be used in combination with other techniques such as in vivo SPME [151,152], SPR-based biosensors [153,154] and atomic and molecular MS [155]. All these techniques can be powerful tools in the future study of the complex metabolic pathways associated with plant hormones.

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Supplementary material

Table S1 – Chromatography/mass spectrometry methods used in auxin quantification: GC and GC/MS based methods.

GC and GC/MS methods								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Arabidopsis tissues	IAA-Glc	60% Isopropanol 40% 0.2 M Imidazole	1) LLE (ethyl acetate) 2) Gel filtration (Sephadex LH-20) 3) HPLC purification	GC/MS	$[^{13}C_6]$IAA-Glc $[^{3}H]$IAA-Glc	Acylation with (1:1) acetic anhydride : 1% DMAP in pyridine 60°C 1h	7 - 17 ng g^{-1} FW	[1]
	IAA-Asp IAA-Glu	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) Gel filtration (Sephadex LH-20) 3) HPLC purification	GC/MS	$[^{3}H]$IAA-Asp $[^{13}C_6]$IAA-Asp $[^{3}H]$IAA-Glu $[^{13}C_6]$IAA-Glu	Diazomethane	(*IAA-Asp*) 7.8 - 17.4 ng g^{-1} FW (*IAA-Glu*) 1.8 - 3.5 ng g^{-1}	
	IAA (free, ester, total)	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) HPLC purification	GC/MS-SIM	$[^{13}C_6]$IAA	Diazomethane	(*Total IAA*) 373 - 1,200 ng g^{-1} FW (*Free IAA*) 2.1 - 30 ng g^{-1} FW	
Arabidopsis tissues	IAA	0.05 M Sodium-phosphate buffer with 0.02% Sodium-diethyldithiocarbamate	Chelating resin (Amberlite XAD-7)	GC/MS/MS (SRM)	$[^{13}C_6]$IAA	1) Diazomethane 2) BSTFA w/ 1% TMCS at 70ºC for 15 min	< 0.3 pg μg^{-1}	[2]
Arabidopsis tissues	IAA	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) HPLC purification	GC/MS-SIM	$[^{13}C_6]$IAA	Diazomethane	18 - 43 ng g^{-1} FW	[3]
Arabidopsis tissues	IAA	0.05 M Sodium-phosphate buffer with 0.02% Sodium-diethyldithiocarbamate	Chelating resin (Amberlite XAD-7)	GC/MS/MS (SRM)	$[^{13}C_6]$IAA	1) Diazomethane 2) BSTFA w/ 1% TMCS at 70ºC for 15 min	≈ 2 - 300 pg mg^{-1} FW	[4]
Arabidopsis tissues	IAA	Methanol in vibrating mill (homogenization), 1h incubation at RT, Diethyl ether and new extraction	Microscale SPE (custom-made)	GC/MS/MS	$[^{2}H]_2$-IAA	Diazomethane	1.75 < IAA < 1,750 ng g^{-1} FW	[5]
Arabidopsis tissues	IAA	0.05 M Sodium-phosphate buffer with 0.02% Sodium-diethyldithiocarbamate	Chelating resin (Amberlite XAD-7)	GC/MS-SIM	$[^{13}C_6]$IAA	1) Diazomethane 2) BSTFA w/ 1% TMCS at 70ºC for 15 min	0.4 - 500 pg mg^{-1} FW	[6]

GC and GC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Arabidopsis tissues	IAA	1-propanol/H_2O/ HCl (2:1:0.002) + DCM	1) Methylation with tms-diazomethane 2) VPE (vapour-phase extraction)	GC/MS-SIM	[$^2H_5$]MeIAA	Trimethylsilyl diazomethane	≈ 5 - 30 ng g^{-1} FW	[7]
Arabidopsis tissues	IAA	80% Methanol with 250 mg L^{-1} BHT	SPE (Sep-pak C18)	GC/MS/MS (SRM)	[$^{13}C_6$]IAA	1) Diazomethane 2) BSTFA/pyridine 80°C 3) BSTFA 80°C	≈ 20 - 400 ng g^{-1} FW	[8]
Arabidopsis tissues	IAA IAA-Asp	80% Methanol	1) DEAE ion exchange 2) SPE (C18) 3) HPLC fractioning	GC/MS-SIM	[$^{13}C_6$]IAA, [$^{13}C_6$]IAA-Asp	2,3,4,5,6-pentafluorobenzyl bromide	(*IAA*) < 10 pg mg^{-1} FW (*IAA-Asp*) ≈ 5 - 20 pg mg^{-1} FW	[9]
Arabidopsis tissues	IAA IAA-Trp	85% Methanol with 100 ng mL^{-1} BHT	1) DEAE resin 2) LLE ($CHCl_3$)	GC/MS-SIM	[$^{13}C_6$]IAA	1-ethylpiperidine and 2,3,4,5,6-pentafluorobenzyl bromide for 30 min at 55°C	*(IAA)* 2.9 - 62.1 pmol g^{-1} FW (*IAA-Trp*) 1.7 - 23.5 pmol g^{-1} FW	[10]
Arabidopsis tissues	IAA	Methanol / formic acid / H_2O (15:1:4)	1) dual-mode SPE - Sep-Pak Plus C18 - Oasis MCX 2) HPLC purification	GC/MS/MS	Not mentioned	Not mentioned	≈ 20 - 40 pmol g^{-1} FW	[11]
Arabidopsis tissues	[$^{13}C_8$-$^{15}N_1$]IAA [$^{13}C_8$-$^{15}N_1$]IBA	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) SPE (PMME resin)	GC/MS-SIM	[$^{13}C_6$]IAA and [$^{13}C_8$-$^{15}N_1$]IBA	Diazomethane 5 min at room temperature	(*IAA*) ≈ 0.5 - 6.5 ng g^{-1} FW	[12]
Arabidopsis tissues	IAA	1) Methanol 2) Diethyl ether 3) Methanol	Microscale SPE (custom-made)	GC/MS/MS	[^2H]$_2$-IAA	Diazomethane	40 - 80 pmol g^{-1} FW	[13]
Arabidopsis tissues	IAA IBA Indole IAA precursors	65% Isopropanol 35% 0.2 M Imidazole	TopTips SPE with NH_2 and PMME resins	GC/MS/MS (SRM)	[$^{13}C_6$]IAA, [$^{13}C_8$,^{15}N]IBA [$^{13}C_8$,^{15}N]indole [$^{13}C_{11}$,^{15}N]IPyA [$^{13}C_{11}$,$^{15}N_2$]Trp	Diazomethane 5 min at room temperature	(*IAA*) 5.87 - 12.91 ng g^{-1} FW (*IBA*) 1.05 ng g^{-1} FW (*IPyA*) 21.58 ng g^{-1} FW	[14]

GC and GC/MS methods (cont.)								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Arabidopsis tissues	IAA, IBA	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (TopTips NH_2 resin) 2) LLE (ethyl acetate)	GC/MS/MS (SRM)	$[^2H_4]$IAA, $[^{13}C_8,^{15}N_1]$IBA	Diazomethane 5 min at room temperature	n.q.	[15]
Arabidopsis thaliana	IAA, IBA	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) HPLC purification	GC/MS-SIM	$[^{13}C_1]$IBA $[^{13}C_6]$IAA	Diazomethane	n.q.	[16]
Arabidopsis thaliana	IAA IBA 4-Cl-IAA IPA	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) SPE (PMME resin)	GC/MS-SIM	$[^{13}C_6]$IAA	Diazomethane 5 min at room temperature	(*IAA*) 5.2 ± 0.5 ng g^{-1} FW	[17]
Arabidopsis thaliana	IAA	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) SPE (PMME resin)	GC/MS/MS (MRM)	$[^{13}C_6]$IAA	Diazomethane 5 min at room temperature	7.54 ng g^{-1} FW	[18]
Arabidopsis thaliana, Zea mays, Nicotiana tabacum, Lycopersicon esculentum	IAA	1-propanol / H_2O / HCl (2:1:0.002) + DCM	1) Methylation with tms-diazomethane 2) VPE (vapour-phase extraction)	GC/MS	$[^2H_5]$MeIAA	Trimethylsilyl diazomethane	< 10 - 100 ng g^{-1} FW	[19]
Daucus carota	IAA (free, conjugated and total)	65% Isopropanol 35% 0.2 M Imidazole	1) SPE 2) HPLC fractioning	GC/MS-SIM	$[^{13}C_6]$IAA	Diazomethane	(*Free IAA*) 15 - 2,060 ng g^{-1} FW (*Conjugated IAA*) 796 - 7,490 ng g^{-1} FW (*Total IAA*) 823 - 9,550 ng g^{-1} FW	[20]
Fraxinus excelsior	IAA	80% Methanol with 20 mg L^{-1} BHT	1) LLE (Hexane, DCM) 2) DEAE Sephadex A-25 3) SPE (C18 Sep-Pak) 4) TLC separation	GC/MS	IAA	Diazomethane + BSTFA	n.q.	[21]
Lilac and Forsythia	IAA	80% Methanol with 200 mg L^{-1} BHT	1) Filtration 2) LLE (DCM) 3) SPE (C18 Sep-Pak) 4) LLE (ethyl acetate)	TLC-UV followed by silylation for GC/MS	$[^{13}C_6]$IAA	1) Diazomethane 2) Tri-Sil BSA	≈13 - 136 ng g^{-1} FW	[22]
Lycopersicon esculentum	IAA	50% Isopropanol	1) LLE (ethyl acetate) 2) SPE (C18 Ultracarb 5 ODS 30)	GC/MS-SIM	$[^{13}C_6]$IAA	Diazomethane	4.2 - 10.6 ng g^{-1} FW	[23]

GC and GC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Nicotiana glauca, *Nicotiana langsdorffii*	IAA	Modified Bieleski MeOH/H_2O/formic acid (75:20:5)	Dual-mode SPE - Sep-Pak Plus C18 - Oasis MCX	GC/MS-SIM	$[D_5]$IAA	BSTFA at 100°C, 60min	See results obtained with LC/MS/MS	[24]
Olea europaea	IAA	70% Acetone	1) SPE (C18) 2) LLE (diethyl ether) 3) HPLC purification	GC/MS-TIC	$[^{13}C_6]$IAA	Silylation	63.4 - 366.9 ng g^{-1} FW	[25]
Pelargonium leaves	IAA	80% Methanol with 2.5 mM Sodium diethyldithiocarbamate	1) C18 clean-up 2) SPE (C18)	GC/MS	MeIAA	Diazomethane, 20 min	84 ng g^{-1} FW	[26]
Petunia hybrida	IAA	Methanol	1) LLE (diethyl ether) with ultrasounds 2) SPE (Chromabond NH_2)	GC/MS/MS (MRM)	$[^2H]_2$-IAA	Diazomethane	≈ 10 - 350 pmol g^{-1} FW	[27]
Phaseolus coccineus	IAA (free, conjugated and total)	70% Acetone	1) LLE (diethyl ether) 2) HPLC purification	GC/MS-TIC	$[^{13}C_6]$IAA	Silylation	(*free IAA*) 0.23 - 13.03 µg g^{-1} FW (*ester-conjugated*) 0.05 - 6.5 µg g^{-1} FW (*amide-conjugated*) 0.15 - 30.7 µg g^{-1} FW (*total*) 0.52 - 50.23 µg g^{-1} FW	[28]
Pinus sylvestris	Free IAA	0.05 M Sodium-phosphate buffer with 0.02% Sodium-diethyldithiocarbamate	Chelating resin (Amberlite XAD-7)	GC/MS/MS (SRM)	$[^{13}C_6]$IAA	1) Diazomethane 2) BSTFA w/ 1% TMCS at 70°C for 15 min	≈ 20 - 750 ng g^{-1} FW	[29]
	Conjugated IAA	According to [130]	According to [130]	According to [130]		According to [130]		
Pisum sativum	IAA	80% Methanol with 250 mg L^{-1} BHT	1) SPE (Sep-Pak C18) 2) HPLC purification	GC/MS-SIM	$[^{13}C_6]$IAA	1) Diazomethane 2) Pyridine + BSTFA 80°C 3) BSTFA 80°C	85 - 138 ng g^{-1} FW	[30]

GC and GC/MS methods (cont.)								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Pisum sativum	IAA	80% Methanol with 250 mg L^{-1} BHT	SPE (Sep-Pak C18)	GC/MS/MS	$[^{13}C_6]$IAA	1) BSTFA 80°C, 20min 2) BSTFA 80°C, 15min	9.68 – 76.27 ng g^{-1} FW	[31]
Ricinus communis infected with *A. tumefaciens*	IAA	80% Methanol with 1 g L^{-1} BHT	SPE (C18)	GC-ECD and GC/MS	IPA	EDAC + PFPH	≈ 0.2 - 3 nmol g^{-1} FW	[32]
Roses and lilies	IAA MeIAA	70% Acetone with 50 mM Citric acid	LLE (diethyl ether)	GC/MS-SIM	MeOAA and OAA (*o*-anisic acid)	BSTFA + TMCS at 80°C 30 min	n.q.	[33]
Solanum tuberosum	IAA (free and conjugated)	70% Acetone	1) LLE (diethyl ether) 2) SPE (C18) 3) HPLC purification	GC/MS-TIC	$[^{13}C_6]$IAA	BSTFA + TMCS	(*free IAA*) < 50 - 250 ng g^{-1} FW (*ester-conjugated*) < 10 - 200 ng g^{-1} FW (*amide-conjugated*) < 50 - 1300 ng g^{-1} FW	[34]
Tropaeolum majus	IAA, IBA, PAA	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) HPLC purification	GC/MS	$[^{13}C_6]$IAA $[^{13}C_1]$IBA $[^{13}C_1]$PAA	Diazomethane	(*IAA*) 12 - 19 ng g^{-1} FW (*IBA*) 11 - 61 ng g^{-1} FW (*PAA*) 1.5 - 1.9 ng g^{-1} FW	[35]

Table S2 – Chromatography/mass spectrometry methods used in auxin quantification: LC and LC/MS based methods.

LC and LC/MS methods								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Arabidopsis tissues	IAA and conjugates: IAA-Ala IAA-Asp IAA-Leu IAA-Glu	60% Isopropanol with 2.5 mM Diethyl dithiocarbamate	1) LLE (diethyl ether) 2) SPE (Env+)	LC/MS/MS (MRM)	[$^{13}C_6$]IAA, other [indole-$^{13}C_6$] standards and unlabeled amide conjugates synthesized according to [131])	Diazomethane	(*IAA-Ala*) 0 - 0.08 ng g^{-1} FW (*IAA-Asp*) ≈ 0.4 - 3.8 ng g^{-1} FW (*IAA-Glu*) ≈ 0.8 - 12 ng g^{-1} FW (*IAA-Leu*) ≈ 0.03 - 0.15 ng g^{-1} FW (*IAA*) ≈ 7 - 25 ng g^{-1} FW	[36]
Arabidopsis tissues	IBA IBA-Glc IAA IAA-Glc oxIAA IAA-Glu IAA-Asp IAA-Ala MeIAA	80% Methanol	1) SPE (C18) 2) Dessalting 3) DEAE-Sephadex 4) SPE (C18)	microLC/ESI-MS/MS (MRM)	[$^{13}C_6$]IAA IBA-Glc	Diazomethane	(Amounts in pmol g^{-1} FW) (*IBA*) 0.48 - 7.33 (*IBA-Glc*) 1,980 - 31,770.65 (*IAA*) 58.21 - 111.47 (*IAA-Glc*) 193.51 - 2,081.62 (*oxIAA*) 305.29 - 407.48 (*IAA-Glu*) 13.94 - 21.68 (*IAA-Asp*) 6.22 - 7.89 (*IAA-Ala*) 4.06 - 5.21 (*MeIAA*) 82.97 - 99.52	[37]
Arabidopsis thaliana	IAA IAA-Asp	Isopropanol : glacial AcOH (99:1)	SPE (Sep-Pak C18)	HPLC/ESI-MS/MS (MRM)	[$^{2}H_5$]IAA d5-IAA	----	(*IAA*) ≈ 50 - 160 ng g^{-1} DW (*IAA-Asp*) ≈ 20 - 490 ng g^{-1} DW	[38]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Arabidopsis thaliana	IAA IBA ICA MeIAA	1-propanol /H_2O/ HCl (2:1:0.002)	1) LLE (DCM) 2) Centrifugation	LC/ESI–MS/MS (MRM)	[$^2H_5$]IAA [$^2H_5$]MeIAA	----	(*IAA*) ≈ 5 - 60 ng g^{-1} FW (*ICA*) ≈ 10 – 1,200 ng g^{-1} FW (*IBA*) < 10 ng g^{-1} FW (*MeIAA*) ≈ 4 - 6 ng g^{-1} FW	[39]
Arabidopsis thaliana	IAA ICA IBA MeIAA	2-propanol/H_2O/ HCl (2:1:0.002)	1) LLE (DCM) 2) Centrifugation	LC/ESI–MS/MS	[$^2H_5$]IAA [$^2H_5$]MeIAA	----	(*IAA*) ≈ 100 ng g^{-1} FW (*ICA*) < 100 ng g^{-1} FW (*IBA*) ≈ 1.8 ng g^{-1} FW (*MeIAA*) ≈ 2 ng g^{-1} FW	[40]
Arabidopsis thaliana	IAA IBA IAA-Asp IAA-Glu IAA precursors oxIAA	50 mM Sodium phosphate buffer containing 1% Diethyldithiocarbamic acid sodium salt	SPE (HLB)	LC/MS/MS (MRM)	[$^{13}C_6$]IAA-Ala [$^{13}C_6$]IAA-Asp [$^{13}C_6$]IAA-Glu [$^{13}C_6$]IAA-Leu [$^{13}C_1$]IBA [$^{13}C_6$]IAA [$^{13}C_6$]oxIAA	Cysteamine 0.25 M, pH 8, 1h RT	(*IAA*) 11.9 - 303 pmol g^{-1} FW (*IBA*) n.d. (*IAA-Asp*) ≈ 10 – 7000 pmol g^{-1} FW (*IAA-Glu*) ≈ 4 – 2000 pmol g^{-1} FW	[41]
Arabidopsis thaliana	IAA	Modified Bieleski CH_3OH/H_2O/HCOOH (15:4:1) overnight at -80°C and double re-extraction at -20°C	Dual-mode SPE: 1) Sep-Pak Plus C18 2) Oasis MCX	LC/ESI-MS/MS	[$^{13}C_6$]IAA	----	749.76 pmol g^{-1} FW	[42]
Arabidopsis thaliana	IAA oxIAA IAA-Asp IAA-Glu	50 mM Sodium phosphate buffer with 0.02% Sodium diethyldithiocarbamate	SPE (Oasis MAX)	LC/MS/MS	[$^{13}C_6$]IAA [$^{13}C_6$]oxIAA [$^{13}C_6$]IAA-Asp [$^{13}C_6$]IAA-Glu	Diazomethane	(*IAA*) < 1 – 3 pmol g^{-1} FW (ox*IAA*) < 1 – 5 pmol g^{-1} FW (*IAA-Asp*) < 1 – 4 pmol g^{-1} FW (*IAA-Glu*) < 1 – 3 pmol g^{-1} FW	[43]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Arabidopsis thaliana	IAA	10% Methanol with ceria-stabilized zirconium oxide beads in a vibration mill	SPE (Oasis HLB)	UPLC/MS/MS (MRM)	[$^2H_5$]IAA	----	20 – 50 pmol g^{-1} FW	[44]
Arabidopsis thaliana, Triticum aestivum, *Nicotiana tabacum*	IAA	MeOH/H_2O/AcOH (15:4:1)	SPE (Oasis MAX)	2D-HPLC/FLD	[5-^{3}H]IAA	----	5.86 – 54.97 pmol g^{-1} FW	[45]
Arabidopsis thaliana, Nicotiana tabacum	IAA	Modified Bieleski Methanol / H_2O / formic acid (75:20:5)	SPE: - Oasis HLB - Oasis MCX	nanoflow LC/ESI-IT-MS/MS (MRM)	[$^2H_5$]IAA	----	25 - 260 pmol g^{-1} FW	[46]
Arabidopsis thaliana, Zea mays	IAA MeIAA	Pre-chilled acetonitrile	1) Centrifugation 2) Filtration	HPLC/ESI-QTOF-MS	IAA, MeIAA	----	(*IAA*) 50 - 720 ng g^{-1} (*MeIAA*) 7 - 290 ng g^{-1}	[47]
Arabidopsis thaliana, Zea mays	IAA MeIAA IAAInos isomers	Pre-chilled acetonitrile (*Z. mays*) Pre-chilled methanol (*A. thaliana*)	1) Centrifugation 2) Filtration	HPLC/ESI-QTOF-MS	IAA MeIAA synthetic isomers of IAAInos	----	(Amounts in µg g^{-1}) *(IAA)* *0.82 – 1.4* *(MeIAA)* *0.197 – 0.9* *(IAAInos P1)* *0.276 – 23.7* *(IAAInos P2) 0.284±0.016* *(IAAInos P3)* *0.608 – 16.9* *(IAAInos P4) 0.494±0.022*	[48]
Banana	IAA IBA IPA NAA	Ultrasound-assisted using methanol : H_2O (85:15)	MIM-SPE (MISPE)	HPLC-UV	IAA IBA IPA NAA	----	(*IAA*) 0.06 - 0.44 µg g^{-1} (*IBA*) 0.06 - 0.32 µg g^{-1} (*IPA*) 0.07 - 0.41 µg g^{-1} (*NAA*) 0.06 - 0.43 µg g^{-1}	[49]
Betula platyphylla	IAA	Methanol	Filtration	LC-MS/MS (MRM)	IBA	----	0.359 - 2.91 µg g^{-1} FW	[50]

LC and LC/MS methods (cont.)								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Brassica napus	IAA IAA-Asp	80% Isopropanol with 1% glacial acetic acid	SPE (Sep-Pak C18)	LC/ESI-MS/MS (MRM)	[$^2H_5$]IAA	----	(*IAA*) < 100 - 600 ng g^{-1} DW (*IAA-Asp*) < 100 ng g^{-1} DW	[51]
Brassica napus	IAA IBA	1-propanol / H_2O / HCl (2:1:0.002)	1) LLE (DCM) 2) SPE (C18) 3) Filtration	LC/ESI-Qtrap-MS/MS (MRM)	IAA, IBA	----	(*IAA*) 11.43 ng g^{-1} FW (*IBA*) n.d.	[52]
Brassica napus	IAA	Acetonitrile / H_2O / formic acid (80:19:1)	1) SPE (Oasis MCX) 2) LLE (ethyl acetate)	HPLC/ESI-MS/MS (MRM)	[$^2H_5$]IAA	----	6.21 - 11.8 ng g^{-1} FW	[53]
Castanea sativa × *Castanea crenata* clone 'M3'	IAA IAAsp IBA	5 mM K-phosphate buffer, pH 6.5, with BHT	SPE (C18)	HPLC-FLD	NAA	----	(*IAA*) ≈ 1 - 35 nmol g^{-1} DW (*IAA-Asp*) ≈ 5 - 50 nmol g^{-1} DW (*IBA*) ≈ 1 - 35 nmol g^{-1} DW	[54]
Cherry rootstock 'GiSelA 5' (*P. cerasus* x *P. canescens*)	IAA IAA-Asp	BHT-Methanol solution (0.5 g L^{-1})	SPE (Strata C18-E)	HPLC-FLD	IAA IAA-Asp	----	(*IAA*) 3 - 558 ng g^{-1} FW (*IAA-Asp*) 250 – 11,860 ng g^{-1} FW	[55]
Chickpea, field pea and lentil	IAA IAA-Ala IAA-Asp IAA-Glu IAA-Leu IBA	Isopropanol : glacial acetic acid (99:1)	SPE (Sep-Pak C18)	UPLC/ESI–MS/MS (MRM)	d5-IAA d3-IAA-Ala d3-IAA-Asp d3-IAA-Leu d3-IAA-Glu	----	(Amounts in nM g^{-1} DW) (*IAA*) 0.42 - 8.91 *(IAA-Asp*) 0.04 - 1284.2 (*IAA-Glu*) 0.01 – 60.77 (*IAA-Ala*) n.d. – 0.35	[56]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Chickpea, field pea, faba bean and lentil	IAA IAA-Asp IAA-Glu IAA-Ala IBA 4-Cl-IAA	Isopropanol : glacial acetic acid (99:1)	SPE (Sep-Pak C18)	HPLC/ESI-MS/MS (MRM)	d5-IAA d3-IAA-Ala d3-IAA-Asp d3-IAA-Leu d3-IAA-Glu	----	(Amounts in nmol g^{-1} DW) (*IAA*) 0.05 - 507.88 (*IAA-Asp*) 0.01 - 919.95 (*IAA-Glu*) 0.01 - 42.19 (*IAA-Ala*) 0.01 - 0.53 (*IBA*) 0.02 – 3.77 (*4-Cl-IAA*) 0.01 - 17.63	[57]
Cicer arietinum, Cicer anatolicum	IAA IAA-Ala IAA-Asp IAA-Glu IAA-Leu	Isopropanol : glacial acetic acid (99:1)	SPE (Sep-Pak C18)	HPLC/ESI-MS/MS (MRM)	d5-IAA	----	(*IAA*) ≈ 20 - 108 nM g^{-1} DW (*IAA-Asp*) ≈ 1.5 - 134 nM g^{-1} DW (*IAA-Glu*) < 3 nM g^{-1} DW (*IAA-Leu*) 0.04 - 0.05 nM g^{-1} DW	[58]
Chinese cabbage	IAA IBA IPA NAA	80% Methanol	LLE (acetic ether)	LC/ESI-IT-MS/MS (MRM)	IAA IBA IPA NAA	----	Not mentioned	[59]
Chlorella vulgaris	IAA IBA IPA NAA	80% Methanol with 1 mM BHT	DLLME	HPLC-FLD	IAA IBA IPA NAA	----	(*IAA*) 37.0 ng g^{-1} FW (*IBA*) n.d. (*IPA*) n.d. (*NAA*) n.d.	[60]
Citrus clementina, *Hordeum vulgare*, *Carica papaya*	IAA	Water	LLE (diethyl ether)	LC/ESI-MS/MS (MRM)	$[^2H_2]$IAA	----	143.67 - 994.22 pmol g^{-1}	[61]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Cocos nucifera (coconut water)	IAA IBA NAA 2,4-D	1) Methanol / acetic acid (100:1) 2) Methanol / H_2O / acetic acid (50:50:1) 3) Methanol / H_2O / acetic acid (30:70:1)	SPE (C18)	HPLC-PDA (MRM to confirm ID)	IAA	----	(*IAA*) 0.122 µM	[62]
Coconut juice (water)	IAA	----	HF-LLLME	HPLC-UV	IAA	----	0.25 - 1.46 µg mL^{-1}	[63]
Courgette samples	IAA 2,4-D NAA	QuEChERS	QuEChERS	UPLC/MS/MS	IAA 2,4-D NAA	----	(*IAA*) 38 ng g^{-1} (*NAA*) < LOQ (*2,4-D*) n.d.	[64]
Cucumber, lettuce, and tomato (from local market)	IBA NAA 2,4-D	Acetonitrile	Filtration	HPLC-FLD	IBA NAA 2,4-D	EDC + APF 60°C for 1h in the dark	n.d.	[65]
Datura metel	IAA	Methanol in sonication bath	Filtration	HPLC/MS/MS with ESI, APCI, or APPI	IAA	----	Melatonin was the focus of the study; IAA was only detected in 10% of flowers	[66]
Dimocarpus longan	IAA	Methanol / formic acid / H_2O (15:4:1)	1) Mixed-mode SPE - C18 - Cation exchange 2) Filtration	LC/ESI-MS	Alizarin	----	5 - 35 ng g^{-1} DW	[67]
Eucalyptus globulus	IAA IAA-Asp	MeOH / formic acid / H_2O (15:1:4)	1) SPE (96-well) - Oasis HLB - Oasis MCX 2) DEAE cellulose column	UPLC/ESI-qMS/MS	d5-IAA d2-IAA-Asp	MS-probe reaction (bromocholine in 70 % acetonitrile and triethylamine for 130 min at 80°C)	(*IAA*) < 200 - 800 pmol g^{-1} FW (*IAA-Asp*) < 500 – 2,000 pmol g^{-1} FW	[68]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Helleborus niger	IAA	50 mM Phosphate buffer with 0.02% Sodium-diethyldithiocarbamate	1) SPE (C8) 2) Immunoaffinity column with polyspecific polyclonal antibodies against IAA	UPLC/MS/MS (MRM)	[$^{2}H_5$]IAA [$^{15}N,^{2}H_5$]IAA-Ala [$^{15}N,^{2}H_5$]IAA-Asp [$^{15}N,^{2}H_5$]IAA-Glu [$^{15}N,^{2}H_5$]IAA-Gly [$^{15}N,^{2}H_5$]IAA-Leu [$^{15}N,^{2}H_5$]IAA-Phe [$^{15}N,^{2}H_5$]IAA-Val	Diazomethane	(Amounts in pmol g^{-1} FW) (*IAA*) 313 – 7378 (*IAA-Asp*) 62.5 – 2089 (*IAA-Glu*) 2.86 - 44.9 (*IAA-Gly*) 3.35 (*IAA-Leu*) 1.60 - 2.24 (*IAA-Phe*) 1.17 (*IAA-Val*) 1.02 (*IAA-Ala*) 0.44	[69]
Helleborus niger	IAA (free and amide-conjugated)	50 mM Phosphate buffer with 0.02% Sodium-diethyldithiocarbamate	1) SPE (C8) 2) Methylation with diazomethane 3) Immunoaffinity column with polyspecific polyclonal antibodies against IAA	UPLC/MS/MS (MRM)	[$^{2}H_5$]IAA [$^{15}N,^{2}H_5$]IAA-Ala [$^{15}N,^{2}H_5$]IAA-Asp [$^{15}N,^{2}H_5$]IAA-Glu [$^{15}N,^{2}H_5$]IAA-Gly [$^{15}N,^{2}H_5$]IAA-Leu [$^{15}N,^{2}H_5$]IAA-Phe [$^{15}N,^{2}H_5$]IAA-Val	----	(*IAA*) ≈ 7 – 50,000 pmol g^{-1} FW (*IAA-Glu*) ≈ 0.1 – 1,000 pmol g^{-1} FW (*IAA-Ala*) ≈ 0.5 – 20,000 pmol g^{-1} FW (*IAA-Val, -Phe, -Leu, -Gly, -Ala*) 0 - 35 pmol g^{-1} FW	[70]
Hordeum vulgare	IAA IPyA	Methanol	LLE (ethyl acetate) vs. SPE (ODS-C18)	HPLC-FLD	IAA IPyA	----	LOD: (*IAA*) 1.82 ng mL^{-1} (*IPyA*) 5.16 ng mL^{-1} LOQ: (*IAA*) 5.51 ng mL^{-1} (*IPyA*) 15.64 ng mL^{-1}	[71]
Lactuca sativa	IAA IAA-Asp	Isopropanol : glacial acetic acid (99:1)	SPE (Sep-Pak C18)	HPLC/ESI-MS/MS (MRM)	d5-IAA	----	(*IAA*) ≈ 10 – 1,300 ng g^{-1} DW (*IAA-Asp*) ≈ 50 - 100 ng g^{-1} DW	[72]
Linum usitatissimum	IAA	Methanol	1) SPE 2) Dilution	HPLC-UV/MS	IAA NAA	----	n.q.	[73]
Lycopersicon esculentum	IAA	80% Ethanol with soluble PVPP	1) Filtration 2) LLE: - Petroleum ether - Diethyl ether	HPLC-FLD and HPLC/MS	IPA [$^{13}C_6$]IAA	----	55 – 300 pmol g^{-1} FW	[74]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Lycopersicon esculentum	IAA	Methanol overnight	SPE (Oasis MAX)	UPLC/MS/MS (MRM)	[$^2H_2$]-IAA	----	31.22 ± 1.72 pmol g^{-1} FW	[75]
Lupinus albus	IAA	50 mM Sodium phosphate buffer with 5 µM BHT	LLE (ethyl acetate)	LC-ECD (GC/MS for ID)	5-^{3}H-IAA	----	145 - 647 ng g^{-1} FW	[76]
Macadamia integrifolia	IAA IBA	80% Methanol	1) SPE (C18 Sep-Pak) 2) LLE (diethyl ether saturated with 0.2N acetic acid)	LC/QTOF-MS/MS	[$^{13}C_6$]IAA	----	(*IAA*) n.d. (*IBA*) 139.66 - 192.75 pmol g^{-1} FW	[77]
Maize	IAA IAA-Asp IAA-Glu IAA-Gly IAA-Lys IAA-Ala IAA-Glc IAA-Ileu IAA-Gln ICA IPA IBA	Acetonitrile + 20 mM Sodium phosphate buffer + 20 mM Sodium perchlorate, pH 5.7 + sonication	Centrifugation	2D-HPLC/FLD	IAA IAA-Asp IAA-Glu IAA-Gly IAA-Lys IAA-Ala IAA-Glc IAA-Ileu IAA-Gln ICA IPA IBA	----	n. d.	[78]
Mung bean	IAA IBA	80% Methanol	LLE (ethyl acetate)	HPLC-CL	IAA IBA	----	(*IAA*) 0.76 - 0.91 µg g^{-1} (*IBA*) 0.57 - 0.61 µg g^{-1}	[79]
Vigna radiata	IAA IBA	Methanol with 10% PVPP	LLE (diethyl ether)	HPLC	IAA IBA	----	(*IAA*) < 50 ng g^{-1} FW (*IBA*) < 10 ng g^{-1} FW	[80]
Musa basjoo, Viola baoshanensis	IAA IBA NAA	Methanol / potassium phosphate buffer (pH 3, 8:2)	SPME	HPLC-UV/Vis	IAA IBA NAA	----	(*IAA*) 3.9 µg L^{-1} (*IBA*) 2.14 µg L^{-1} (*NAA*) 0.93 µg L^{-1}	[81]
Nicotiana tabacum	IAA	Bieleski solvent (MeOH:$CHCl_3$:H_2O:AcOH, 12:5:2:1)	Dual-mode SPE - Sep-Pak Plus C18 - Oasis MCX	2D-HPLC	[^{3}H]IAA	----	< 240 – 1,022 pmol g^{-1} FW	[82]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Nicotiana glauca, N. langsdorffii	IAA	Modified Bieleski Methanol / H_2O / formic acid (75:20:5)	Centrifugation	LC/MS/MS	d5-IAA	----	16 - 200 ng g^{-1} FW	[24]
Nicotiana benthamiana, Solanum lycopersicum	IAA IAA-Asp IAA-Glu	Isopropanol : glacial acetic acid (99:1)	SPE (Sep-Pak C18)	HPLC/ESI-MS/MS (MRM)	d5-IAA	----	(*IAA-Asp*) 17 - 225 ng g^{-1} DW (*IAA-Glu*) 11 - 24 ng g^{-1} DW	[83]
Olea europaea	IAA IAN	80% Ethanol with PVPP and 100 mg L^{-1} BHT	1) Filtration 2) LLE - Petroleum ether - Diethyl ether	HPLC-FLD (GC/MS used to ID)	IPA	----	(*IAA*) 82 - 720 ng g^{-1} DW (*IAN*) 56 - 80 ng g^{-1} DW	[84]
Oryza sativa	IAA and conjugates: IAA-Ala IAA-Asp IAA-Ile IAA-Glu IAA-Phe IAA-Val	80% Acetone with 2.5 mM Diethyl dithiocarbamate	SPE (C18)	LC/ESI-MS/MS	[$^{13}C_6$]IAA	----	(*IAA*) 16 – 6,500 pmol g^{-1} FW (*IAA-Ala*) 0 - 9 pmol g^{-1} FW (*IAA-Asp*) 41 - 178 pmol g^{-1} FW (*IAA-Glu*) 11 - 79 pmol g^{-1} FW	[85]
Oryza sativa	IAA IAA-Asp IAA-Glu IAA-N-Glc IAA-Asp-N-Glc IAA-Glu-N-Glc	Acetone / H_2O (4:1) with 2.5 mM Diethyldithiocarbamic acid	SPE (Sep-Pak Plus C18)	LC/ESI-MS/MS (MRM)	IAA-N-[6,6-$^2H_2$]Glc IAA-Asp-N-[6,6-$^2H_2$]Glc IAA-Glu-N-[6,6-$^2H_2$]Glc	----	Amounts in nmol g^{-1} FW (*IAA*) < 0.5 (*IAA-Asp*) < 0.5 (*IAA-Glu*) < 0.5 (*IAA-N-Glc*) ≈ 0.2 (*IAA-Asp-N-Glc*) < 0.8 (*IAA-Glu-N-Glc*) ≈ 0.4	[86]
Oryza sativa	IAA IAA-Ala IAA-Asp IAA-Phe IAA-Ile IAA-Leu IAA-Trp	Methanol : formic acid : water (15:1:4)	1) SPE (96-well) - Oasis HLB - Oasis MCX 2) DEAE cellulose column	UPLC/ESI-qMS/MS	d5-IAA d2- IAA-Ala d2-IAA-Asp d2-IAA-Phe d2-IAA-Ile d2-IAA-Leu d2-IAA-Phe	MS-probe reaction (bromocholine in 70% acetonitrile and triethylamine for 130 min at 80°C)	(*IAA*) 9.68 - 290.46 pmol g^{-1} FW (*IAA-Ala*) 22.16 - 33.51 pmol g^{-1} FW (*IAA-Asp*) 606.1 pmol g^{-1} FW	[87]
Oryza sativa	IAA	65% Isopropanol 35% 0.2 M Imidazole	1) SPE (NH_2 resin) 2) SPE (PMME resin)	HPLC/ESI-MS-MS (MRM)	[$^{13}C_6$]IAA	----	84 ng g^{-1} FW to 2.4 µg g^{-1} FW	[88]

LC and LC/MS methods (cont.)								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Oryza sativa	IAA IBA	Acetonitrile	Magnetic SPE coupled with *in situ* derivatization (MSPE-ISD)	UPLC/MS/MS (MRM)	[$^2H_5$] IAA	BTA + TEA	(*IAA*) 39.52 ng g^{-1} FW (*IBA*) n.d.	[89]
Oryza sativa	IAA	Acetonitrile	Magnetic SPE (MSPE)	UPLC/MS/MS (MRM)	[$^2H_2$] IAA	----	50 – 150 ng g^{-1} FW	[90]
Pisum sativum	IBA	5 mM Phosphate buffer pH 7.0	[C4mim][PF6]	HPLC-UV	IBA	----	(IBA) 5.2 - 100.3 ng g^{-1} (spiked samples)	[91]
Pisum sativum	IAA 4-Cl-IAA	MeOH/H_2O (4:1) with 250 mg L^{-1} BHT	SPE	UPLC/MS/MS (MRM)	[$^{13}C_6$]IAA [D_4]4-Cl-IAA	----	(*IAA*) < 200 ng g^{-1} FW (*4-Cl-IAA*) 200 – 1,200 ng g^{-1} FW	[92]
Pea, wheat, rice	IAA IBA	Methanol with 0.1% BHT	1) LLE - Petroleum ether - *n*-Hexane 2) mag-MIP beads	HPLC-UV	IAA IBA	----	(*IAA*) 7.5 – 19.3 ng g^{-1} FW (*IBA*) n.d.	[93]
Pea and rice	IAA IBA	Vacuum microwave-assisted extraction (VMAE): 80% methanol with 0.01% BHT (10 min, 25°C)	1) LLE (ethyl acetate) 2) Mag-MIP beads	HPLC-FLD	IAA IBA	----	(*IBA*) n.d. (*IAA*) ≈ 7 - 53 ng g^{-1}	[94]
Prunus subhirtella	IAA IBA IAA-Asp	BHT methanolic solution + 5 mM Potassium phosphate buffer pH 6.5	SPE (Strata C18-E)	HPLC-FLD	IAA IBA IAA-Asp	----	(*IBA*) 6.3 µg g^{-1} FW (*IAA*) 0.7 – 10.7 µg g^{-1} FW (*IAA-Asp*) 16 – 30 µg g^{-1} FW	[95]
Seaweed (*Ecklonia maxima* and *Macrocystis pyrifera*)	IPA ILA IPya IAA IAA-Asp IAA-Gly IAA-Ala IAA-Leu	70% Ethanol for 3h and re-extraction	1) DEAE-cellulose ODS column 2) Immunoaffinity chromatography with polyclonal antibodies against auxins	LC/ESI- MS-SIM	[$^{13}C_6$]IPA [$^{13}C_6$]ILA [$^{13}C_6$]Ipya [$^{13}C_6$]IAA [^{15}N]IAAsp [^{15}N]IAGly [^{15}N]IAAla [^{15}N]IALeu	Diazomethane	(Amounts in pmol mL^{-1}) *IAA* 7.09 - 11.67 *IAA-Asp* 3.89 - 7.58 *IAA-Ala* < LOD - 0.31 *IAA-Gly* 3.10 - 4.56 *IAA-Leu* 0.10 - 0.48 *ILA* 1.23 - 1.35 *IPA* 2.26 - 2.73 *IPya* 1.09 - 5.96	[96]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Rosmarinus officinalis	IAA	Methanol : isopropanol (20:80) with 1% of glacial acetic acid, using ultra sonication (4-7°C)	Filtration	UPLC/ESI-MS/MS (MRM)	d5-IAA (=[$^2H_5$]IAA)	----	30 -70 pmol g^{-1} DW	[97]
Solanum lycopersicum	IAA	80% Methanol	1) Treatment with PVPP 2) LLE (ethyl acetate)	HPLC/UV	IAA	----	≈ 20 - 100 ng g^{-1} FW	[98]
Triticum aestivum	IAA	80% Methanol with 2.5 mM Sodium diethyldithyocarbamate	1) SPE (Strata C18-E) 2) LLE (diethyl ether)	qTrap LC/MS/MS	[$^2H_5$]-IAA	----	11.6 - 29.6 pmol g^{-1} FW	[99]
Triticum spp.	IAA	Water	SPE (C18)	LC/MS/MS (SRM)	Benzoic acid	----	3.0 - 3.3 µg g^{-1}	[100]
Tobacco cells, radish seedlings	Cytokinins (IAA was isolated in the process)	MeOH/H_2O/AcOH (15:4:1)	Dual-mode SPE - Sep-Pak Plus C18 - Oasis MCX	HPLC-ELISA or HPLC/MS	Not mentioned	----	Not mentioned	[101]
Transgenic tobacco	IAA	Overnight at -20°C with Bieleski solvent	Dual-mode SPE - Sep-Pak Plus C18 - Oasis MCX	2D-HPLC (according to [132])	[^{3}H]IAA	----	61.3 - 177.4 pmol g^{-1} FW	[102]
Tobacco BY-2 cells	IAA IAA-Asp IAA-Glu	Cell homogenate	1) SPE 2) Immunoaffinity extraction	UPLC/MS/MS	Not mentioned	----	(*IAA*) ≈ 2 - 10 pmol g^{-1} FW (*IAA-Asp*) ≈ 100 - 300 pmol g^{-1} FW (*IAA-Glu*) ≈ 3 - 6 pmol g^{-1} FW	[11]

LC and LC/MS methods (cont.)

Sample	Analyte	Extraction	Purification	Analysis	Std used	Derivatization	Amounts detected	Reference
Vitis berlandieri x *Vitis riparia* *Chasselas* x *Vitis berlandieri* *Vitis berlandieri* x *Vitis riparia*	IAA	70% Methanol	1) Filtration 2) LLE - Ethyl acetate - Diethyl ether 3) Anhydrous sodium sulfate	HPLC-FLD	IAA	----	< 20 - 140 ng kg^{-1}	[103]
Vitis vinifera (Grape juice, grape must and wine)	IAA	Hydrolysis (from conjugates) and neutralization	1) SPE (C18/OH) 2) SAX	HPLC-FLD	IPA	----	MUSTS (*Free IAA*) < 3 µg L^{-1} (*Bound IAA*) <12 - 120 µg L^{-1} WINE (*Free IAA*) < 3 - 90 µg L^{-1} (*Bound IAA*) < 40 µg L^{-1}	[104]
Vitis vinifera	IAA IAA-Asp	60% Isopropanol with 2.5 mM Diethyl dithiocarbamate	1) LLE (diethyl ether) 2) SPE (Env+)	LC/ESI-MS/MS (MRM)	d5-IAA d5-IAA-Asp	----	(*IAA*) < 400 – 1,600 pmol g^{-1} FW (*IAA-Asp*) 20 – 5,000 pmol g^{-1} FW	[105]

Table S3 – Electrokinetic methods used in auxin quantification.

Electrokinetic methods								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Arabidopsis tissues	IAA IPA NAA	80% Methanol	1) SPE (ODS-C18) 2) Filtration	pCEC-UV	IAA IPA NAA	----	(*IAA*) n.d.	[106]
Arthrobacter sp. (MKA20), *Bacillus* sp. (YA21) and *Enterobacter* sp. (CNB26)	IBA 2,4-D IAA PAA	Supernatant of cell culture was diluted 5-fold	Ultrafiltration	CEC-UV	IAA	----	(*IAA*) 3.16 - 38.6 μg mL^{-1}	[107]
Banana	IAA IBA NAA 2,4-D	Acetonitrile	Centrifugation	CE-LIF	IAA IBA NAA 2,4-D	6-oxy-(acetypiperazine) fluorescein (APF) + 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at 60°C for 60 min	(*IAA*) 99.6 - 119.1 ng g^{-1} (*IBA*) 101.5 - 280.1 ng g^{-1} (*NAA*) n.d. (*2,4-D*) n.d.	[108]
Corn	IAA	Methanol	Centrifugal filtration	pCEC-UV	IAA	----	1.94 μg g^{-1}	[109]
Mung bean and acacia	IAA IBA	dCPE (dual-cloud point extraction)	dCPE (dual-cloud point extraction)	CE-ECL	IAA IBA	2-(2-aminoethyl)-1-methylpyrrolidine (AEMP) labeling	(*IAA*) 0.69 - 1.03 μg g^{-1} (*IBA*) n.d.	[110]
Oryza sativa	IAA IBA	80% Methanol	1) SPE (C18) 2) LLE (ethyl ether)	CE-TOF-MS	[$^2H_5$] IAA	BTA + TEA	(*IAA*) 14.3 ng g^{-1} FW (*IBA*) 67.1 ng g^{-1} FW	[111]
Tobacco tissues	IAA NAA 2,4-D	70% Methanol	LLE (ethyl acetate)	MECC	IAA NAA 2,4-D	----	n.d.	[112]
Tomato	IAA IBA NAA IPA	Acetone	LLE - Dichloromethane - Petroleum ether	CZE	IAA IBA NAA IPA	----	n.q.	[113]

Table S4 – Immunoassays and methods involving other types of detection used in auxin quantification.

Immunoassays								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Brassica juncea	IAA	80% Methanol with 1% BHT	SPE (C18)	ciELISA	IAA	----	7 - 15 ng g^{-1}	[114]
Douglas fir	IAA, IAA-Asp	Acidified water : Methanol (1:4) with 2 mM BHT	1) Nitrocellulose filter attached to a Sep-Pak C18 column, which was in turn attached to a 0.2 µm Teflon filter 2) HPLC fractionation	ELISA	[^{3}H]-IAA	Diazomethane	(*IAA*) < 500 ng g^{-1} DW (*IAA-Asp*) ≈ 500 – 4,700 ng g^{-1} DW	[115]
Medicago truncatula, Sinorhizobium meliloti	IAA	80% Methanol 2% glacial acetic acid 10 mg L^{-1} BHT	SPE (ODS C18)	ELISA	[^{3}H]IAA	Diazomethane	1.9 - 5.2 µmol g^{-1} FW	[116]
Oryza sativa	IAA	80% Methanol with 1 mM BHT	SPE (C18 Sep-Pak)	ELISA	----	----	243 ng g^{-1} FW	[117]
Oryza sativa	IAA	80% Methanol with 1 mM BHT	SPE (C18 Sep-Pak)	ELISA	----	----	< 8 - 300 pmol g^{-1} FW	[118]
Oryza sativa	IAA	80% Methanol	1) PVPP-DEAE column 2) SPE (C18 Sep-Pak)	Immunosensor	IAA	Diazomethane	29.3 – 44.9 µg g^{-1}	[119]
Ricinus communis infected with *A. tumefaciens*	IAA	80% Methanol with 1 g/L BHT	1) LLE (diethyl ether) 2) Diazomethane 3) Immunoaffinity column	ELISA	IPA as IS	Diazomethane	≈ 100 - 500 nmol g^{-1} FW	[32]
Mung bean sprouts	IAA	80% Methanol	SPE (C18 Sep-Pak)	Immunosensor	----	----	12.7 – 32.2 ng g^{-1}	[120]
Seeds (wheat, corn, soybean)	IAA	80% Methanol	SPE (C18 Sep-Pak)	Immunosensor	----	----	16.8 – 759.2 ng g^{-1}	[121]
Seeds (wheat, corn, mung bean, soybean, millet and brown rice)	IAA	80% Methanol	SPE (C18 Sep-Pak)	Immunosensor	----	----	16.6 – 769.4 ng g^{-1}	[122]

Other methods								
Sample	**Analyte**	**Extraction**	**Purification**	**Analysis**	**Std used**	**Derivatization**	**Amounts detected**	**Reference**
Gladiola, apple and phoenix tree leaves	IAA	Ethyl acetate	Not mentioned	Carbon-nanotube biosensor	Not mentioned	----	3.02 – 5.61 µg g^{-1}	[123]
Zea mays	IAA	Not mentioned	Not mentioned	Carbon-nanotube biosensor	Not mentioned	----	52.5 ng g^{-1}	[124]
Zea mays	IAA	Not mentioned	Not mentioned	Carbon-nanotube biosensor	Not mentioned	----	Biosensor used to measure IAA fluxes (fmol cm^{-2} sec^{-1})	[125]
Peach, Rosa, and Crape myrtle	IAA	Methanol + PVPP	Filtration	MIM-SPR	IAA	----	0.13 – 0.28 µg g^{-1} FW	[126]
Mung bean sprout leaves	IAA	Methanol	Centrifugation	Amperometric detection	IAA	----	4.03 - 4.22 µg g^{-1}	[127]
Vigna radiata	IAA	Not mentioned	Not mentioned	Fluorimetric assay	IAA	----	9 – 21 ng g^{-1} FW	[128]
Vigna radiata	IAA	Not mentioned	Not mentioned	Fluorimetric assay	IAA	----	9 – 16 ng g^{-1} FW	[129]

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Chapter III

QUANTIFICATION OF FREE AUXINS IN SEMI-HARDWOOD PLANT CUTTINGS AND MICROSHOOTS BY DISPERSIVE LIQUID-LIQUID MICROEXTRACTION / MICROWAVE DERIVATIZATION AND GC/MS ANALYSIS

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Quantification of free auxins in semi-hardwood plant cuttings and microshoots by dispersive liquid-liquid microextraction / microwave derivatization and GC/MS analysis

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Abstract

Several studies have suggested that differences in natural rooting ability of plant cuttings could be attributed to differences in endogenous auxin levels. Hence, during rooting experiments, it is important to be able to routinely monitor the evolution of endogenous levels of plant hormones. This work reports the development of a new method for the quantification of free auxins in auxin-treated *Olea europaea* (L.) explants, using dispersive liquid-liquid microextraction (DLLME) and microwave assisted derivatization (MAD) followed by gas chromatography / mass spectrometry (GC/MS) analysis. Linear ranges of 0.5 – 500 ng mL^{-1} and 1 – 500 µg mL^{-1} were used for quantification of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), respectively. Determined by serial dilutions, limits of detection (LOD) and quantification (LOQ) were 0.05 ng mL^{-1} and 0.25 ng mL^{-1}, respectively for both compounds. When using the calibration curve for determination, LOQ corresponded to 0.5 ng mL^{-1} (IAA) and 0.5 µg mL^{-1} (IBA). The proposed method proved to be substantially faster than other alternatives, and allowed free auxin quantification in real samples of semi-hardwood cuttings and microshoots of two olive cultivars. Concentrations found in the analyzed samples are in the range 0.131 – 0.342 µg g^{-1} (IAA) and 20 – 264 µg g^{-1} (IBA).

Keywords: Adventitious rooting, Auxins, DLLME, MAD, GC/MS, *Olea europaea* (L.)

1. Introduction

Olive (*Olea europaea* L.) is one of the main crop species in the Mediterranean region and is mainly propagated by cuttings[1]. The success of plant propagation by cuttings mostly depends on the capacity of the explants to form adventitious roots. Treatment of explants with plant growth regulators such as indole-3-butyric acid (IBA) is a widely adopted procedure in vegetative plant propagation protocols[2,3] and particularly crucial in difficult-to-root species or cultivars where otherwise the rooting process may never occur[4]. This is the case of some of the most important olive cultivars[5]. Differences in the rooting ability of cuttings have been related with the metabolism of the absorbed auxins[2,6] and the effectiveness of exogenously applied IBA has been related to the ability of the cuttings to convert it into indole-3-acetic acid (IAA)[3,5]. Consequently, monitoring the evolution of auxin levels at the base of treated cuttings, during the adventitious root formation process, has become an essential topic in agronomical comparative studies involving cuttings with different rooting behaviors. Indeed, the analysis of endogenous auxin levels and their evolution during the adventitious root formation process as a result of root-inducing treatments, must be simple enough to be used as a routine practice. Typically, root-inducing treatments used in olive propagation involve very high concentrations of IBA (500-6000 mg L^{-1})[5]. However, the natural concentrations of plant hormones in plant tissues are inherently low, usually ng/g, raising challenges in their quantification and requiring optimization of sample preparation, from grinding to derivatization. These issues were recently reviewed by Porfirio *et al.*[7], where sample preparation procedures, including extraction solvents, purification and derivatization, are reviewed, discussed and critically compared. The very high concentrations of IBA used in root-inducing treatments pose another analytical challenge as the methods developed for quantification of IAA and IBA in auxin-treated tissues have to be robust enough to allow quantification of two analytes present at concentrations an order of magnitude apart.

Dispersive liquid-liquid microextraction (DLLME) is a technique used in aqueous samples allowing high enrichment factors[8,9]. Being a microextraction technique, it has several advantages compared with classical extraction methods including lesser volume of solvents, shorter extraction time, and minimum sample loss. Although its application in solid matrices is not very common, some reports can be found in the literature[10], in which cases solid samples had to be submitted to a classic solvent extraction in order to become suitable for DLLME. Previous work by Lu *et al*.[11] used DLLME for auxin extraction from *Chlorella vulgaris* (a unicellular green algae) and *Duranta repens* (an evergreen shrub). However, due to “severe background

interference", the optimized DLLME procedure that was successful for the extraction of auxins from *Chlorella vulgaris* was not effective in *Duranta repens* and only a semi-qualitative analysis was performed in these tissues. Indeed, in this earlier report, auxin quantification following DLLME was only performed in unicellular algae. Thus our approach is, as far as we know and reported[7], the first successful application of DLLME to auxin extraction from plant tissues.

Gas chromatography / mass spectrometry (GC/MS) has been widely used for the analysis of plant hormones and is more frequently reported in literature because it is more sensitive than liquid chromatography / mass spectrometry (LC/MS)[7]. The development of LC/MS instruments with improved sensitivity has increased the popularity of this technique and, in fact, non-volatile compounds like auxins can be more easily analyzed by LC methods, which has been done in several cases[12,13]. However, while LC/MS also offers high-throughput analysis, its instrumentation is far more expensive than GC/MS equivalents, and is less prevalent in many agronomical laboratories. Although significant improvements in sensitivity were introduced by selected reaction monitoring (SRM)[14], this kind of instrument and its operation is not affordable by many labs and this work also aims to provide a method that can be applied in common benchtop GC/MS instruments. Thus GC/MS still continues to be the most preferred analytical method to perform quantitation whenever compound volatility is achievable[7,15].

Auxins are not naturally volatile and need to be derivatized before GC/MS analysis. So far, two main derivatization reactions have been used: methylation with diazomethane[16] and silylation with several reagents[17–19]. However, both these methods have drawbacks; methylation with diazomethane is fast, but the reagent is highly toxic and explosive[20], while silylation can take up to 1h, which is significant when working with large numbers of samples. Nevertheless, silylation proved to be the most suitable derivatization procedure for profiling plant hormones[19].

Microwave-assisted derivatization (MAD), which has been widely used in chemical synthesis, has been recently applied to the preparation of derivatives for GC/MS analysis, greatly reducing derivatization time and improving reaction efficiency[21]. So far most MAD applications use domestic or ordinary microwave ovens[21], which may not provide optimal conditions for a chemical reaction to occur because of inaccurate temperature and pressure setting. However, the results obtained have been impressive and promising, making MAD by domestic microwave oven a viable, affordable and practical alternative. Furthermore, the recent development of silicon carbide (SiC)-

based microtiter plates/rotor systems equipped with GC vials will likely contribute to high-throughput sample processing by minimizing temperature differences among vials[22].

Here a fast DLLME-MAD sample preparation method for free auxin quantification by GC/MS analysis is presented and optimization of DLLME and MAD conditions is described. The method was successfully applied to randomly selected olive (*Olea europaea* L.) samples resulting from rooting trials.

2. Experimental

2.1. Reagents and materials

Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and indole-3-propionic acid (IPA) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). A combined stock solution of IAA and IBA [100 μg mL^{-1}] was prepared with methanol and stored at -20 ^{o}C. To maintain the quality of the standards, several aliquots were taken from the stock solution, designated as working solutions and kept at -20 ^{o}C for storage. [$^{13}C_6$]IAA (Cambridge Isotopes Laboratories (Cambridge, MA, USA)) was used as internal standard for IAA quantification. A stock solution of 1 mg mL^{-1} was originally prepared with methanol and stored in an amber vial at -20 ^{o}C. A working solution of 1 μg mL^{-1} was prepared by stock dilution and stored under the same conditions. IPA was used as internal standard for IBA semi-quantification. A stock solution of 10 mg mL^{-1} was originally prepared with methanol and stored in an amber vial at -20 ^{o}C. A working solution of 1 mg mL^{-1} was prepared by stock dilution and stored under the same conditions. 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. Sample origin and preparation

Olive (*Olea europaea* L.) explants were prepared at Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Évora, Portugal. Two types of cuttings were used: semi-hardwood cuttings of the cultivars 'Galega vulgar' and 'Cobrançosa', and microshoots of cv. 'Galega vulgar'. In order to stimulate adventitious root formation, the base of the explants was dipped for 10 s in an IBA solution at 3.5 g L^{-1} (17.15 mM) (in the case of semi-hardwood cuttings) and 3 g L^{-1} (14.7 mM) (in the case of microshoots). Semi-hardwood cuttings (12-15 cm from the middle region of year growing branches) were collected from field grown plants and were transferred after IBA treatment into a water-cooling greenhouse, being 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 cuttings' leaves was reduced by removing all leaves except the 4 on the top and by automatically sprinkling water at regular intervals throughout the rooting trial. For the *in vitro* rooting trial, microshoots with 4-5 nodes of the cv. 'Galega vulgar' were obtained from the apical portion of *in vitro* pre-cultured explants and prepared according to Peixe *et al.*[23]. After IBA treatment, the explants were inoculated into OM basal medium[24], according to the procedure proposed by Macedo *et al.*[25].

At designated time points after the treatment, the basal portions of the explants (approx. 1 cm from explant base) were collected and lyophilized in preparation for auxin analysis. Ten semi-hardwood explants and 30 microshoots were collected for each sample. Each sample was ground in a mortar and pestle while frozen in liquid nitrogen. About 100 mg of the powdered plant tissue was transferred into a solvent rinsed 5 mL screw-cap glass tube. Briefly, 3 mL of 80% methanol containing 1 mM BHT (stored at 4 °C before use) was added to each sample to eliminate oxidation processes, and extraction was performed by end-over-end shaking in the dark at 4 ^{o}C overnight. After extraction, each tube was centrifuged (Beckman-Coulter Allegra 6R) at 3000 rpm, 4 ^{o}C for 10 min with the supernatant being transferred into a solvent rinsed conical glass tube. The residual pellet was re-extracted with 1 mL of methanol for 1 hour under the same conditions as described above. Subsequently, the extracts were combined, dried under a stream of nitrogen, redissolved with 420 µL of methanol and diluted with water to a final volume of 3 mL. The extract was prevented from being exposed to light at all stages of extraction.

2.3. Dispersive liquid-liquid microextraction (DLLME)

For DLLME optimization purposes, a blank mixture of 420 µL of a concentrated BHT methanolic solution [714 µg mL^{-1}] and 2.58 mL of nanopure water was used as starting aqueous sample. This aqueous sample was then spiked with an aliquot of the auxin standard mixture containing 1 µg each of IAA and IBA, corresponding to a final concentration of 10 µg mL^{-1}, and with an equivalent amount of internal standard (IS). Optimization included choice of pH and salt concentration, volumes of extraction and disperser solvents, and effect of vortex-assisted extraction, ultrasounds-assisted extraction and re-extraction.

Once the procedure was optimized, DLLME was performed in samples by adding 0.450 g of NaCl to the aqueous sample and adjusting the pH to 4 with 100 mM HCl. A solvent mixture containing 200 µL of chloroform ($CHCl_3$) (extractant) and 1 mL acetone (disperser) was injected into the sample via a glass syringe forming a cloudy solution. The mixture was briefly shaken manually, sonicated in ice for 1 min (Bransonic® Ultrasonic Cleaner 1510R-MT, Branson Ultrasonics Corporation, Danbury, CT, USA) 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 tapered base amber GC vial (9mm Target DP Micro-V Tapered MicroVial with 150µL reservoir, ThermoScientific, Rockwood, TN, USA).

2.4. Microwave-assisted derivatization (MAD)

MAD optimization was accomplished using an aliquot of the auxin standard mixture containing 1 µg each of IAA and IBA, corresponding to a final concentration of 10 µg mL^{-1} and with an equivalent amount of IS. The standard mixture and plant extracts were dried under a stream of N_2 prior to derivatization. Briefly, 100 µL of BSTFA was added to the standards and plant extracts. The vials were tightly capped, placed in a Mini Combi-Rac™ (Analytical Sales and Services, Inc, NJ, USA) and heated at 630 watts (W) for 5 min in a commercially available microwave oven (Hamilton Beach P70B20AP-G5W) for derivatization. After cooling, excess reagent was evaporated under a mild stream of N_2 and, immediately after drying, the derivatized standards and plant extracts were dissolved with 100 µL hexane for subsequent GC/MS analysis. In each experiment three replicates were included and the average and standard deviation of the replicates was considered.

2.5. GC/MS analysis

The derivatized standards and plant extracts were analyzed for IAA and IBA by GC/MS 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 × 0.250 mm with 0.25 µm film thickness d_f). The injected volume was set at 2 µL in splitless mode for 1 minute. The front inlet injector temperature was 250 ºC, and the transfer line temperature was 280 ºC. The ion source temperature was set at 250 °C. The oven conditions used were the following: initial temperature of 80 ºC held for 2 min, temperature was ramped to 140 ºC at 20 ºC/min and held for 2 min, temperature was ramped to 200 ºC at 2 ºC/min and held for 5 min and finally, temperature was ramped to 250 ºC at 30 ºC/min and held for 10 min. A post-run at 270 ºC for 5 min was included to completely clean the column. Helium was the carrier gas flowing at 1 mL min^{-1}. Samples were analyzed both in full scan and selected ion monitoring (SIM) modes. Given the different reactivity of the analytes' functional groups to BSTFA, two derivatives were observed for each phytohormone (-tms1 and -tms2, corresponding to the mono- and disilylated derivatives, respectively) (**Table S1 in ESI**). Similar results were obtained when using other reagents such as hexamethyldisilazane (HDMS): trimethylsilyl chloride (TMCS) : Pyridine, or even when adding pyridine to BSTFA as a catalyst. Therefore, and as previously described by Giannarelli *et al.*[26], the peak areas of both derivatives were added (-tms1 plus –tms2) and quantification was accomplished by getting the total area of each analyte in relation to the total area of the respective IS. This ratio was then translated into concentration through a calibration curve described in the next section. All derivatives were analyzed within 24h of derivatization. Hence, no hydrolysis products were ever found in chromatograms.

2.6. Method validation

The proposed DLLME-MAD method was validated using an adaptation of a previously described procedure[27]. Two sets of standard curves, each containing 6 concentration points, were prepared. **Set A:** three standard curves were constructed in the starting aqueous samples (see description of DLLME optimization) spiked *after* extraction. **Set B:** three standard curves were constructed in the starting aqueous samples (see description of DLLME optimization) spiked *before* extraction. A constant concentration

[20 μg mL^{-1}] of [$^{13}C_6$]IAA was used in both sets. Because the plant samples were treated with high levels of IBA (see Sample origin and preparation), the standard curves were not constructed in real olive samples as proposed by Matuszewski *et al*.[27]. Given the abnormally high levels of IBA in the samples, the addition of low concentrations of standards, such as the lower points of the calibration curves, would be impossible to distinguish. Recovery (RE) was calculated according to Matuszewski *et al*.[27], as RE (%) = B/A x 100. The terms A and B are the slopes of the calibration curves obtained for sets A and B, respectively. Linearity was determined by plotting the total (sum of two TMS derivatives) peak area ratio of IAA to [$^{13}C_6$]IAA and IBA to IPA vs. concentration ratio of IAA to [$^{13}C_6$]IAA and IBA to IPA, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were determined by serial dilution of standards analyzed following DLLME-MAD. LOD corresponded to a signal-to-noise ratio (S/N) of 3 and LOQ to a S/N of 10, based on the signal of m/z 202 (representative chromatogram in **Fig. S1 - ESI**). However, based on the obtained calibration curves[28,29], the practical LOQ values correspond to 0.5 ng mL^{-1} (IAA) and 0.5 μg mL^{-1} (IBA). Validation results are shown in **Table 1**.

2.7. Statistical analysis

Student's *t*-tests, analysis of variance (ANOVA) and post-hoc Tukey HSD test were performed using R Studio software (version 0.98.1083). Significant differences were considered at $p < 0.05$. All experiments were performed in triplicate.

3. Results and discussion

3.1. Optimization of MAD - Optimum power level and reaction time

A commercially available microwave oven with a maximum power of 700 W was used to perform the derivatization reaction, in which 630 W was determined as the optimum power level for MAD reaction (data not shown). To find the optimum reaction time, IAA and IBA (1 μg each) were derivatized at 630 W for 1 to 7 min (**Fig. 1a)**, but no significant differences were found among reaction times. Because higher reaction times at 630 W could result in microwave overheating, longer reaction times at a lower power level were also tested (**Fig. 1b**). Again, no differences were found between reaction times and the chromatographic response obtained under these conditions was

consistently lower than at 630 W. Given that the focus of this method is IAA quantification, the conditions that favored a higher IAA chromatographic response were chosen and thus 5 min at 630 W were considered optimal.

3.2. Comparison with conventional silylation

Conventional silylation protocols include heating the analytes with the derivatizing reagent at high temperatures (70 – 100 ºC) for at least 15 min, an example of which is the work of Birkemeyer *et al.*[19] who optimized a silylation protocol using N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) for phytohormone profiling and found that 100 ºC for 1h was the combination that best suited all tested plant hormones. We compared the efficiency of the presented MAD method with that of conventional silylation (convection). One microgram of IAA and IBA standards was derivatized with N,O-Bistrifluoroacetamide (BSTFA) at 630 W for 5 min, and, in parallel, at 100 ºC for 1 h. No significant differences in derivatization products were found between treatments. Therefore MAD reveals a drastically improved performance over that of conventional silylation procedures by producing an equivalent reaction product in a fraction of the time (**Fig. 1c**).

Even though there are already several reports of MAD technique[30], this is, to the best of our knowledge, the first report of MAD applied to auxin quantification by GC/MS. The method developed herein is simple, fast and practical to most laboratory situations. Furthermore, the proposed MAD-BSTFA method has been proven suitable to derivatize auxins from plant tissues that are known to have low phytohormone concentrations (IAA 0.131 – 0.342 µg g^{-1} dry weight (DW); IBA 20 - 264 µg g^{-1} DW).

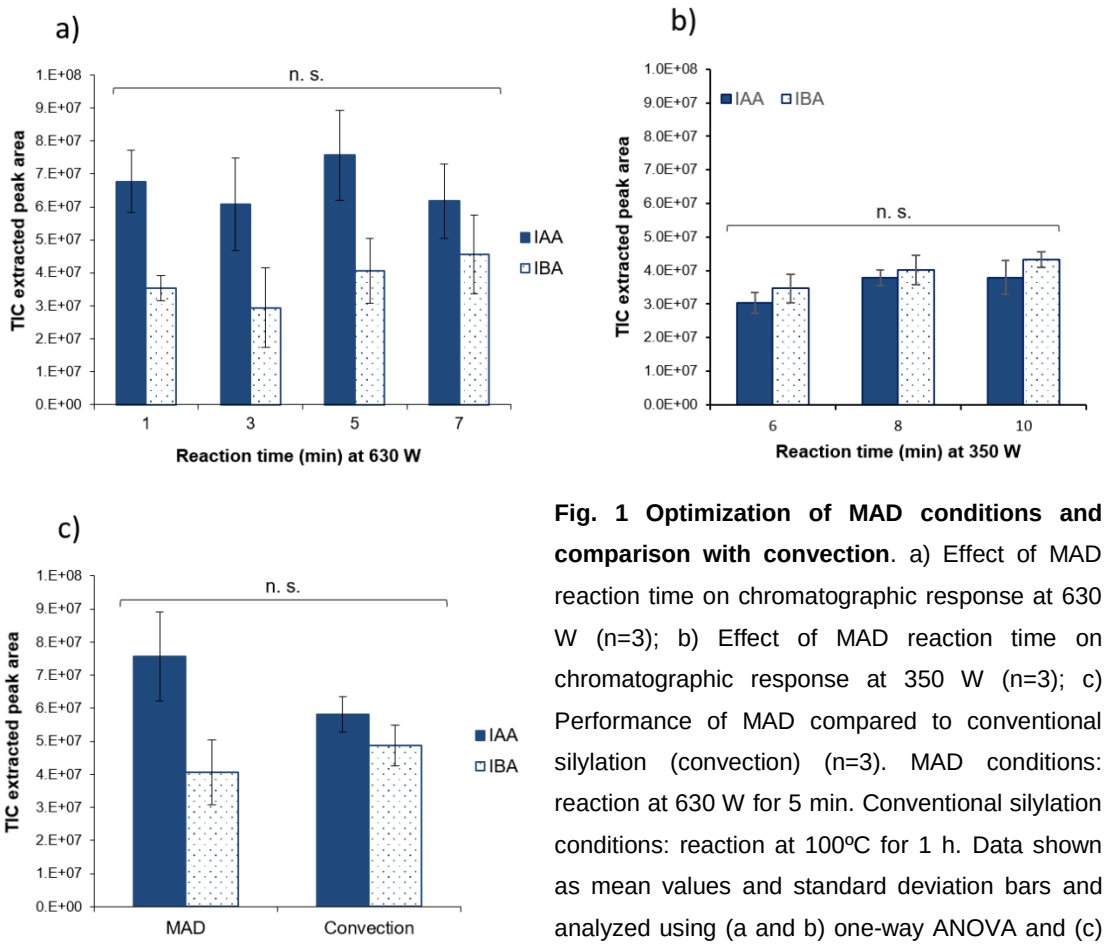

Fig. 1 Optimization of MAD conditions and comparison with convection. a) Effect of MAD reaction time on chromatographic response at 630 W (n=3); b) Effect of MAD reaction time on chromatographic response at 350 W (n=3); c) Performance of MAD compared to conventional silylation (convection) (n=3). MAD conditions: reaction at 630 W for 5 min. Conventional silylation conditions: reaction at 100ºC for 1 h. Data shown as mean values and standard deviation bars and analyzed using (a and b) one-way ANOVA and (c) Student's *t*-test. Statistical analysis of the data corresponding to each analyte was performed separately n.s. = Non significant differences at 95% confidence interval

3.3. Pre-treatment conditions

Although many types of solvents have been reported in the literature, methanol is the most commonly used solvent for the extraction of phytohormones[7,15]. Some reports can be found that discourage the use of primary alcohols like methanol as extraction solvents as they can form esters with IAA[16], resulting in artifacts. However, such esterification-derived compounds were never found in the chromatograms of the analyzed standards of IAA and IBA in the studied concentration ranges. Thus methanol was used as extraction solvent (see Experimental for details).

3.4. Optimization of DLLME

3.4.1. Effect of pH and ionic strength

Recovery of analytes from the aqueous phase can be severely influenced by pH and salt concentration. Generally, in DLLME, molecules should be in their neutral form to enhance extraction from the aqueous layer by the extractant. Indeed, adjusting the pH near the analyte's pK_a (≈ 4.8) promotes the neutral species and ultimately improves extraction yield. Also, an increase in ionic strength leads to a decrease in the solubility of analytes in the aqueous sample, improving the extraction. Using as a starting point the solvents and respective volumes optimized by Lu *et al*.[11], the optimal pH and ionic strength conditions were determined in a 2 factorial experimental design. In this experiment, fifty microliters of chloroform ($CHCl_3$) were used as extractant and 1 mL of acetone was used as disperser in all cases. Three pH levels were tested: 2, 4 and 6, the latter representing a control where no pH adjustments were done. Simultaneously, three sodium chloride (NaCl) concentrations were investigated: 0, 7.5 and 15% (w/v) NaCl. Results are shown in **Fig. 2a**.

While the control showed very poor extraction efficiency, significant improvements were achieved when the pH was adjusted to acidic conditions. However no significant differences were observed between pH 2 and pH 4, as could be predicted by the pK_a values for IAA (4.75) and IBA (4.80) and as previously reported by other authors while optimizing DLLME for the extraction of non-steroidal non-inflammatory drugs from water samples[31].

Further improvements were achieved by increasing salt concentration (**Fig. 2a**). Even though there were no significant differences between 7.5 and 15% NaCl, the highest chromatographic response for both auxins was attained with the combination of pH 4 and 15% (w/v) NaCl. Moreover, this method was developed for the quantification of auxins in plant samples that were treated with very high concentrations of IBA, therefore the probability of detecting IBA in real samples, even with lower recovery rates, is much higher than to detect IAA, which is present in endogenous amounts in samples. Therefore, our choice was based on the conditions that favored IAA extraction. For that reason, optimum conditions for extraction were determined to be pH 4 and 15% (w/v) NaCl with recovery rates of 99 ± 1% and 115 ± 1% for IAA and IBA, respectively.

3.4.2. Extraction solvent and disperser solvent

The next step in DLLME optimization was the selection of extraction and dispersion solvents. The proper choice of this pair of solvents has a major contribution to the success of DLLME, and both solvents must meet specific criteria[9,31]. Using the pH and salt conditions optimized in the previous experiment, several volumes of $CHCl_3$ (50 – 200 µL) and acetone (400 – 1000 µL) were tested using a 2 factorial experimental design. As shown in **Fig. 2b**, optimal volumes of $CHCl_3$ (extractant) and acetone (disperser) were determined as 200 µL and 1 mL, respectively.

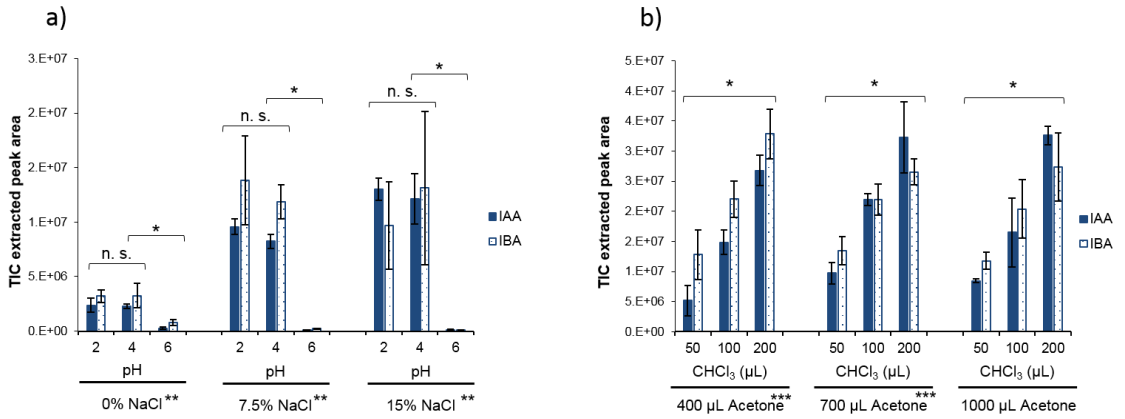

Fig. 2 Optimization of DLLME conditions. a) Effect of DLLME's pH and ionic strength on chromatographic response (n=3). Other DLLME conditions: 3 mL sample, 50 µL $CHCl_3$, 1000 µL acetone; b) Effect of DLLME's solvent volume on chromatographic response (n=3). Other DLLME conditions: 3 mL sample, pH 4, NaCl 15% (w/v).

Blanks spiked with [10 µg/mL] of standards were used. Mean peak areas correspond to extracted total ion chromatogram (TIC). Data analyzed using two-way ANOVA and shown as mean values and standard deviation bars. Independent factors used for two-way ANOVA are NaCl and pH (panel a) and chloroform and acetone volumes (panel b).

n.s. = Non significant differences at 95% confidence interval

* Significant differences at 95% confidence interval

** Significant differences at 95% confidence interval were found among all concentrations of NaCl

*** Significant differences at 95% confidence interval were found between these volumes of acetone

3.4.3. Effect of re-extraction, ultrasounds and vortex-assisted extraction

Since the introduction of the original DLLME technique, in 2006[9], many improvements have been added including the expansion of extraction and dispersion solvents

together with the development of several techniques to assist dispersion, as already reviewed[32,33]. Vortex-assisted and ultrasound-assisted extractions are among the most widely used techniques[34] by promoting the formation of a homogenous emulsion. In addition, two-step DLLME (ie. re-extraction after DLLME) has also been reported to significantly enhance recovery of analytes[31]. Our experiments tested three additional steps in the optimization process: vortex-assisted emulsification, ultrasound-assisted emulsification and re-extraction. Re-extraction did not produce any results since, at the salt concentration used (15% NaCl), an upper organic layer was observed. The collection of this upper layer was very difficult and, in all attempts, a significant volume of the residual aqueous phase was also collected. Nevertheless, to assure that single extraction was not associated with analyte loss and that the best conditions had been chosen, a calibration curve was performed using double extraction at 7.5% NaCl for comparison. Indeed, the slope of the curve obtained under these conditions was lower than the equivalent slope at 15% NaCl, confirming the higher efficiency of this salt concentration (data not shown). When comparing vortex-assisted and ultrasounds-assisted extraction, the latter was considered superior (**Fig. S2 in ESI**).

3.5. Chromatographic analysis and quantification

Upon developing an analytical GC/MS strategy, incomplete derivatization[35] and artifacts deriving from the silylating reagents[36] must be taken into account. Once the possible artifacts are identified by GC/MS in the full scan mode, accurate data interpretation can be done using selected ion monitoring (SIM) analysis and isotopically-labeled analytes as IS. SIM scans will only show the ions of interest and the mass difference between the isotopically-labeled and naturally occurring analytes will allow for ion extraction and accurate quantification of the reference compound. Nevertheless, it should be noted that the referred mass difference between analyte and IS must be enough to avoid isotopic interference[37]. In the developed method, quantitation was accomplished using [$^{13}C_6$]IAA as IS for IAA, which has a 6-unit mass difference in relation to IAA (**Fig. S3 in ESI**). Here IBA was intentionally added to the samples in very high concentrations in order to evaluate subsequent formation of IAA. Therefore, IBA was only semi-quantitated, since a non-labeled compound (indole-3-propionic acid (IPA)) was used as IS for IBA quantitation by GC/MS. Structural similarities between IPA and IBA guarantee a similar behavior during extraction, yet the mass difference between the two compounds allows good chromatographic separation (**Fig. S3 in ESI**).

3.6. Method validation

The proposed DLLME-MAD method was developed for the quantification of IAA in samples treated with abnormally high concentrations of IBA. Because IBA levels are far above endogenous amounts, a semi-quantification strategy was used to evaluate the evolution of IBA levels between time-points in rooting studies. On the other hand, IAA levels in the samples are in the same order of magnitude as endogenous levels. Therefore, IAA quantification was accurately accomplished using an isotopically-labeled IS. In reality two separate calibration curves were prepared and used for quantification. The calibration curve used for IAA quantification was constructed using 100 ng mL^{-1} of [$^{13}C_6$]IAA as IS and had linear ranges of 0.5 – 500 ng mL^{-1}. The calibration curve used for IBA semi-quantification was constructed using 100 µg mL^{-1} of IPA as IS and had linear ranges of 1 – 500 µg mL^{-1}.

Results of method validation are summarized in **Table 1** and the detailed procedure is described in the Experimental section.

Table 1 Linearity, Recovery, and Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed DLLME-MAD method.

Analyte	Regression eq.	Linear range	r^2	Recovery (%)	LOD* (ng mL^{-1})	LOQ* (ng mL^{-1})
IAA	y = 1,6105(±0.0349)x + 0,0709(±0.0704)**	0.5 – 500 ng mL^{-1}	0.997	99 ± 1	0.05	0.25
IBA	y = 0,7953(±0.0306)x + 0,0078(±0.0736)**	1 – 500 µg mL^{-1}	0.990	115 ± 1	0.05	0.25

* Determined by serial dilution of standard solutions analyzed after DLLME-MAD (see Methods for details);

** Equations shown as y = slope(±error)x + intercept(±error)

3.7. Sample analysis

To prove the reliability of the developed method, we proceeded to analyze samples of semi-hardwood cuttings and microcuttings of olive (*Olea europaea* L.). Samples included cuttings with different responses to IBA treatments: 'Cobrançosa' is considered an easy-to-root cultivar, while 'Galega vulgar' is described as a difficult-to-

root cultivar. Samples were subjected to the optimized DLLME-MAD conditions, adding 10 µL of [$^{13}C_6$]IAA working solution (100 ng mL^{-1} in the sample) and 10 µL of IPA working solution (100 µg mL^{-1} in the sample) to each sample for quantitation purposes. **Fig. 3** shows sample SIM chromatograms simplified with ion extraction for the fragment at m/z 202 shared by the di-silylated form of IAA, IBA and IPA. Results of sample quantification are shown in **Table 2**. Quantification was performed by ion extraction of SIM chromatograms, while peak identification was accomplished through TIC chromatogram analysis, after full scan mode GC/MS (**Fig. S4 in ESI**).

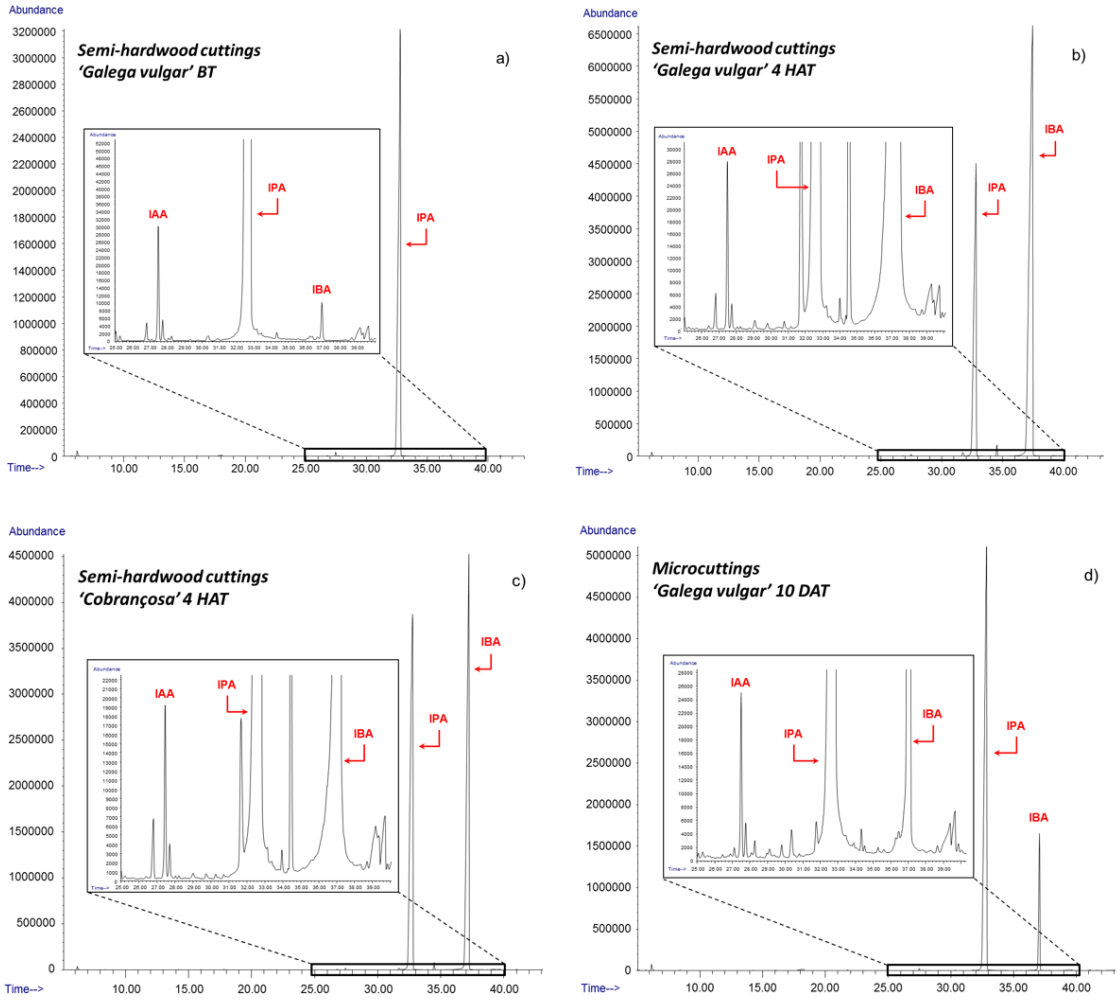

Fig. 3 SIM chromatograms (m/z 202) of *Olea europaea* (L.) samples. a) 'Galega vulgar' semi-hardwood cuttings before treatment (BT); b) 'Galega vulgar' semi-hardwood cuttings 4 hours after treatment (HAT); c) 'Cobrançosa' semi-hardwood cuttings 4 hours after treatment; d) 'Galega vulgar' microcuttings 10 days after treatment (DAT).

Table 2 Quantification of IAA and IBA in olive cuttings and comparison with values found in literature also for olive samples.

Sample	Sample dry weight (DW) (mg)	Estimated concentration (µg mL^{-1})		Concentration in sample (µg g^{-1} DW ± 1SD)	
		IAA	IBA	IAA	IBA
Semi-hardwood cuttings					
'Galega vulgar' BT[a]	104.3	0.357	Below calibration limits	0.342 ± 0.084	n.q.[d]
'Galega vulgar' 4 HAT[b]	100.6	0.214	265.5	0.212 ± 0.084	263.94 ± 0.15
'Cobrançosa' 4 HAT[b]	100.8	0.171	123.8	0.170 ± 0.084	122.86 ± 0.15
Microshoots					
'Galega vulgar' 10 DAT[c]	97.0	0.127	19.4	0.131 ± 0.084	19.97 ± 0.15
Semi-hardwood 'Koroneiki' [38]	-	-	-	0.082 ± 0.030[e]	-
Semi-hardwood unknown cultivar[39]	-	-	-	108.7 ± 14.7	n.d.

[a] BT = before treatment; [b] HAT = hours after treatment; [c] DAT = days after treatment; [d] n.q. = not quantified; [e] Mean ± S.E.

Our results show that the developed method is a simple, accessible and reliable form of routine quantification of free auxin levels in olive samples subjected to root-inducing treatments, a common practice in studies on adventitious root formation. The process of adventitious root formation is poorly understood and the exact reason behind the low rooting ability of some genotypes is still an unanswered question, although it has been widely related with auxin levels in the cuttings[5]. For this reason, comparative studies

including easy- and difficult-to-root cultivars are very important to understand the biochemical differences that may explain different rooting behaviors. These are especially important for olive, one the most important crops in the Mediterranean basin, as some of the most economically relevant olive cultivars are recalcitrant to rooting. Given the large number of samples resulting from such studies, a simple method which requires no specialized laboratory equipment and that can be used routinely is extremely important.

Furthermore, we demonstrate that the method is applicable to very different types of plant tissues, being useful both in studies using semi-hardwood cuttings and using more juvenile microcuttings. This is a particularly important advantage, as the tissues differ considerably regarding their physical properties. As shown in **Table 3**, most published methods for auxin quantification use *Arabidopsis* or other herbaceous tissues[16–18]. However, semi-hardwood cuttings represent a more complex matrix, as a result of their higher lignin and phenolic content which hinder the analysis. Hence few methods have been applied to semi-hardwood olive cuttings[39]. While it was our goal to compare the analytical performance of this method with others reported in the literature in terms of LOD and/or LOQ, in many cases such parameters are not described. For this reason, we compare the techniques used in methods described elsewhere and the amounts of auxins determined in such cases (**Table 3**). Thus, to the best of our knowledge, this is the only method that has been applied simultaneously to microcuttings and semi-hardwood olive cuttings, and is the first successful application of DLLME-MAD auxin extraction to plant tissues.

Table 3 Comparison of DLLME-MAD with other methods found in the literature.

Sample	Purification	Derivatization	Quantification	Analyte	Detected amounts	Ref.
Arabidopsis thaliana	SPE	Methylation with diazomethane	GC/SRM-MS	IAA	5.87 ± 1.23 ng g^{-1} FW	14*
				IBA	1.05 ± 0.15 ng g^{-1} FW	
Arabidopsis thaliana	SPE	Methylation with diazomethane	GC/SRM-MS	IAA	7.4 ng g^{-1} FW	16*
Arabidopsis thaliana	SPE	Methylation with diazomethane	GC/MS-SIM	IAA	5.2 ± 0.5 ng g^{-1} FW	40*
Arabidopsis thaliana	VPE	Silylation with BSTFA	GC/MS-SIM	IAA	10 ± 0.5 ng g^{-1} FW	41*
Viola baoshanensis	SPME	-	HPLC-UV	IAA	1.47 µg g^{-1}	42*
				IBA	0.65 µg g^{-1}	
Chlorella vulgaris	DLLME	-	HPLC-FLD	IAA	37.0 ng g^{-1} FW	11*
				IBA	n.d.	
Olea europaea	DLLME	Silylation with BSTFA (MAD)	GC/MS-SIM	IAA	130.85 – 342.23 ng g^{-1} DW	Current work
				IBA	19.97 – 263.94 µg g^{-1} DW	

FW = fresh weight; SPE = solid-phase extraction; VPE = vapor-phase extraction; SPME = solid-phase microextraction; * LOD and LOQ not reported in these references

4. Conclusion

Plant hormone quantification is a crucial component of agronomical studies. Classic extraction techniques such as solid-phase extraction (SPE) have been widely used in the extraction and purification of plant hormones[14,18], although entailing disadvantages

like consumption of large volumes of organic solvents, costly consumables, and multi-step purification procedures associated with sample losses[33]. To avoid these problems, a DLLME-MAD method was developed for the quantification of IAA and IBA in olive samples. DLLME, like other microextraction techniques, can solve most of the abovementioned issues by substantially reducing the volumes of hazardous organic solvents and the length of the overall protocol. In addition, fewer steps are involved in the process which minimizes sample loss. Moreover, the overall cost of operation is also reduced due to the lack of non-reusable consumables. The proposed method is applicable under the optimized conditions: ultrasounds-assisted DLLME using 200 µL $CHCl_3$ and 1 mL acetone at pH 4 and 15% NaCl followed by MAD with BSTFA for 5 min at 630 W. Determined by serial dilution, LOD and LOQ are 0.25 ng mL^{-1} and 0.5 ng mL^{-1}, respectively. However, linear ranges are above those concentrations and, according to the determined calibration curves, the actual LOQ values correspond to 0.5 ng mL^{-1} and 0.5 µg mL^{-1} for IAA and IBA, respectively. Even though there is a gap between the obtained LOQ values, they are consistent with the auxin amounts found in samples, which fall within the linear calibration ranges and are far above the LOQ. Furthermore, this method markedly reduced the derivatization time from 1 h to 5 min, a considerable advantage over conventional procedures which cannot be disregarded when dealing with large amounts of samples.

Despite the many advantages of microextraction techniques, an important advantage of classical SPE over DLLME is the possibility of automation. Indeed, DLLME automation is not currently possible although efforts are being made to turn this into a reality. Recent reports describe fully automated DLLME procedures, also called “Lab in a syringe” [43,44]. Furthermore, microextraction techniques allow performing extractions in a solvent-free manner[45], a clear advantage over classical techniques. Hopefully the improvement of these techniques will lead to simpler and more environmentally friendly analytical methods in the future.

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Supplementary material

Table S1. Ions used in auxin quantification. The suffix –tms1 and –tms2 corresponds to the mono-silylated and di-silylated derivatives, respectively.

Phytohormone	m/z (molecular ion)	m/z_1 (fragment ion)
IAA-tms1	247	130
IAA-tms2	319	202
IBA-tms1	275	130
IBA-tms2	347	202
[$^{13}C_6$]IAA-tms1	253	136
[$^{13}C_6$]IAA-tms2	325	208
IPA-tms1	261	130
IPA-tms2	333	202

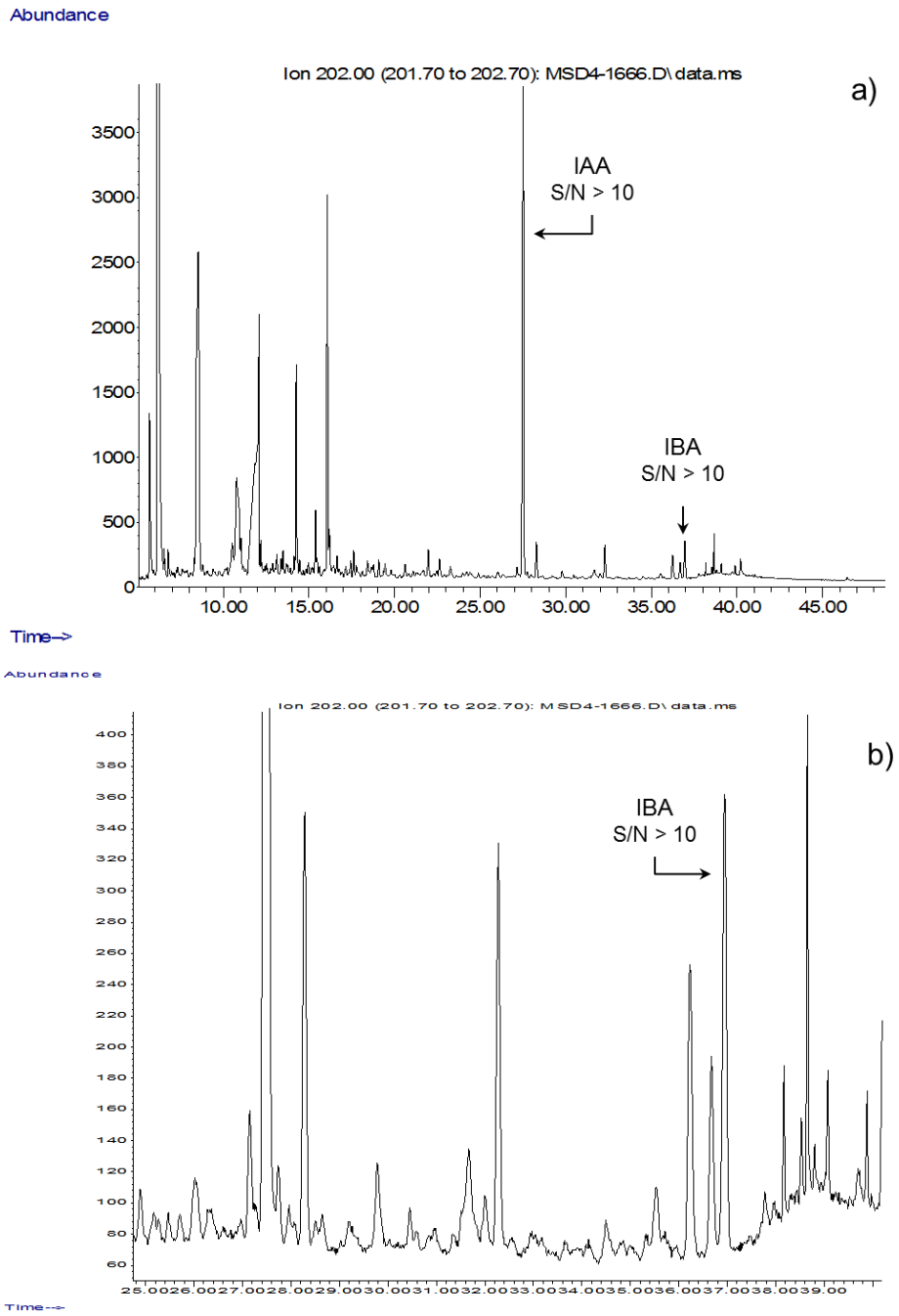

Fig. S1. SIM chromatogram (m/z 202) of a [0.25 ng/mL] standards mixture following DLLME-MAD. a) Full view of the chromatogram; b) Detail of the chromatogram shown in a) where IBA peak is visible. IAA and IBA peaks are identified as well as the respective S/N. This concentration corresponds to the LOQ, based on the S/N ratios for IAA (S/N 15) and IBA (S/N 10), determined by serial dilution of standard solutions analyzed following DLLME-MAD.

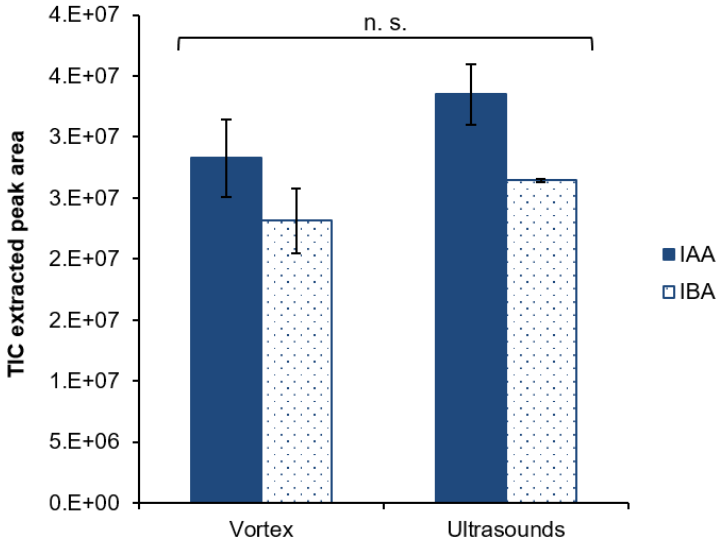

Fig. S2. Effect of vortex- and ultrasounds-assisted extraction on chromatographic response (n=3). Blanks were spiked with [10 µg/mL] of standard mixture. Other DLLME conditions: 3 mL sample, 200 µL $CHCl_3$, 1000 µL acetone, pH 4, NaCl 15% (w/v), one-step extraction. Mean peak areas correspond to extracted TIC signal. Data analyzed using Student's t-test and shown as mean values and standard deviation bars.

n.s. = Non significant differences at 95% confidence interval

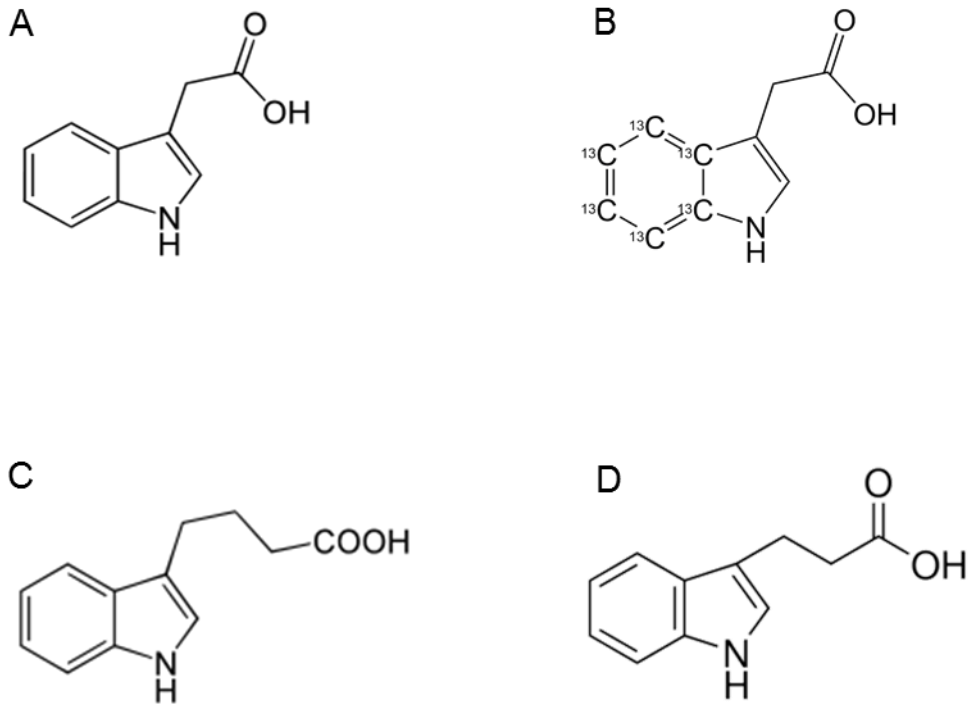

Fig. S3. Chemical structures of the analytes and their respective internal standards. (A) Indole-3-acetic acid (IAA); (B) [$^{13}C_6$]IAA; (C) Indole-3-butyric acid (IBA); (D) Indole-propionic acid (IPA)

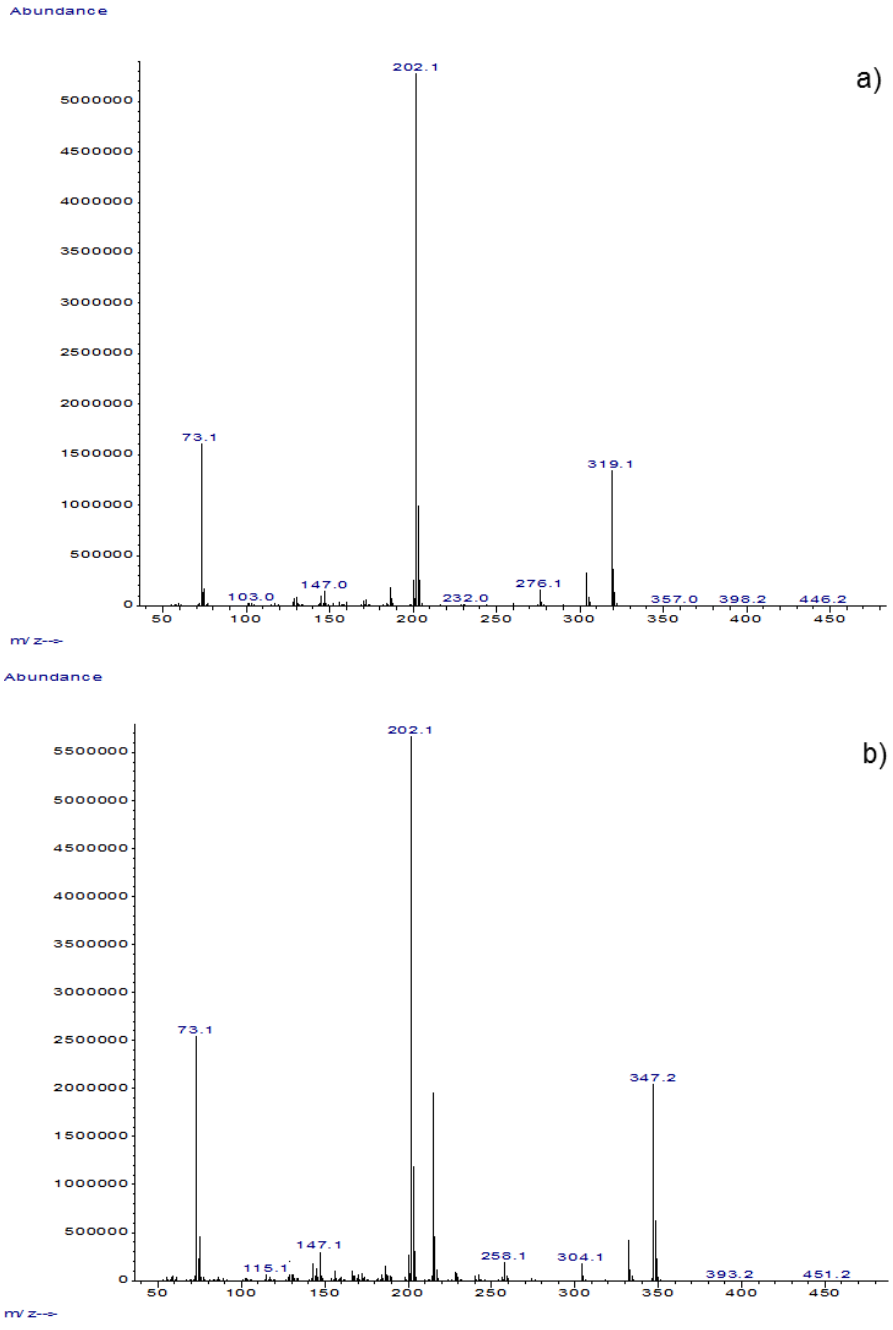

Fig. S4. Mass spectra (MS) of IAA (a) and IBA (b) peaks found in TIC chromatograms of samples.

Chapter IV

TRACKING BIOCHEMICAL CHANGES DURING ADVENTITIOUS ROOT FORMATION IN OLIVE (*Olea europaea*)

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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, 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**), which can be quantified by various methods, as recently reviewed 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).

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 (H_2O_2) 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). In *Arabidopsis*, the gene *FtSH4* was suggested to mediate auxin metabolism, transport or signaling. The *ftsh4-4* mutants showed growth and developmental deficiencies, including lower IAA levels and higher H_2O_2 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 aimed at comparing 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). [$^{13}C_6$]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'.

2.3. *In vitro* rooting experiments

Microshoots were 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.

2.4. *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.

2.5. Sample collection

During *in vitro* rooting, ten segments from the basal portion (approx. 1 cm from the base) of the explants were collected in triplicate at 4, 8, 24, 48, 96, 144, 192, 240, 336, 432, 528, 624 and 720 h after auxin treatment. In addition, ten segments were collected in triplicate before auxin treatment (0 hours after treatment) and were used as

control samples. A total of 840 explants were collected. All samples were flash frozen in liquid nitrogen and stored at –80°C for subsequent enzyme assays.

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

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

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

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

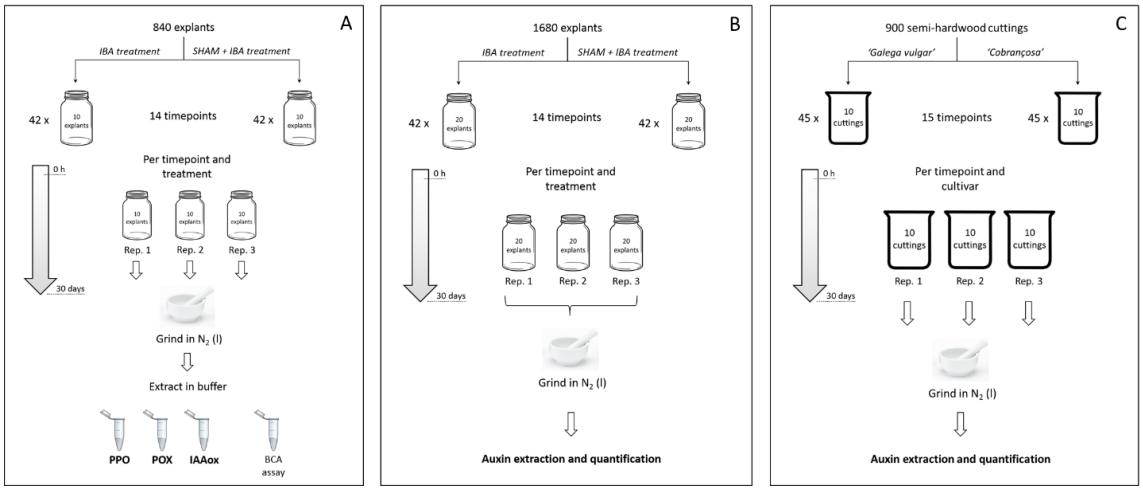

Figure 1. Schematic representation of the experimental design used for sample collection. (A) Sample collection for enzymatic activities in microshoots; (B) Sample collection for auxin quantification in microshoots; (C) Sample collection for auxin quantification in semi-hardwood cuttings.

2.6. 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 in 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.

2.7. 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.

2.7.1. PPO activity

100 µL of crude extract was added to 900 µL 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 ΔAbs490 min^{-1} protein $(mg)^{-1}$.

2.7.2. POX activity

100 µL of crude extract was added to 900 µL 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 ΔAbs490 min^{-1} protein $(mg)^{-1}$.

2.8. 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 µL of crude extract was added to 650 µL 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 µL 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).

2.8.1. 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, Muttenz, 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, Muttenz, 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, Muttenz, 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, Muttenz, Switzerland) for confirmation of IAA bands (**Supplementary Figure S1**). Plates were scanned (TLC Scanner 2, CAMAG, Muttenz, Switzerland) under monochromatic light in fluorescence mode and residual IAA was quantified by classical densitometry using CATS software version 3.20 /1998 (CAMAG, Muttenz, Switzerland). IAAox activity was expressed in terms of residual IAA (ng) protein $(mg)^{-1}$.

2.9. 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.

2.10. Auxin quantification by gas chromatography/mass spectrometry (GC/MS)

The collected material (twenty microshoot segments and ten semi-hardwood segments per replicate per time-point) was lyophilized in preparation for auxin extraction. Given the low mass of each replicate, composite samples were used for extraction and quantification of auxins from microshoot samples. Each sample was ground and homogenized in a mortar with liquid nitrogen. About 100 mg of the powdered plant tissue was transferred into a solvent-rinsed 5 mL screw-cap glass tube and extracted according to the protocol described below, using [$^{13}C_6$]IAA and IPA as internal standards for IAA and IBA, respectively. The resulting methanolic extracts were further submitted to dispersive liquid-liquid microextraction (DLLME) followed by microwave derivatization (MAD). Finally, free IAA and IBA quantification was performed by GC/MS-SIM, as described below.

Briefly, 3 mL of 80% methanol containing 1 mM BHT (stored at 4 °C before use) was added to each sample to eliminate oxidation processes, and extraction was performed by end-over-end shaking in the dark at 4°C overnight. After extraction, each tube was centrifuged (Beckman-Coulter Allegra 6R) at 3000 rpm, 4°C for 10 min with the supernatant being transferred into a solvent rinsed conical glass tube. The residual pellet was re-extracted with 1 mL of methanol for 1 h under the same conditions as described above. Subsequently, the extracts were combined, dried under a stream of nitrogen, redissolved with 420 µL of methanol and diluted with water to a final volume of 3 mL. The extract was prevented from being exposed to light at all stages of extraction. The sample were further purified by DLLME by adding 0.450 g of NaCl to the aqueous sample and adjusting the pH to 4 with 100 mM HCl. A solvent mixture containing 200 µL of chloroform ($CHCl_3$) (extractant) and 1 mL acetone (disperser) was injected into the sample via a glass syringe forming a cloudy solution. The mixture was briefly shaken manually, sonicated 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 µL 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 N_2 and, immediately after drying, the derivatized samples were dissolved with 100 µL 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 × 0.250 mm with 0.25 µm film thickness df). The injected volume was set at 2 µL 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.

2.11. 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 (**Figure 2**). Nevertheless, no visual negative traits were observed on growth and nutritional status of the microshoots treated with SHAM (**Figure 2A** and **2B**). SHAM also had no effect on calli formation (**Figure 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 manifested 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 (**Figure 2D** and **2E**).

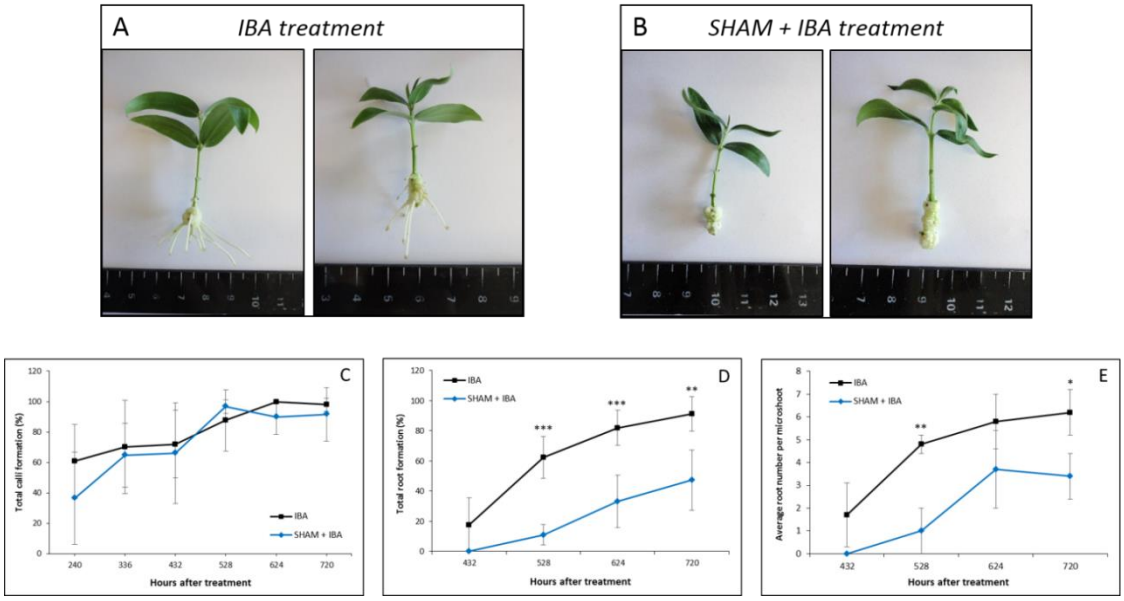

Figure 2. Inhibitory effect of SHAM on adventitious root formation in olive microshoots. (A) Microshoots collected 30 days after treatment with 14.7 mM IBA; (B) Microshoots collected 30 days after treatment with 14.7 mM IBA + 100 mM SHAM; (C) Effect of SHAM on calli formation; (D) Effect of SHAM of rooting 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 (**Figure 3**), significant differences in the activity of particular enzymes were detected between treatments (**Figure 4**).

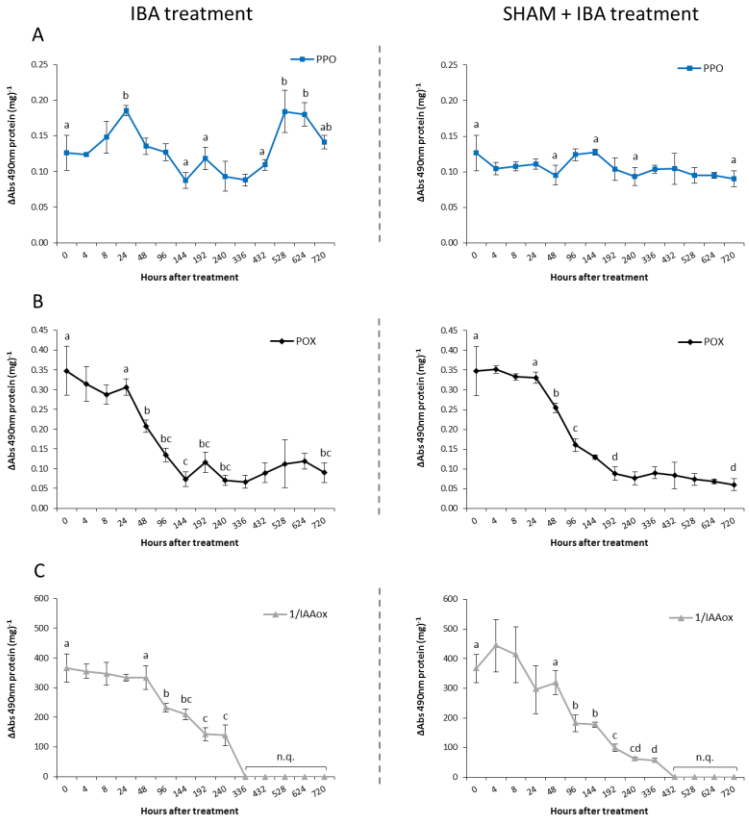

Figure 3. Effect of SHAM treatment on activity levels of oxidative enzymes during adventitious root formation in olive microshoots. Activity levels of PPO (A), POX (B) and IAAox (C) were measured on microshoots treated with IBA (left) and SHAM + IBA (right). Different lower-case letters correspond to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

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

On the other hand, microshoots treated with SHAM + IBA (**Figure 3**), showed a nearly constant PPO activity throughout root formation. In this case POX activity also

decreased significantly during root formation, at a more constant pace than in IBA treatment, and it stabilized after 192 h. IAAox activity also increased throughout adventitious rooting. Whereas such an increase was not significant until 48 h, two significant increases were observed at 96 h and 192 h. Finally, IAAox activity reached its maximum at 432h (no detectable residual IAA), exactly 96 hours later than in IBA treatment, remaining stable until the end of the trial, at 720 h.

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

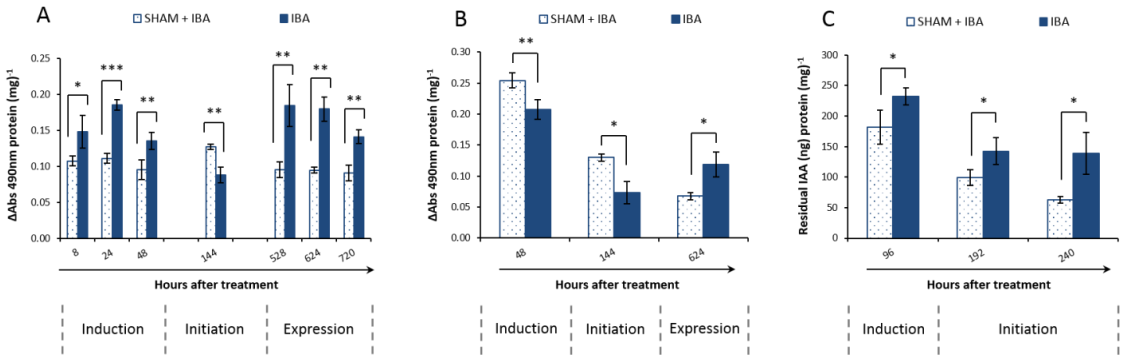

Figure 4. Effect of SHAM treatment on individual enzyme activities. (A) PPO activity, (B) POX activity, (C) IAAox activity (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). The indicated rooting stages were previously determined by Macedo et al. (2013). The timepoints shown here do not indicate the length 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 alone and with SHAM + IBA (**Figure 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 (**Figure 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 (**Figure 5B**). A similar trend was observed for IBA levels in microshoots treated with SHAM + IBA. (**Figure 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 (**Figure 5C**).

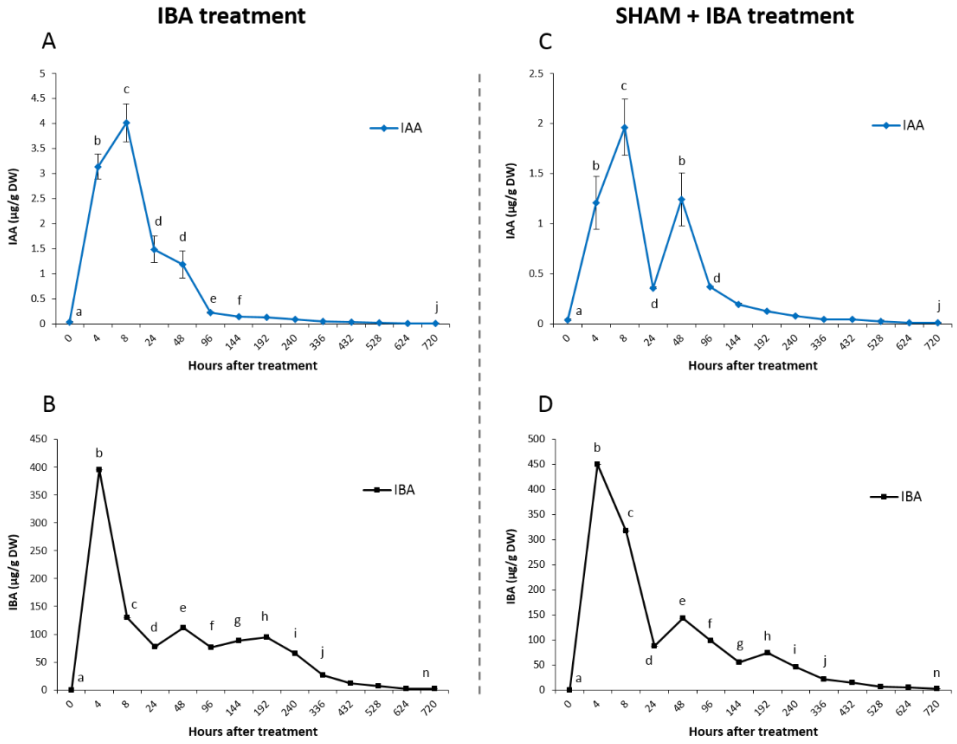

Figure 5. Changes in free auxin levels during adventitious root formation in olive microshoots treated with IBA (left) and with SHAM + IBA (right). (A) IAA levels in microshoots treated with IBA; (B) IBA levels in microshoots treated with IBA; (C) IAA levels in microshoots treated with SHAM + IBA; (D) 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 (**Figure 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 Figure 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 (**Figure 6A**). Actually, at 96 and 144 h IAA levels were significantly higher in SHAM + IBA microshoots (inset in **Figure 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 8h (317 ± 6 µg/g compared with 130 ± 1 µg/g). From 144 - 240 h this trend was reversed and from 336 to 720 h IBA levels decreased progressively to a minimum in both treatments (**Figure 6B**).

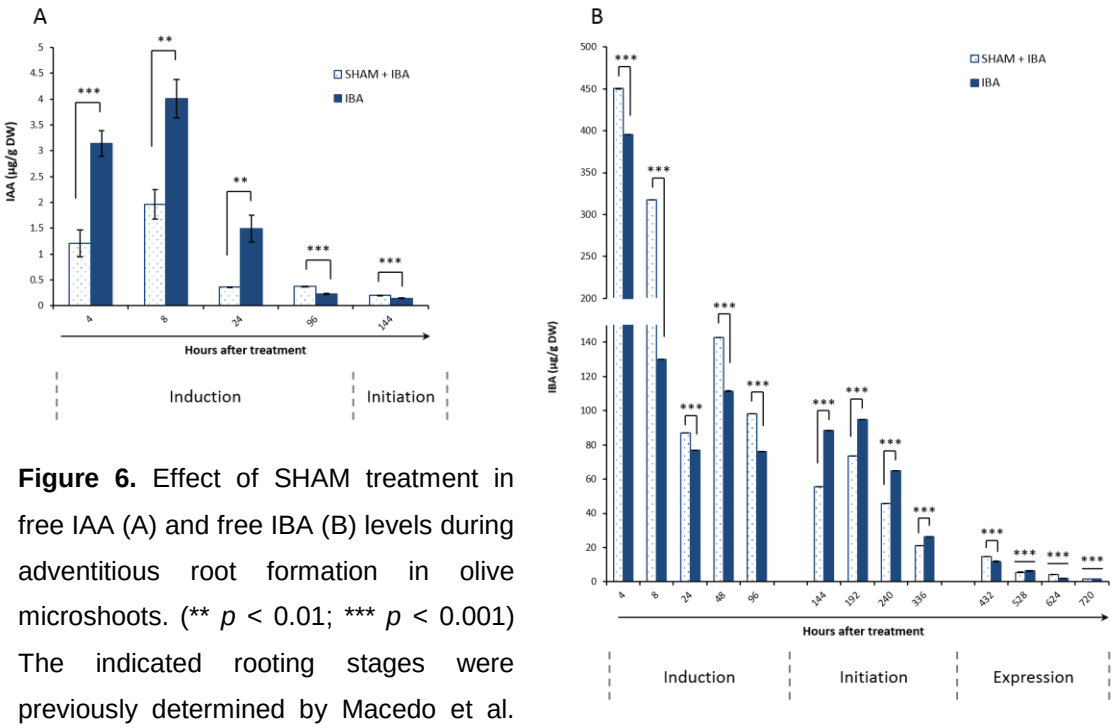

Figure 6. Effect of SHAM treatment in free IAA (A) and free IBA (B) levels during adventitious root formation in olive microshoots. (** $p < 0.01$; *** $p < 0.001$) The indicated rooting stages were previously determined by Macedo et al. (2013). The timepoints shown here do not indicate the length of each phase.

3.3.2. Free auxin levels in semi-hardwood cuttings

Changes in free IAA and IBA levels were evaluated over the rooting period (**Figure 7** and **Figure 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, 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 (**Figure 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 (**Figure 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 (**Figure 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 (**Figure 7D**).

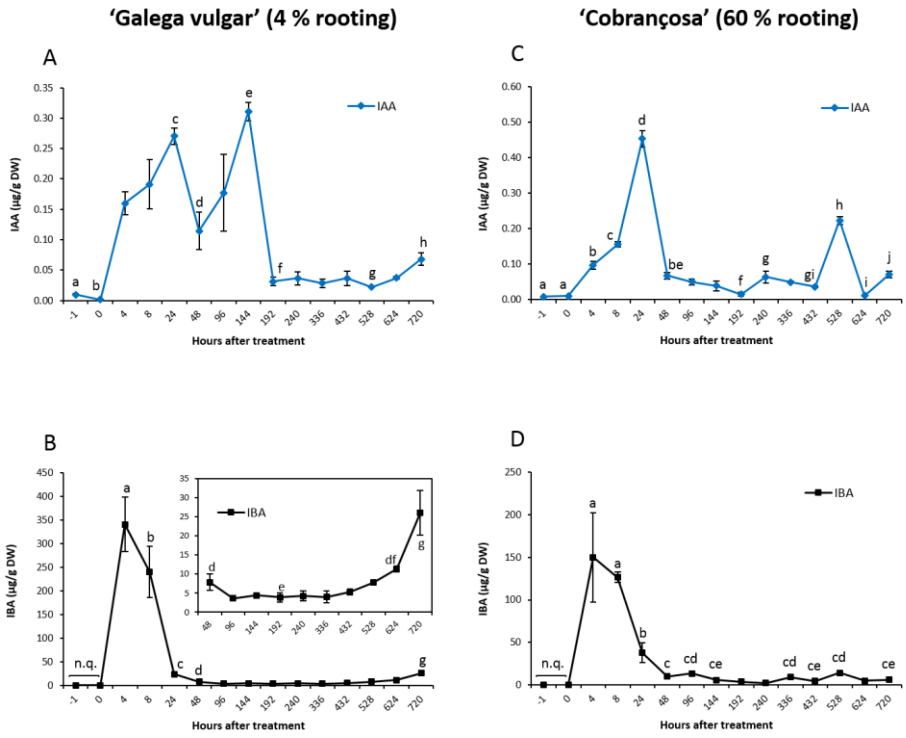

Figure 7. Changes in free IAA and IBA levels during rooting of semi-hardwood cuttings. (A) IAA levels of 'Galega Vulgar' cuttings; (B) IBA levels of 'Galega Vulgar' cuttings; (C) IAA levels of 'Cobrançosa' cuttings; (D) IBA levels of 'Cobrançosa' cuttings. Different lower-case letters correspond to statistically 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 (**Figure 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 (**Figure 8B**).

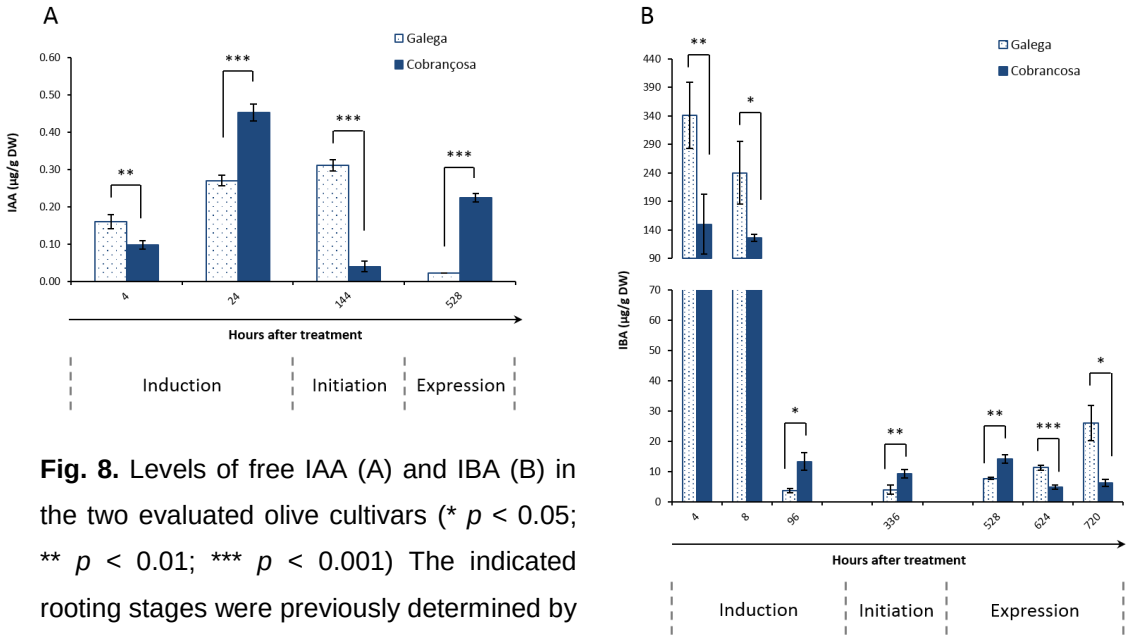

Fig. 8. Levels of free IAA (A) and IBA (B) in the two evaluated olive cultivars (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) The indicated rooting stages were previously determined by Macedo et al. (2013). The timepoints shown here do not indicate the length of each phase.

4. Discussion

4.1. Rooting performance as affected by treatments and cultivars

Confirming previous results on this subject (Santos Macedo et al., 2009, 2012), the treatment of olive microshoots with SHAM significantly reduced the rooting percentage and the average number of roots per microshoot, therefore inhibiting the formation of adventitious roots. Also in agreement with previous results obtained in olive (Santos Macedo et al., 2009, 2012), the inhibitory effects of SHAM did not affect calli percentage because in both treatments, calli formation always preceded root development at the site of treatment. These results suggest the inhibitory effect of SHAM is likely related with the later stages of adventitious root induction rather than with cell dedifferentiation. SHAM has been suggested to suppress adventitious rooting by inhibiting AOX activity (Santos Macedo et al., 2009, 2012), which could lead to an increased production of reactive oxygen species (ROS), as documented for tobacco, soybean and pea (Maxwell et al., 1999; Popov et al., 1997; Van Aken et al., 2009).

However the exact action of SHAM in adventitious rooting is not well understood and other molecular mechanisms may be involved in the process, as discussed below.

4.2. Temporal changes in oxidative enzymes activity

Monophenols, such as SHAM, have been reported to stimulate the enzymatic degradation of IAA by IAAox (Grambow and Langenbeck-Schwich, 1983; Lee, 1980) and this effect is dependent on the type of monophenol. *p*-substituted monophenols (as the case of *p*-coumaric acid, used here in the determination of IAAox activity) are described to be more active in IAAox 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 Fe^{3+}-IAAox isoform during IAA degradation (Pedreño et al., 1990). Therefore, *m*-monophenols like SHAM could 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, H_2O_2-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 activity to control IAA levels and facilitate the development of new adventitious roots by stimulating lignin formation and cross-linking of cell wall components (Passardi et al., 2004, 2005). This would also explain why IBA-treated explants have higher POX activity during expression phase, since more roots are produced in response to this treatment, in agreement with Tonon et al. (2001). A decrease in POX activity during root formation, also described by Tartoura et al. (2004) and Fekete et al. (2002), could be a result of the root-inducing treatment itself. IBA treatments significantly decreased POX activity in mung bean seedlings (Li et al., 2009b) and naphtaleneacetic acid (NAA) has been described to have a suppressive effect on POX gene expression in soybean hypocotyls (Chen et al., 2002). This suppressive effect could be a result of auxin responsive elements that are regulated by exogenously applied auxins, as suggested by results from *Cinnamomum kanehirae* (Cho et al., 2011).

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

The biggest changes in enzyme activity were observed in PPO activity. While in SHAM+IBA-treated microshoots no major changes were detected during root formation, in IBA-treated explants PPO activity significantly increased during induction, decreased during initiation and increased again during expression. These results are in agreement with results from Qaddoury and Amssa (2003), Satisha et al. (2008) and

Cheniany et al. (2010), who observed a larger magnitude of changes in PPO activity in easy-to-root cultivars of *Phoenix dactylifera*, *Vitis* sp. and *Juglans regia*. Sharp increases and higher PPO activity have also been related with enhanced rooting in other species (*Bruguiera parviflora*, *Cynometra iripa*, *Excoecaria agallocha*, *Heritiera fomes*, *Thespesia populnea*, *Eucalyptus urophylla*) (Basak et al., 2000; Li et al., 1999). The increased PPO activity during rooting, previously described in olive (Macedo et al., 2013), could be associated with lignification processes and/or phenolic metabolism (Batish et al., 2008), or could be related to H_2O_2 levels. H_2O_2 has been suggested to work as signaling molecule, acting downstream in the auxin signaling pathway, mediating auxin responses prior to adventitious rooting in cucumber (Li et al., 2007, 2009a). Li et al. (2009c) reported an increase in endogenous H_2O_2 levels in mung bean seedlings after IBA treatment and removal of the primary root, suggesting that IBA may induce rooting indirectly through a pathway involving H_2O_2. Further evidence showed that H_2O_2 treatments, which enhanced adventitious rooting, stimulated PPO activity in *Chrysanthemum* (Liao et al., 2010), possibly through activation of AOX (Santos Macedo et al., 2009). IBA treatments promote AOX gene transcription (Santos Macedo et al., 2012), which in turn can stimulate phenylpropanoid biosynthesis (Sircar et al., 2012; Vogt, 2010) leading to an increased concentration of monophenolic compounds which are natural substrates of PPO. This would also explain why in microshoots treated with SHAM, an AOX inhibitor, no visible changes in PPO were detected. Alternatively, auxin could promote the apoplastic production of ROS that increase cell wall extensibility by promoting the breakdown of polysaccharides and proteins (Schopfer et al., 2002). In 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.

It should be mentioned that, like auxin levels, changes in enzyme activities were also measured in semi-hardwood cuttings. However, considering the inherently high variability of this type of plant material, the results obtained were not conclusive and for that reason they are presented in a separate section of this work (see **Appendix 2**).

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 (**Figure 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 H_2O_2 levels which may negatively affect POX activity at substrate level, allowing phenylpropanoids to be more available for other metabolic pathways such as accumulation of monophenolics. This would facilitate PPO action and the consequent polymerization of the resulting products. Resulting polymers, such as precursors of lignin (which is necessary for cell wall synthesis and expansion) (Hiraga et al., 2001; Vanholme et al., 2010) are susceptible to be metabolized by POX enzymes, which could ultimately act on these substrates once H_2O_2 levels rebound after the initial AOX activity decreases, at the end of induction or early initiation. Moreover, IAA degradation by IAAox during this phase also generates ROS (Schopfer et al., 2002), which may stimulate the production of antioxidant phenolic compounds, also increasing PPO activity.

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

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

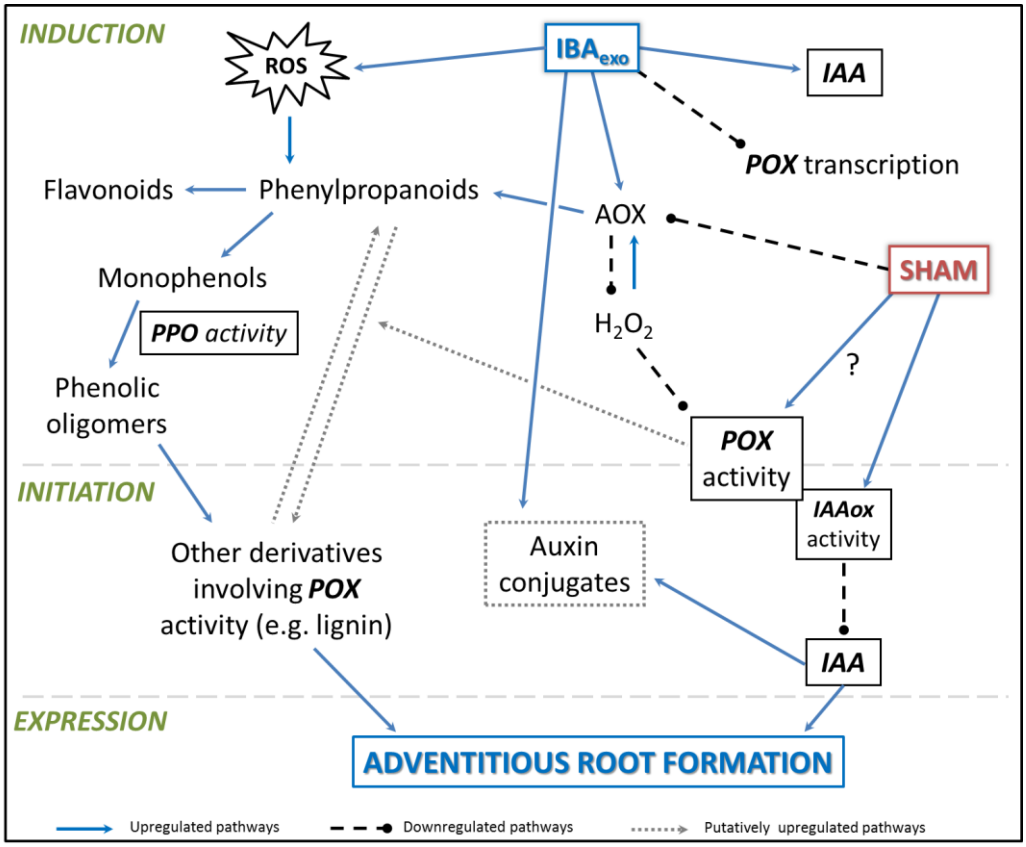

Fig. 9. Schematic representation of the proposed molecular pathways putatively involved in olive adventitious root formation.

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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Supplementary material

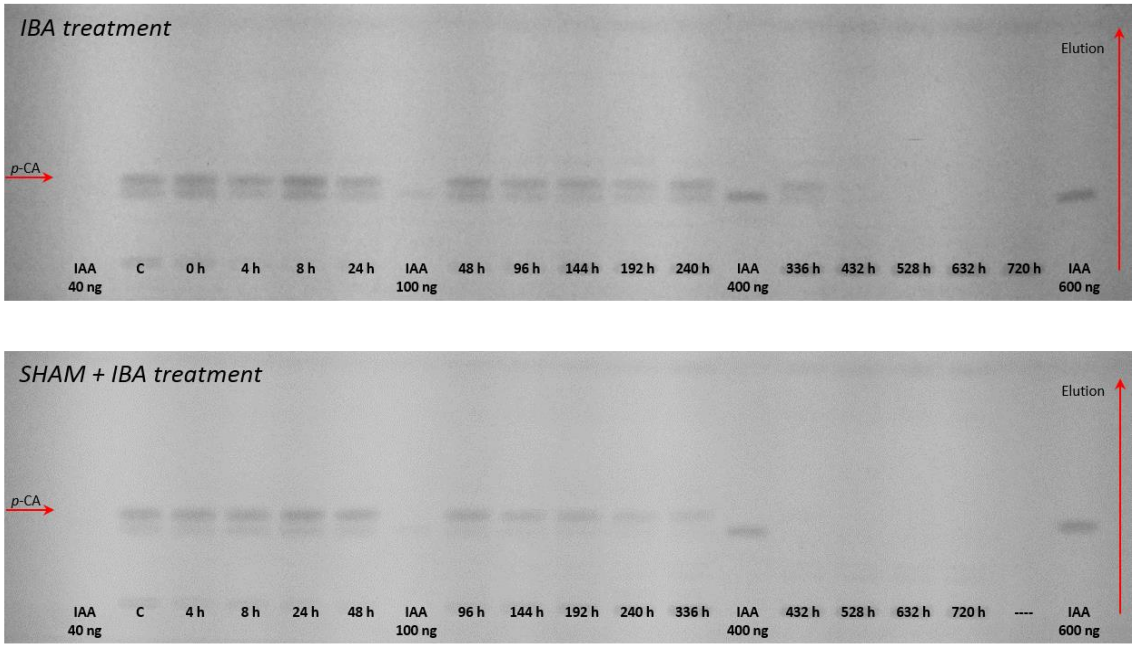


Figure S1. Representative HPTLC results for measurement of IAAox activity. Samples and IAA standard were applied at the base of the plate and eluted with *n*-butanol : isopropanol : ammonium hydroxide : water (2.5 : 10 : 1 : 1, v/v) (arrow indicates the direction of elution). A band of *p*-coumaric acid (*p*-CA) is visible in every sample lane. A control (C) corresponding to null activity, where the reaction was immediately stopped at 0 min incubation time by adding *n*-butanol : formic acid (15:1), was included in every plate for comparison. Known amounts of IAA standard were applied along with the samples to build a calibration curve in each plate, allowing for IAA quantification.

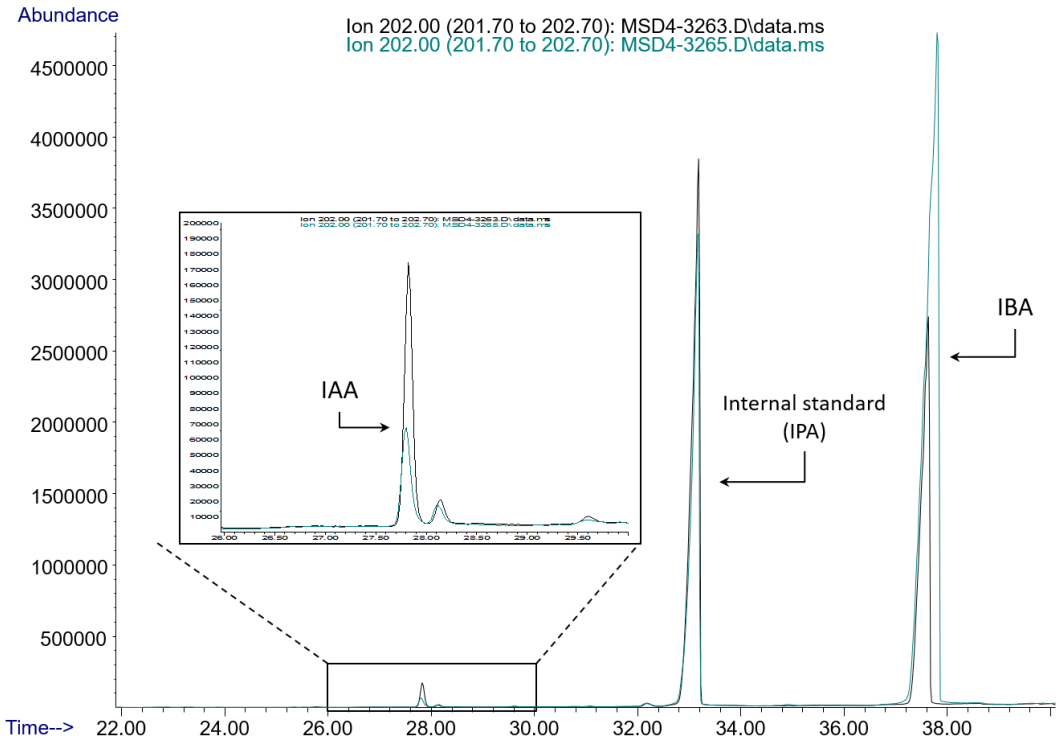


Figure S2. Overlaid SIM chromatograms of olive microshoot samples at 8 h after treatment. Black = IBA treatment; Blue = SHAM + IBA treatment. The chromatograms are extracted for the ion (m/z 202) used for IAA and IBA quantification.

CONCLUSIONS AND FUTURE WORK

Adventitious root formation is a fundamental step in the propagation of many plants, and especially olives. However, as described in **Chapter I**, the current knowledge on this subject is substantially delayed in comparison with other plant developmental processes, such as primary and lateral rooting. Most of the available information is based on trials developed with model species, like *Arabidopsis* sp. or *Tobacco* sp. In woody species, like *Olea europaea* (L.), the anatomy, biochemical background, genetic control of the process, and the action of exogenous factors able to affect it, remains mostly unknown.

In olive, although a lot of information can be found in the literature regarding the effect of several exogenous factors (also described in **Chapter I**), this information is mostly empirical, non-systematic and not consolidated. Furthermore, most studies on adventitious rooting of olive are still performed with semi-hardwood cuttings and therefore are dependent on uncontrollable factors such as environmental conditions. Moreover, the higher structural complexity of semi-hardwood cuttings results in an increased matrix effect that can interfere with the sensitivity of analytical techniques.

Despite the great variety of analytical methods available for auxin quantification (reviewed in **Chapter II**), there still isn't (and likely there will never be) a universal method that can be applied in any type of plant tissue. The complexity of the sample matrix varies with plant material and for that reason analytical methods must be adapted and/or optimized to the plant material available. Furthermore, ideally several families of plant hormones would be analyzed with the same method, allowing a complete and dynamic view of the metabolic processes occurring during adventitious rooting. Although this has been described in the literature, the lack of purification methods able to separate all types of plant hormones is associated with the need for powerful instrumentation capable of distinguishing such compounds. In this work a quantification method for free IAA and IBA was developed (described in detail in **Chapter III**). The developed method is based on DLLME-MAD and GC/MS analysis and it proved to be useful in the analysis of both microshoots and semi-hardwood cuttings. Nevertheless, it is not a perfect method and some pitfalls could be improved. The organic plant extracts resulting from DLLME still contain a lot of phenolic compounds that interfere with quantification and complicate analysis. Hence even after DLLME the plant matrix is highly complex and ultimately decreases instrument maintenance intervals. For that reason, a purification step that would produce cleaner extracts could be introduced before MAD to improve this method. Also, it would be highly desirable to quantify auxin conjugates to better understand the fate of exogenously applied IBA. Is it fully converted into IAA? Is it conjugated? Is the resulting

IAA conjugated? If so, what is it conjugated into? The answer to these questions is fundamental to comprehend the biochemical mechanisms involved in adventitious root formation in olive.

Likewise, oxidative enzymes have been widely related with adventitious rooting (discussed in **Chapter I**), however, despite all the research in this subject, the precise role of PPO, POX and IAAox is still not fully understood. One reason for this may be related with the fact that both PPO and POX are groups of enzymes with similar functions. Hence, although a lot of studies focus on the activity of these enzymes, given their broad substrate specificity each study may be evaluating a different enzyme. On the other hand, very little is known about IAAox. This common denomination has been used for a long time but only recently genes encoding this type of enzyme have been identified in *Arabidopsis* (discussed in **Chapter IV**).

The results presented here, in agreement with results observed in other species (discussed in **Chapter IV**), show that differences in the activity of some oxidative enzymes as well as differences in the endogenous concentrations of free IAA and IBA seem to be related with the rooting ability of the (micro)cuttings. Cuttings with higher PPO activity are more prone to form adventitious roots while higher levels of POX (including IAAox) activity seem to be related with a difficult-to-root behavior. In turn, a deficient IBA-IAA conversion also appears to be associated with the difficulty in forming adventitious roots and high IAA concentrations during initiation phase seem to inhibit root formation, which was observed in both microshoots and semi-hardwood cuttings (discussed in detail in **Chapter IV**).

In fact, and to the best of my knowledge, a putative hypothesis for olive adventitious root formation is presented here for the first time (**Chapter IV**). It is proposed that exogenously applied IBA promotes a decrease in POX activity, either directly or through AOX activation. In turn, IBA-activated AOX may stimulate the phenylpropanoid biosynthetic pathway, producing PPO substrates and leading to an increase in PPO activity. This results in the formation of polymers, such as lignin, that can be POX substrates and may increase its activity possibly through a positive feedback mechanism. On the other hand, the conversion of IBA into IAA putatively increases IAAox activity by increasing the concentration of its substrate (IAA). The degradation of IAA by IAAox generates ROS, which can also stimulate the production of antioxidant phenolic compounds that ultimately will become substrates for PPO activity. This scenario would explain the inhibiting effect of SHAM, an inhibitor of AOX (and a

potential POX substrate) which has also been described to stimulate IAA degradation by IAAox.

In future work, it is essential to study the metabolism of auxin conjugates during adventitious rooting of olive microshoots to determine if these metabolites play a major or rather secondary role, which can't be accomplished without the development of adequate analytical methods. It is also important to understand the exact role of SHAM in order to comprehend the mechanisms that may be inhibited during adventitious rooting of explants treated with SHAM. One of the main conclusions of this work is definitely the need to replace semi-hardwood cuttings as sampling material in research studies. It is senseless to continue using a plant material which, given its intrinsic features, hampers data analysis only because it's more accessible. The genetic homogeneity associated with *in vitro*-cultured microshoots, as well as the possibility to control all the external variables associated with adventitious rooting (humidity, light, temperature, etc.), make this plant material the most reliable option for scientific research studies.

Appendix I

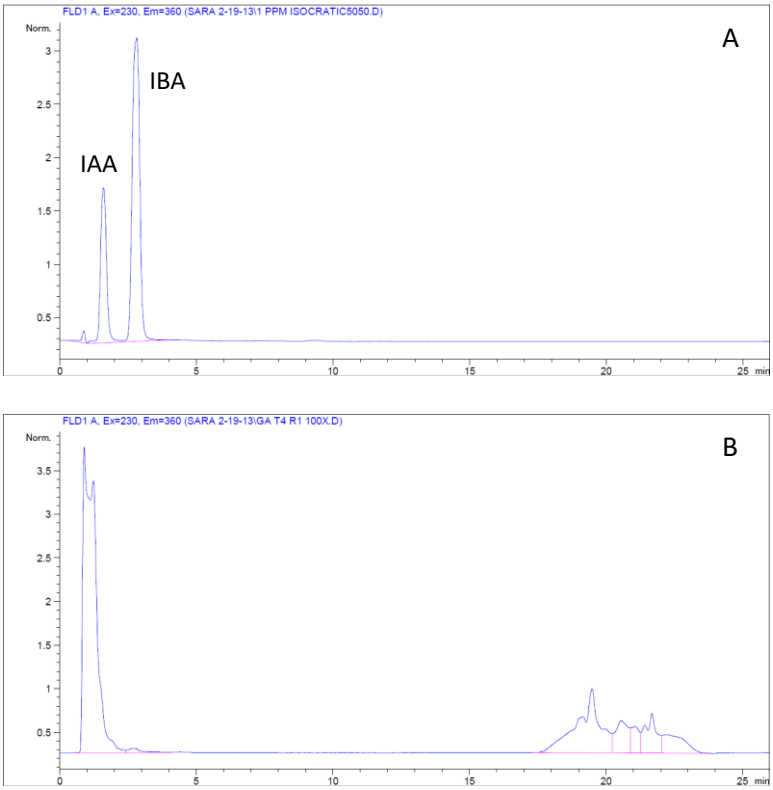
METHOD DEVELOPMENT TOWARDS ANALYTICAL SEPARATION OF AUXINS BY GC/MS

In this work, free IAA and IBA were extracted from olive samples using DLLME-MAD and quantified by GC/MS-SIM. However, prior to the development of the method described in Chapter III, several extraction/purification procedures were investigated and different analytical techniques were evaluated.

HPLC-FLD

Considering the non-volatile nature of auxins, the first approach to the subject was based on high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD, Agilent 1260 Infinity). Using the method developed by Pan *et al*. (2010) for sample preparation, olive samples were separated in a C18 reversed-phase column (Luna 5µm C18(2) 150 x 2.0 mm, Phenomenex, USA), using an adaptation of the conditions described by Nakurte *et al*. (2012): mobile phase consisting of methanol and 1% acetic acid (aq) (50:50 v v^{-1}) under isocratic conditions at a flow rate of 0.5 mL min^{-1}. Detection was monitored at 230 nm (Ex) and 360 nm (Em).

Although under these conditions peaks of pure IAA and IBA standards could be resolved (**Figure 1a**), a very poor separation was obtained for olive samples (**Figures 1b and 1c**).



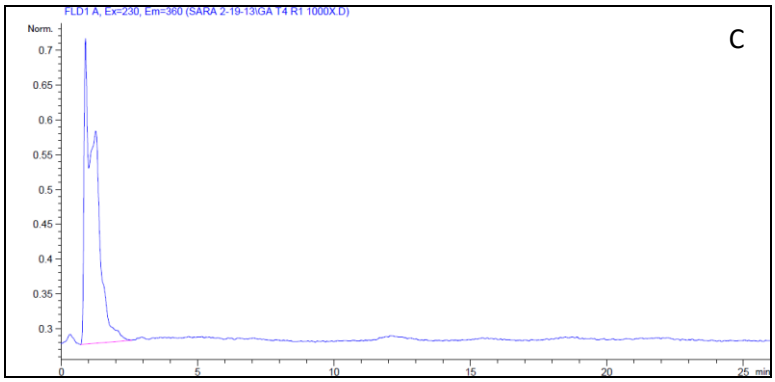


Figure 1. Auxin separation by HPLC-FLD. (A) Chromatogram of a mixture of IAA and IBA standards at 1 ppm; (B) Chromatogram of a sample of semi-hardwood cuttings of olive 'Galega vulgar' (100x diluted); (C) Chromatogram of a sample of semi-hardwood cuttings of olive 'Galega vulgar' (1000x diluted).

To overcome this problem, the elution was changed to a gradient separation (**Table 1**) adapted from Kim *et al*. (2006): the mobile phase consisted of (A) 10% methanol containing 0.3% acetic acid, (B) 90% methanol containing 0.3% acetic acid and (C) acetonitrile, at a flow rate of 0.3 mL min^{-1}.

Table 1. Initial HPLC solvent gradient used for auxin separation (adapted from Kim *et al*. 2006)

Time (min)	A (%)	B (%)	C (%)
0 - 12	50	50	0
12.2 - 23	5	0	95
23.2 - 35	50	50	0

The separation only improved mildly (**Figure 2**) and didn't improve with further adjustments of the gradient, so a different column was tested.

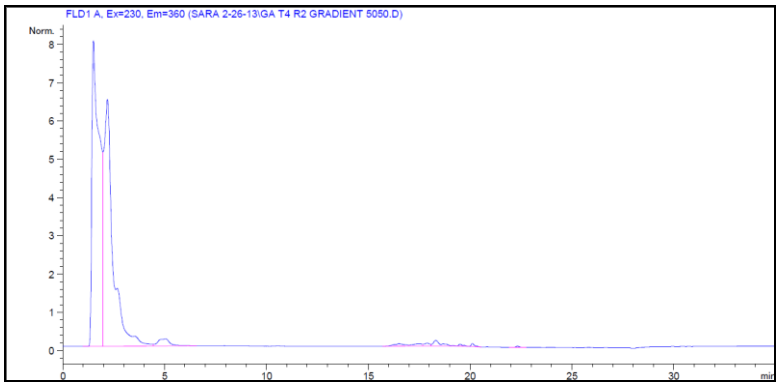


Figure 2. Chromatogram of a sample of semi-hardwood cuttings of olive 'Galega vulgar' (100x diluted) using the gradient described in Table 1

Using a column with a pentafluorophenylpropyl stationary phase (Ascentis Express F5 150 x 4.6 mm, 2.7 µm, Supelco), the mobile phase was changed to (A) 10% methanol containing 0.3% acetic acid, (B) 90% methanol containing 0.3% acetic acid and the solvent gradient was adjusted (**Table 2**), using the same wavelengths for detection. Having both polar and non-polar character, this column has a higher separation power than common C18 columns, which could be very useful for plant extracts given their high matrix complexity.

Table 2. Adjusted HPLC solvent gradient used for auxin separation

Time (min)	A (%)	B (%)
0	50	50
15	20	80
25	3	97
35	50	50

The separation was moderately improved (**Figure 3a**) and a peak potentially corresponding to IBA was found in olive samples (**Figure 3b**).

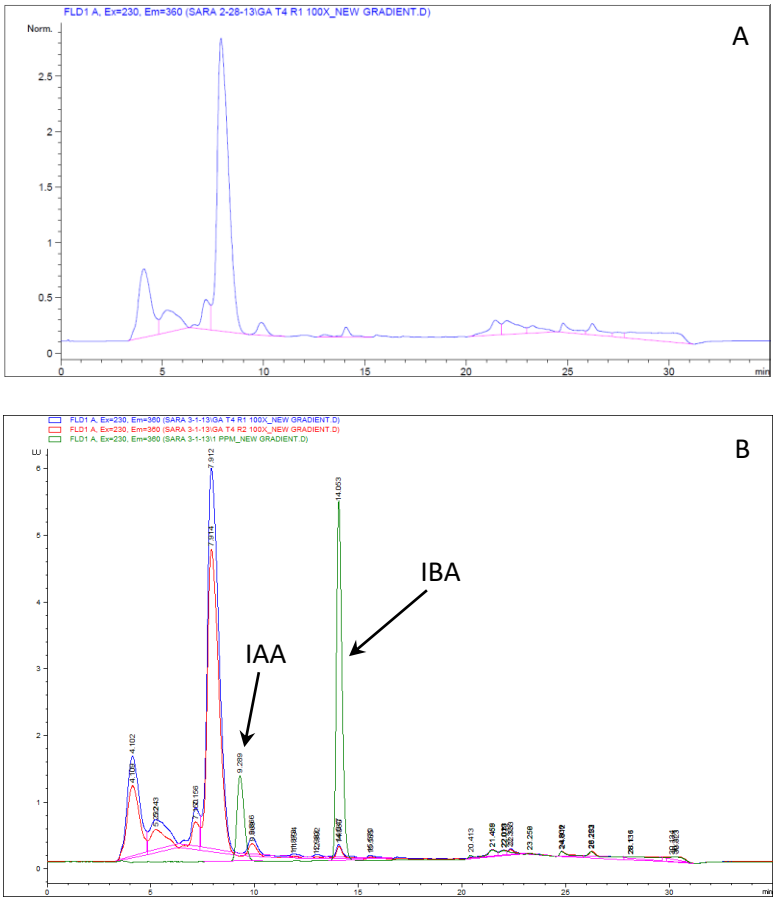


Figure 3. Chromatography results after changing the column and adjusting the solvent gradient. (A) Chromatogram of a sample of semi-hardwood cuttings of olive 'Galega vulgar' (100x diluted); (B) Overlaid chromatograms of two samples of olive semi-hardwood cuttings (blue and red) and a solution of 1 ppm auxin standards (green)

While trying to further improve separation, the gradient was changed again although unsuccessfully (data not shown). Because IAA wasn't found in olive samples, the hypothesis of the presence of methylated IAA (MeIAA) and indole-3-carboxylic acid (ICA) was considered. To test that hypothesis, chromatograms of MeIAA and ICA standards were firstly compared with chromatograms of IAA and IBA standards and it was shown that this gradient wouldn't be able to distinguish these compounds because, due to high structural similarities, their retention times were too similar and they would co-elute (**Figure 4**).

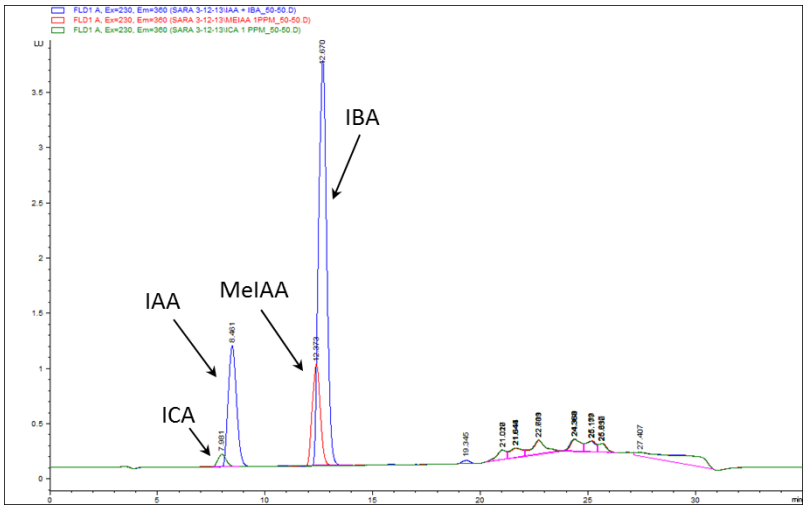


Figure 4. Overlaying chromatograms of 1 ppm ICA (green), IAA (blue, first peak), MeIAA (red) and IBA (blue, second peak).

Given this similarity, and using this gradient, HPLC-FLD wouldn't be able to distinguish these molecules if they were present in real samples. For that reason, and having access to an LC/MS/MS instrument (Thermo Finnigan LTQ MS/MS), pure IAA, IBA, ICA and MeIAA standards were analyzed by LC/MS/MS to determine the mass fragments obtained for each auxin and subsequently look for these fragments in olive samples.

Standards were separated with the same column, mobile phase and solvent gradient used for HPLC-FLD analysis (**Table 2**), at a lower flow rate (0.25 mL min^{-1}) which increased the total analysis time to 1h. Negative ion mode was used for analysis. The results encountered initially indicated that the sensitivity of the technique was lower than expected, as IAA and IBA molecular ions (m/z 174 and 202, respectively) were only observed at 200 ppm, while ICA molecular ion (m/z 160) was only found at 50 ppm. Considering the runs were performed in negative ion mode, MeIAA ions were never observed.

However, after analyzing the ions present in the chromatograms, it was considered the possibility of the presence of IAA- and IBA-acetate adducts, resulting from acetic acid in the mobile phase. Indeed, $[M\text{-}H + CH_3COOH]^-$ ions (m/z 233 and 261) with MS/MS fragments corresponding to auxin-acetate adducts (m/z 189 and 218) were found.

Having identified the MS fragments produced by auxins, a sample where IBA had been potentially identified by HPLC-FLD was analyzed under the same conditions. A

sufficiently abundant (1.88E5) ion was found at m/z 261.77, with an MS^2 fragmentation pattern that matched that of pure IBA (**Figure 5**). Therefore this peak was assigned to IBA-acetate and confirmed IBA's presence in olive samples. However, the ion corresponding to IBA was not very abundant and other major ions were found in the same peak, indicating that the putative IBA peak found in HPLC-FLD (**Figure 3**) was not pure. Furthermore, no ions corresponding to IAA, IAA-acetate or ICA were found in samples.

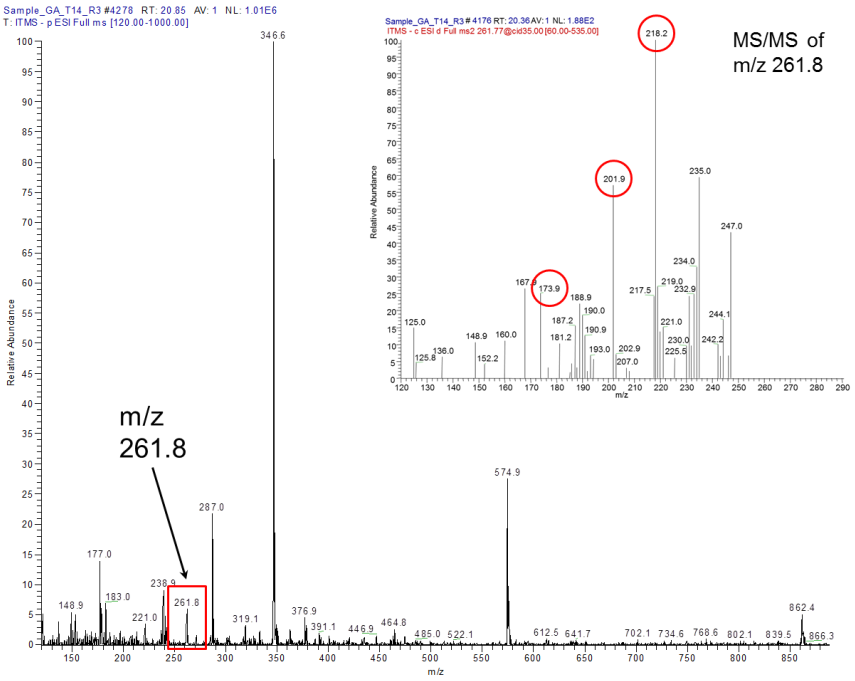


Figure 5. Full MS spectrum of a 'Galega vulgar' semi-hardwood cuttings sample at 20.85 min. The ion corresponding to IBA-acetate adduct is marked in red. The MS/MS spectrum of that ion is shown in the inset. Fragments marked in red in the MS^2 spectrum were also found in the MS/MS spectrum of pure IBA.

LC/MS/MS analysis also allowed the identification of several phenolic acids and related compounds in the samples (**Figure 6**). These compounds were present in large excess compared to IBA, which explains why is hard to identify auxins by HPLC-FLD. Structural similarities determine a similar behavior during extraction and purification, and the peaks found in HPLC-FLD chromatograms, although containing auxins, are impure mixtures of several compounds, as mentioned previously.

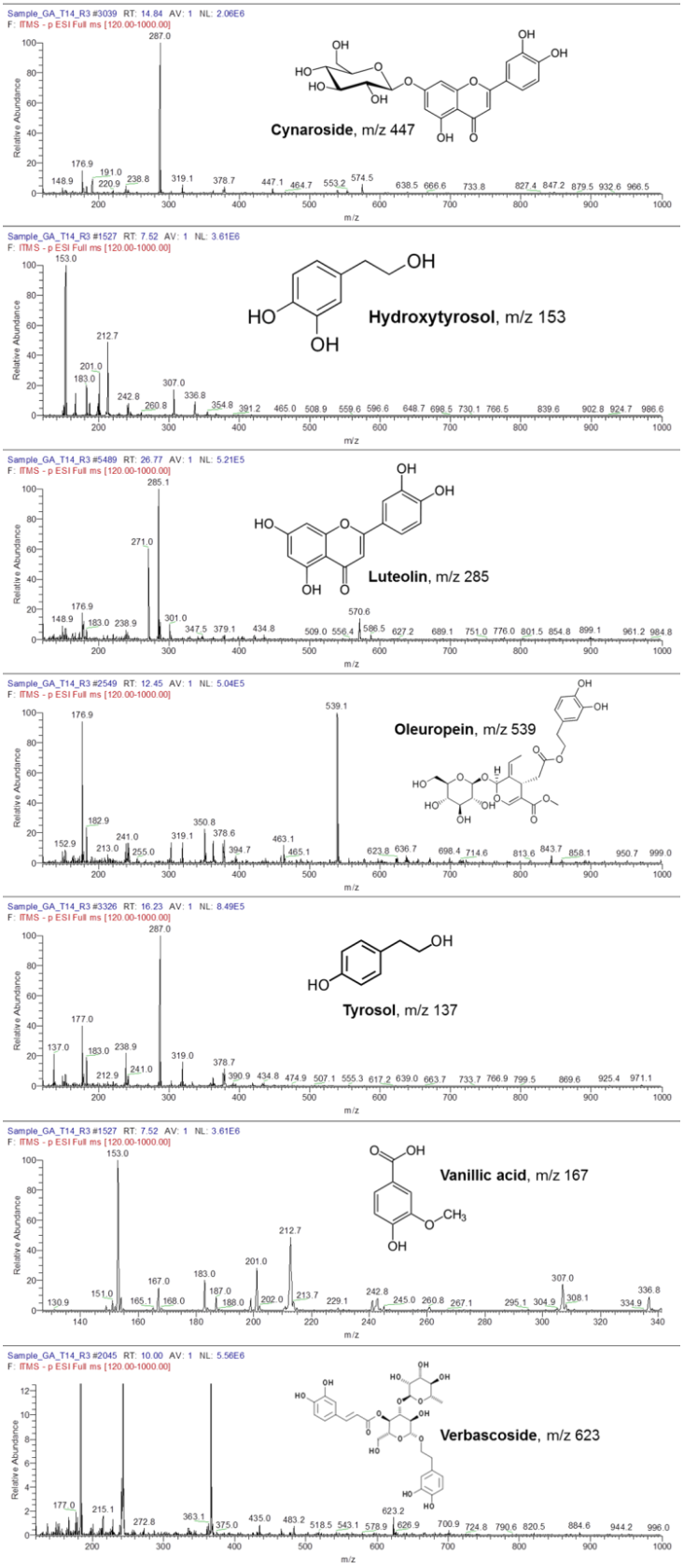


Figure 6. Full MS spectra of compounds found in a chromatogram of a 'Galega vulgar' semi-hardwood cuttings sample.

Having access to the technique, this conclusion was further reinforced by NMR analysis of fractions collected from HPLC chromatograms. To fully confirm the presence (or absence) of IAA in the samples, the fractions putatively containing IAA were collected by HPLC-UV, analyzed by 1H-NMR and compared with pure IAA (**Figure 7**). Two main conclusions originated from NMR analysis:

1) IAA was not found in the collected fractions. Although an indole derivative could be present, this compound would have substitutions in the benzene ring, excluding the possibility of auxin derivatives;

2) The collected fractions were definitely mixtures of compounds. After comparing the NMR results with the compounds identified by LC/MS/MS, vanillic acid was identified among other substances.

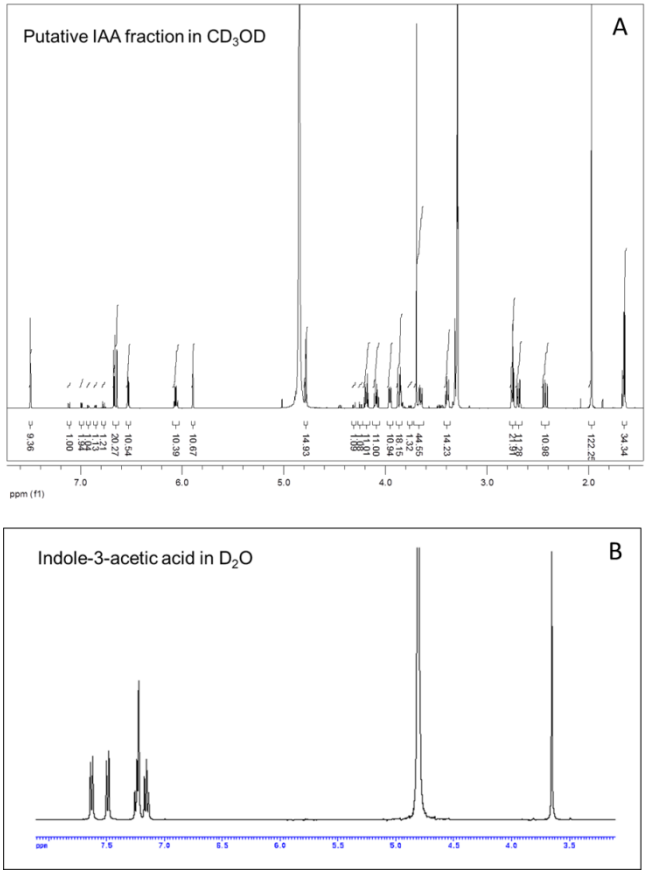


Figure 7. 1H-NMR analysis of a 'Galega vulgar' semi-hardwood cuttings sample**.** (A) 1D-1H spectrum of the collected fraction putatively containing IAA; (B) 1D-1H spectrum of pure IAA found in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu).

At this point, a few considerations should be pointed out. The results obtained by LC/MS/MS and NMR clearly show the importance of comparative analysis using different analytical techniques. The conclusions achieved here allowed confirming the presence of one analyte in olive samples and the absence of another, clarifying the results produced by HPLC-FLD. However, these conclusions were only possible because both techniques were available in the facilities where this work was developed. It should be mentioned that this is often *not* the case, because this type of instrumentation is expensive, while requiring high maintenance and costly consumables. In many lab scenarios a few different types of instrumentation will likely be available and often the choice of analytical methods is not based on which is the *best* method but rather on what is *available*. If LC/MS/MS and NMR hadn't been available, a lot more time and effort would have been put into improving HPLC-FLD conditions until a good separation with a positive correspondence between sample peaks and standard peaks had been achieved.

Considering the results obtained by LC/MS/MS and NMR, a different sample preparation method was used in an attempt to isolate auxins from the predominant phenolic compounds. The method described by Nakurte *et al*. (2012) included a purification step consisting of LLE with pH manipulation in order to separate auxins from the remaining plant matrix. Trying to obtain higher recoveries, we slightly modified the method by increasing the number of LLE cycles at pH 3. Nevertheless, given the similarities between auxins and many phenolic compounds, they will likely have a similar behavior during extraction/purification and for that reason the solvent gradient in HPLC-FLD was also re-adjusted (**Table 3**).

Table 3. Re-adjusted HPLC solvent gradient used for auxin separation

Time (min)	A (%)	B (%)
0	98	2
5	98	2
50	2	98
55	2	98

Olive samples were prepared according to the new extraction method and the results were compared with *Arabidopsis* samples prepared under the same conditions (positive control) (**Figure 8**). The obtained chromatograms were abnormally similar and in both samples a large putative IBA peak could be found. While in olive samples a large IBA peak could be considered feasible as a result of root-inducing treatments, *Arabidopsis* samples were not submitted to such treatments and the amount of IBA present in those samples should be residual. Just like LC/MS/MS and NMR results, this result also led to the suspicion that the separation obtained by HPLC-FLD was not efficient and the peaks found in chromatograms corresponded to mixtures of prevalent compounds existing in large amounts in any plant matrix (for example, phenolic acids).

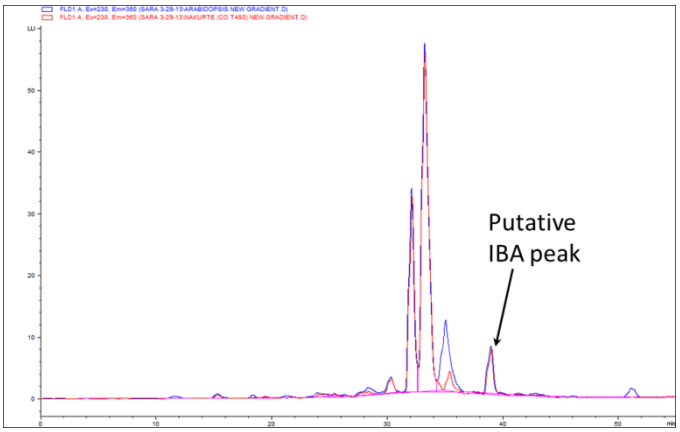


Figure 8. Overlaid HPLC-FLD chromatograms of an olive sample (red) and an *Arabidopsis* sample (blue).

After several adjustments of the solvent gradient which didn't produce any improvements in separation (data not shown), a different extraction method (Matsuda *et al*. 2005) was tested as another attempt of purifying IAA and IBA from other interfering compounds. The method consisted of SPE-C18 purification and proved to be useful in sample cleanup (**Figure 9**).

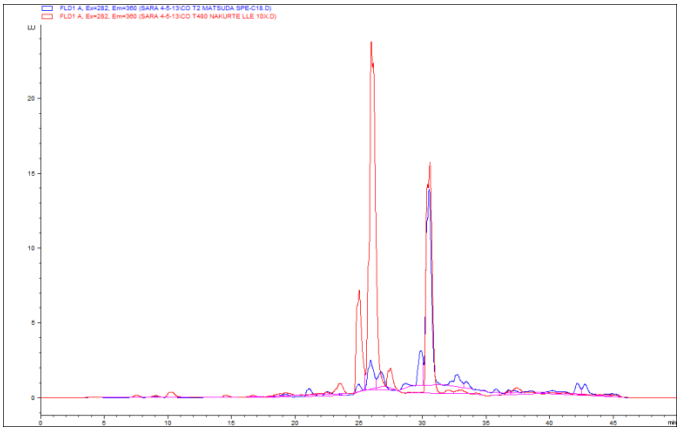


Figure 9. Overlaid chromatograms resulting from two different sample preparation methods: LLE (red), SPE (blue). Note that sample prepared by LLE was diluted 10x before injection.

Despite several extraction/purification methods had been applied, until this point IAA hadn't been identified in olive samples, and because IAA is metabolized by the plant into aminoacid-conjugated forms (see Chapter I) the possibility of IAA being present in the form of IAA-conjugates was considered. To test this hypothesis, samples prepared by SPE C18 (Matsuda *et al*. 2005) were analyzed by HPLC-FLD and the chromatograms were compared with that of IAA-Ala and IAA-Asp standards (**Figure 10**).

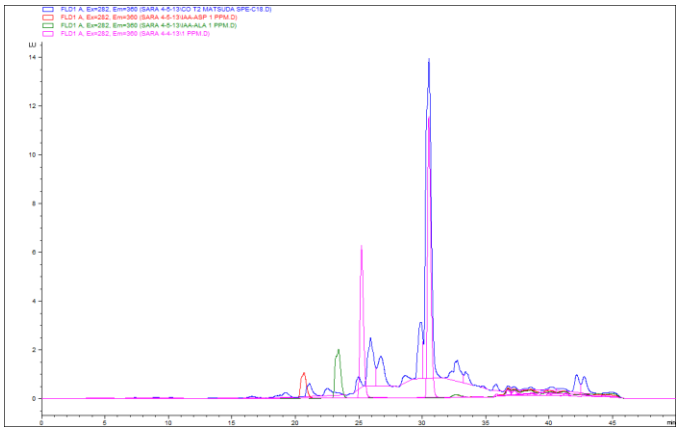


Figure 10. Overlaid chromatograms of a solution of 1 ppm auxin standards and a sample of 'Cobrançosa' semi-hardwood cuttings. IAA-Asp (red), IAA-Ala (green), IAA and IBA (in this order, pink), 'Cobrançosa' sample (blue). Sample was prepared using a SPE-C18 cleanup method according to Matsuda *et al*. (2005).

Although a peak in the sample had a retention time corresponding with IBA standard (**Figure 10**), none of the other standard peaks matched any peak in the sample. To clarify this issue, samples prepared by both LLE and SPE methods were analyzed by LC/MS/MS (LTQ Orbitrap Discovery, Thermo Scientific, USA), using the same column and conditions used in HPLC-FLD. Once again, IBA was found in both samples (m/z 202) but no IAA (m/z 174) or IAA-Asp (m/z 289) ions could be found. In the sample prepared by LLE, a very small ion corresponding to IAA-Ala (m/z 245) was found but the intensity was very close to noise level (**Figure 11**).

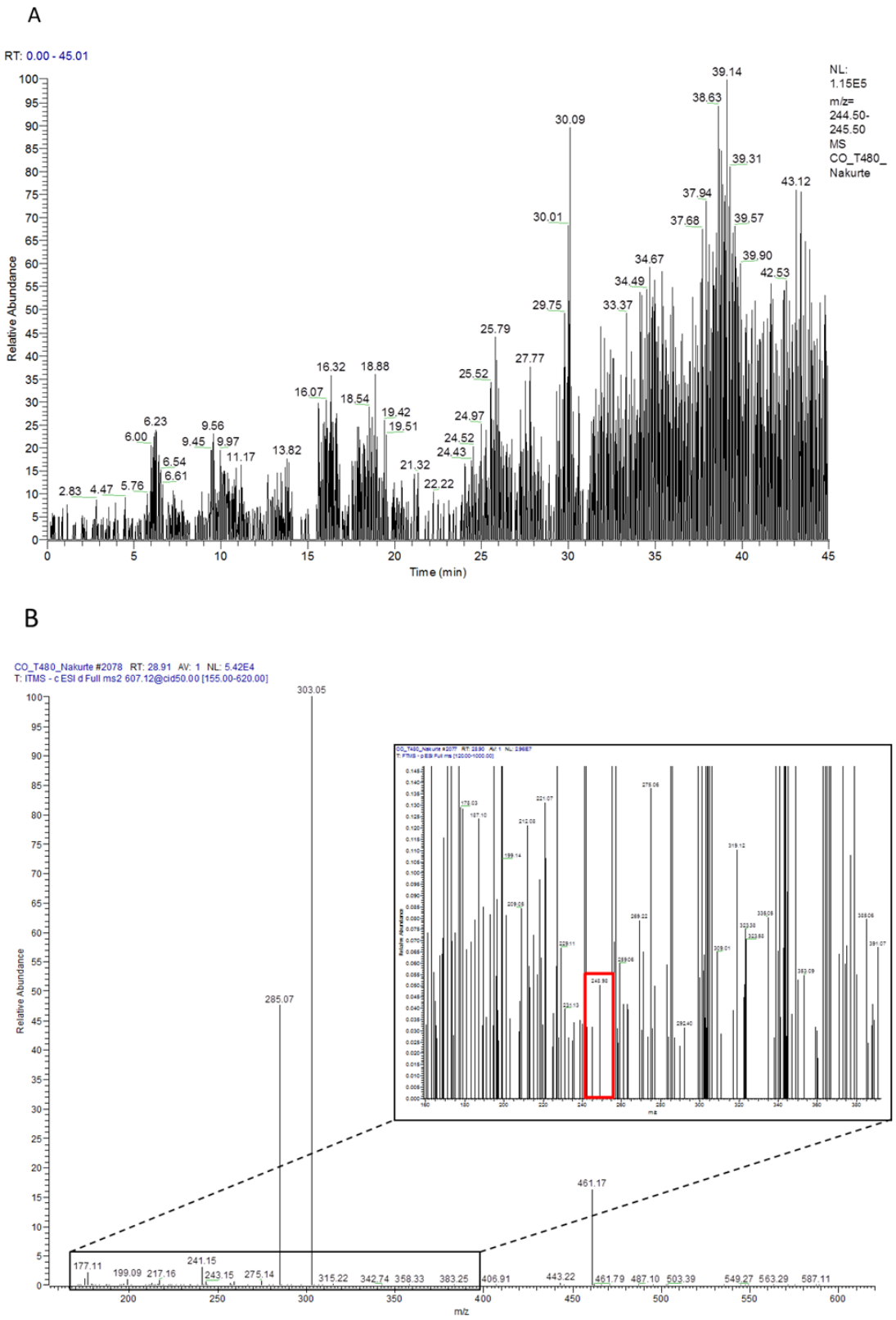


Figure 11. Possible detection of IAA-Ala in a sample 4 h after treatment. (A) Extracted ion (m/z 245) chromatogram of a sample purified by LLE; (B) MS of the peak at 28.91 min. An ion possibly corresponding to IAA-Ala is marked in red in the figure inset.

Although IAA couldn't be identified, the method (Matsuda *et al*. 2005) seemed promising as a cleanup step after extraction and so it was combined with LLE (Nakurte *et al*. 2012): LLE should efficiently extract auxins while contaminants should be eliminated by SPE. This method was in fact applied to samples but the obtained results were not very satisfactory (**Figure 12**). IBA was identified in the samples, but neither free nor conjugated IAA was found. Furthermore, the combined procedure turned out to be too long and not compatible with high-throughput analysis.

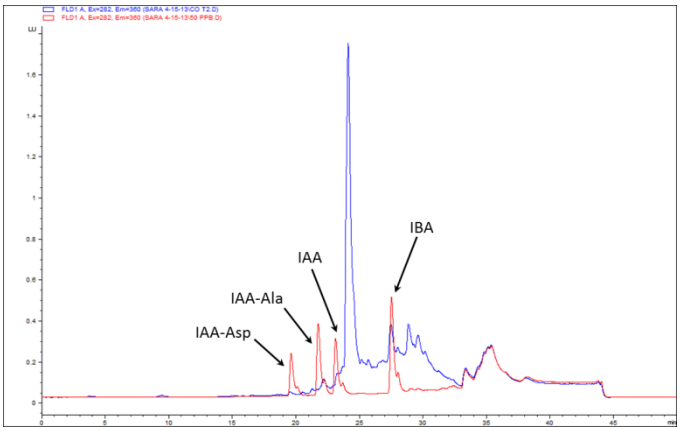


Figure 12. Overlaid chromatograms of a sample of 'Cobrançosa' semi-hardwood cuttings prepared by the combined LLE-SPE method (blue) and a solution of 50 ppb auxin standards (red).

As last attempt to isolate auxins by HPLC-FLD, old extracts (prepared by an adaptation of Pan *et al*. (2010)) were purified by SPE according to Matsuda *et al*. (2005). Even though a better separation was obtained, no peaks in the sample perfectly matched the standard peaks and some compounds overloaded the detector (**Figure 13**).

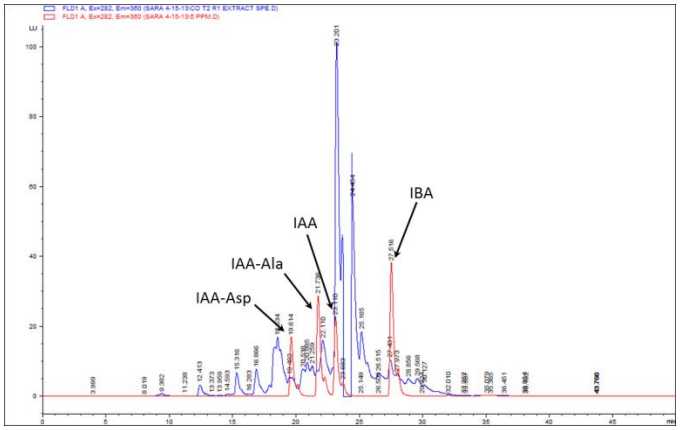


Figure 13. Overlaid chromatograms of an extract of semi-hardwood cuttings of 'Cobrançosa' prepared according to an adaptation of Pan *et al*. (2010) and purified by SPE according to Matsuda *et al*. (2005) (blue) and a solution of 5 ppm auxin standards (red).

GC/MS

In comparison with GC methods, which require a derivatization step before auxin analysis, LC could be viewed as the ideal analytical technique for this type of analyte. Hence, the method development described in this work started with LC separation. However, and despite being the most frequently used technique for auxin quantification, no reliable results were obtained with LC even after several different approaches. GC/MS has been widely applied to auxin quantification (Chapter II) and is actually a more powerful technique than HPLC-FLD. The MS detector allows an unequivocal identification of compounds and the possibility of performing analyses in selected ion monitoring (SIM) significantly increases the sensitivity of the technique.

Although compounds like auxins could be more easily analyzed by LC methods, avoiding the derivatization procedure, GC/MS analysis offers several advantages over LC approaches, where ion suppression of co-eluting compounds is frequent. Additionally, when using full-mode GC/EI-MS, the reproducible fragmentation patterns allow the use of mass spectra database for peak identification, which cannot ever be performed when LC/MS methods are used (Koek *et al*. 2011). This is of particular interest for complex matrices, such as plant samples. Multisector GC/MS instruments can be particularly powerful as they allow performing selected reaction monitoring (SRM), where the specific fragmentation of a given compound can be followed. However, as mentioned above, this kind of instruments is not commonly accessible to many labs. It is worth mentioning that even though SIM is the best method for quantification, full scan is still needed for identification purposes. In real samples, analyte identification can be affected by co-migrating compounds with the same fragment ions (matrix effect). Although this possibility is remote, it can't be precluded and therefore sample analysis in full scan is highly important.

Considering the abovementioned, and once again, having access to this type of instrument, method development proceeded using GC/MS.

The first approach to derivatization consisted in a methylation reaction by methanolysis. In this reaction, the analytes are incubated with 1 M methanolic HCl (MeOH-HCl) for a set amount of time at 80°C to produce methylated derivatives, by esterification of the analytes (**Figure 14**).

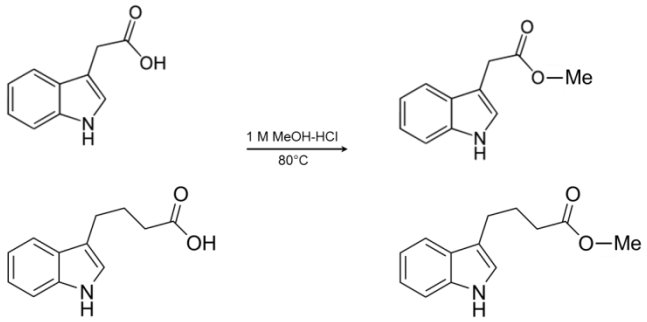


Figure 14. Methanolysis reaction

Aiming to optimize reaction time, preliminary experiments were performed where auxin standards were hydrolyzed with 1 M MeOH-HCl for 1 through 5h (**Figure 15**). No degradation was observed and because peak area increased with reaction time, 4h were used in following experiments.

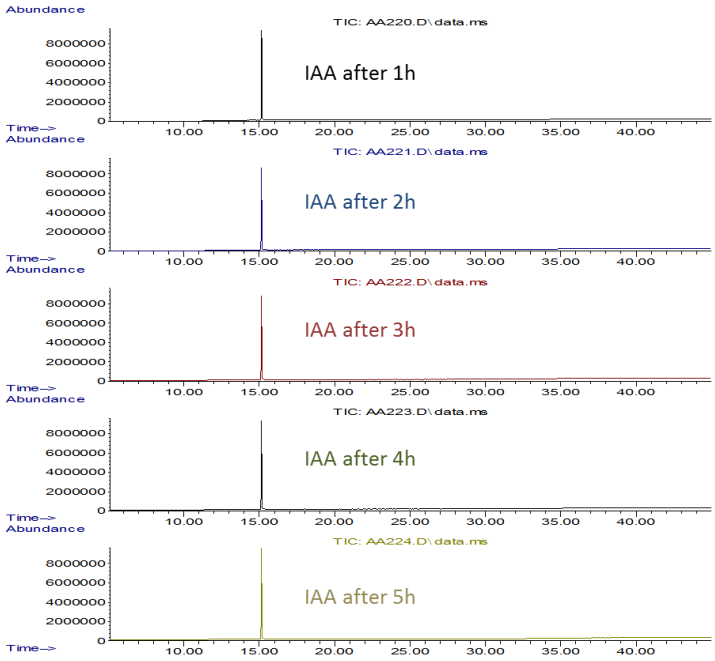


Figure 15. Effect of reaction time on IAA derivatization by methanolysis

To test the applicability of GC/MS to olive samples, an old extract prepared by the method of Pan *et al*. (2010) was derivatized for 4h by methanolysis and analyzed by GC/MS. The obtained chromatogram was much more complex than those obtained by HPLC-FLD, which indicated a better separation. In fact, IAA and IBA fragments were both found in the mass spectrum, and small peaks containing IAA and IBA fragments were found in the chromatogram (**Figure 16**), which was a very positive and promising

result. However, this result wasn't feasible because the sample used in this experiment was a sample collected 1 day after the IBA root-inducing treatment which meant that the amount of IBA in that particular sample should be very high. A problem of degradation was excluded as similar results were obtained with a freshly prepared extract, which indicated that the extraction/purification procedure had to be improved.

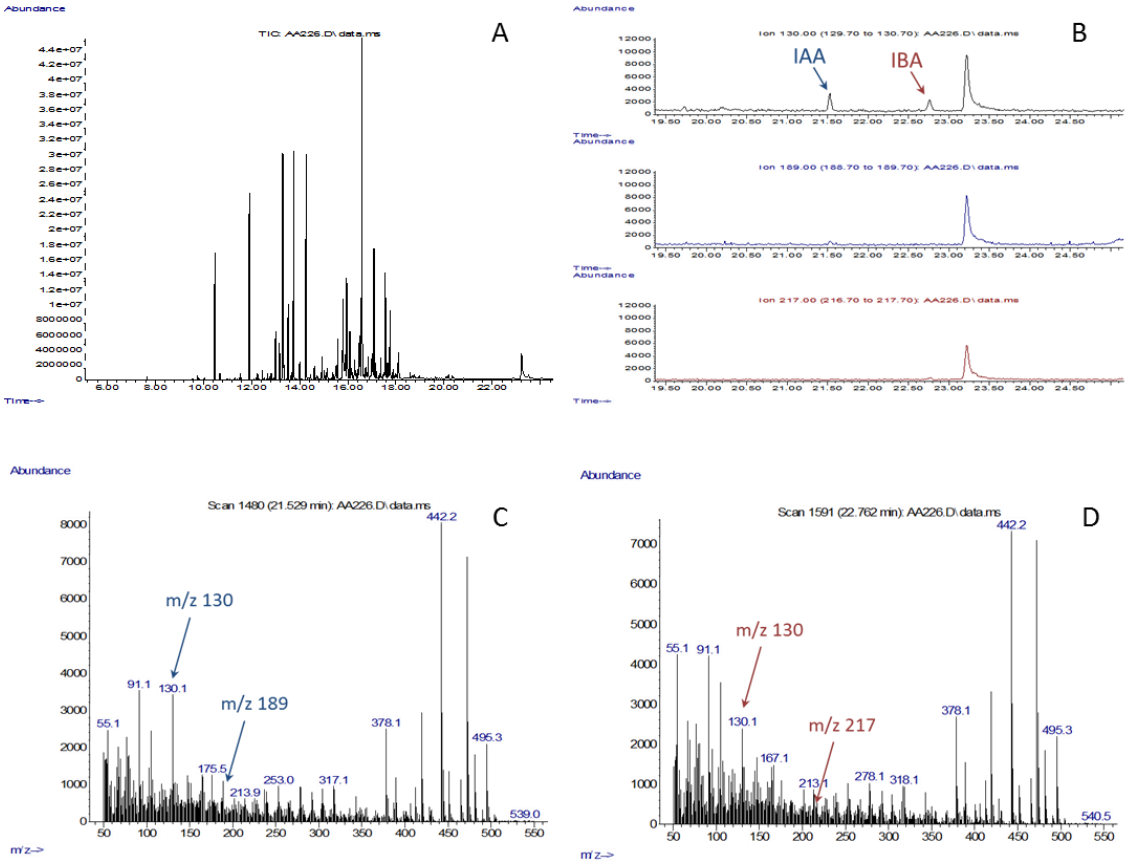


Figure 16. GC/MS analysis of a sample of 'Cobrançosa' semi-hardwood cuttings. (A) TIC chromatogram; (B) Extracted ion chromatogram (m/z 130, 189 and 217) showing to peaks corresponding to IAA and IBA; (C) Mass spectrum of the putative IAA peak in panel (B); (D) Mass spectrum of the putative IBA peak in panel (B).

To improve the extraction method (adapted from Pan *et al*. 2010), an LLE purification step was introduced after derivatization by methanolysis. In this step, the derivatized analytes would be reconstituted in water and partitioned against dichloromethane at neutral pH. This procedure was applied to a 'Cobrançosa' sample previously spiked with 4 µg of IAA and IBA standards. Although IAA and IBA fragments were observed in the resulting mass spectrum, the size of the peaks found in the extracted ion chromatogram (m/z 130) was too small considering the concentration used to spike the

sample (**Figure 17a**). This was associated with a low recovery of the method, as the same amount of pure IAA and IBA produced a signal 10x higher (**Figure 17b**).

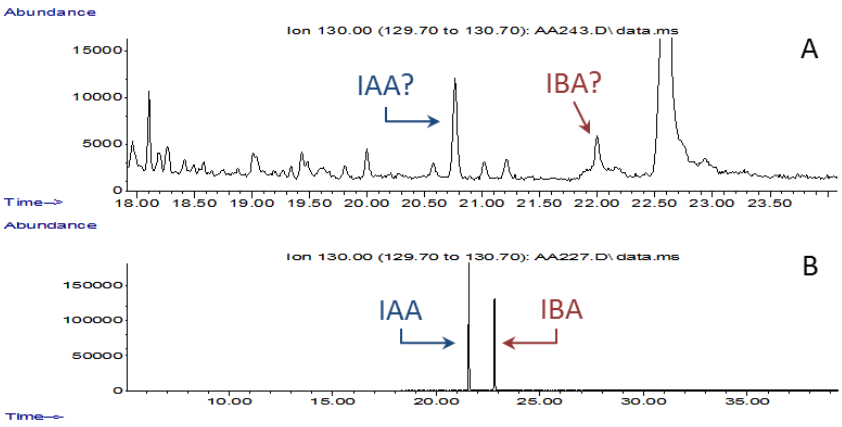


Figure 17. Effect of LLE after methanolysis on recovery. (A) Extracted ion chromatogram (m/z 130) of a 'Cobrançosa' semi-hardwood cuttings sample purified by LLE after derivatization; (B) Extracted ion chromatogram (m/z 130) of pure IAA and IBA standards derivatized by methanolysis. Note: the retention times in the chromatograms are not the same because the GC temperature program was adjusted between runs.

Isopropanol, the extraction solvent used in the method of Pan *et al*. (2010), is a relatively weak solvent, hence that could have been the cause for a low recovery of the method. To investigate this possibility, the same sample was extracted by the method of Pan *et al*. (2010) using 4 different solvents. The performance of isopropanol, methanol, ethanol and acetone was compared in this experiment. Furthermore, in the purification step, dichloromethane was also replaced by a stronger solvent (chloroform). Although IAA and IBA ions were found in all extracts, a clear identification (with acceptable peak shape) was only obtained with acetone (**Figure 18**).

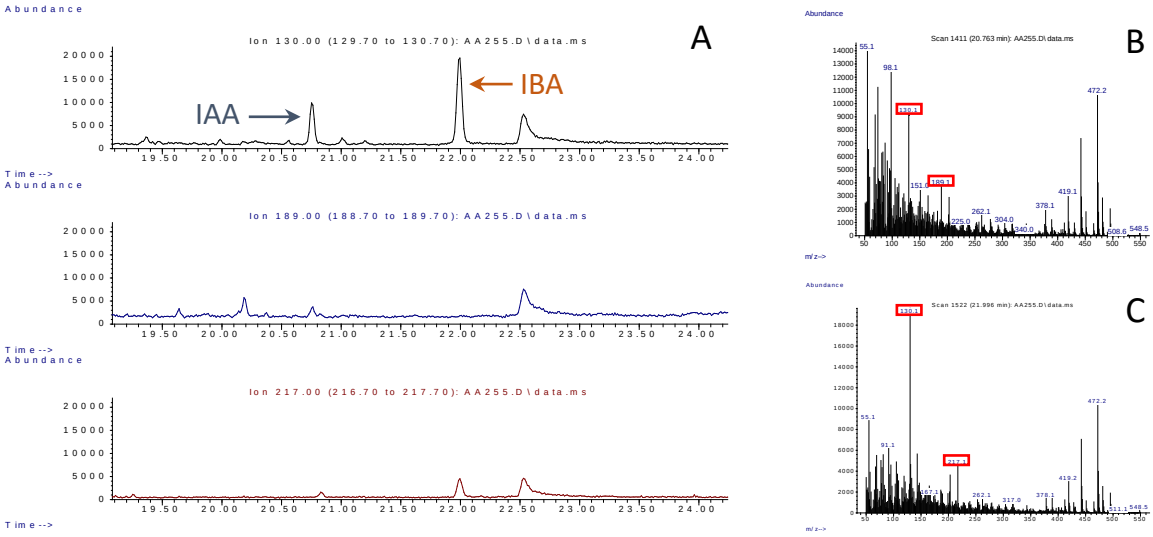


Figure 18. Extraction solvent optimization. (A) Extracted ion chromatogram of a 'Galega vulgar' sample extracted with acetone; (B) MS of IAA peak; (C) MS of IBA peak.

Low recovery could also be related with a post-derivatization LLE. After derivatization the analytes are volatile and they could have been lost in the LLE step. Using the solvents that originated best results in the previous experiment (acetone and methanol), samples were extracted with a mixture of [acetone : methanol (3:1)] : water : HCl (4 : 1 : 0.002), containing 100 µg/mL of BHT and purified by LLE, using chloroform as partition solvent. LLE was performed *before* derivatization. However, no auxin peaks were observed in the resulting chromatogram (**Figure 19**).

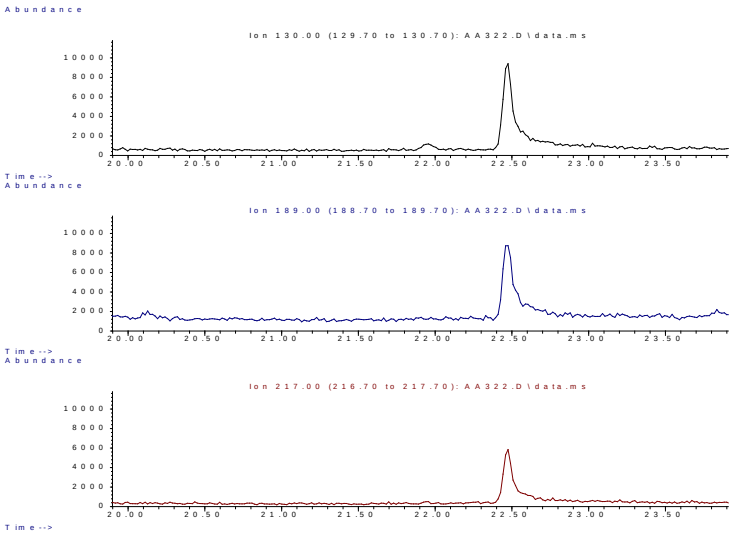


Figure 19. Extracted ion chromatogram of a 'Cobrançosa' sample purified by LLE before derivatization.

Although this method had been successful in extracting IAA and IBA previously (data not shown), the results were not consistent and the amounts of IBA found were systematically smaller than expected, which indicated the method was unreliable.

At the same time, other approaches for sample purification were also tested. It has been shown that the main source of matrix effects in olive samples are phenolic compounds (**Figure 6**), and polyvinylpirrolidone (PVP) has been described to bind to phenolics, acting as a chelating agent (Andersen and Sowers, 1968; Chan *et al*. 2007). Therefore, in a parallel experiment, PVP was incorporated in the extraction solvent as an attempt to purify the sample from phenolic compounds, although no notorious effect was observed (**Figure 20**).

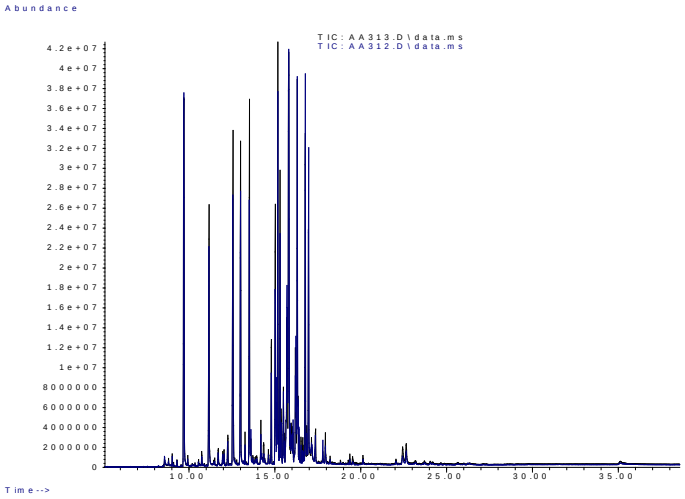


Figure 20. Effect of PVP on sample purification. Overlaid chromatograms of a 'Cobrançosa' sample extracted with (blue) and without (black) PVP.

Considering the low peak areas obtained so far, the method described by Nakurte *et al*. (2012) was applied to olive samples again, with minor adjustments. The original extraction solvent (methanol) was replaced by the mixture described above: [acetone : methanol (3:1)] : water : HCl (4:1:0.002). A sample of 'Galega vulgar' semi-hardwood cuttings collected 8h after IBA treatment, which should contain very high amounts of IBA (close to mg/g DW), was used for analysis. Nonetheless no auxin peaks were found in the resulting chromatogram (**Figure 21**).

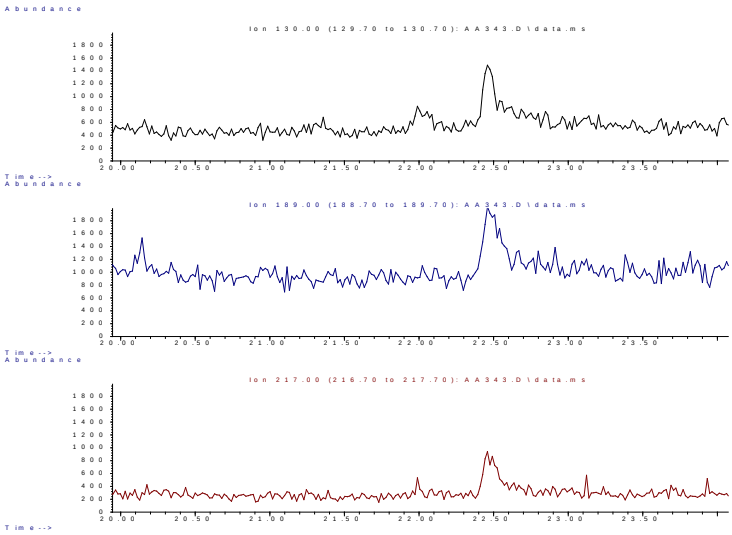


Figure 21. Extracted ion chromatogram of a 'Galega vulgar' sample prepared according to an adaptation of Nakurte *et al*. (2012).

As previously mentioned, one of the main sources of matrix effects found in olive samples are phenolic compounds, but pigments also contribute to this effect. Plant extracts without further purification are typically green, as a result of pigments such as chlorophyll. Considering the chemical structure of chlorophyll and other pigments, adding NaCl to a polar extract could potentially increase pigments' solubility in water by a salting-in effect, while decreasing auxins' solubility by a salting-out effect. By decreasing auxins' solubility in aqueous solutions, they would be more easily extracted by an organic solvent during LLE. Based on this information, an experiment was performed where a 'Cobrançosa' spiked sample was firstly extracted with a saturated NaCl aqueous solution (0.5 M) followed by a longer extraction with an organic solvent. Three solvents were compared (methanol, ethanol and acetone). The obtained extracts were further partitioned against chloroform.

Unlike methanol, acetone and ethanol were considered unsuitable because they are miscible with chloroform, hence no phase separation during LLE was observed when using these solvents. Nevertheless, in all extracts, peaks corresponding to IAA and IBA were found in the resulting chromatograms (**Figure 22**). However, the recovery associated with the extraction procedure was again very low, especially in the case of IBA. The sample used in this experiment, which had been collected 8h after IBA treatment, should contain very high amounts of IBA and that was not observed in the results (**Figure 22**). Furthermore, the sample had been spiked with 25 µg of auxin standards and the peak areas obtained after extraction were much smaller than the

areas obtained from the direct derivatization of the same amount of pure standards (**Table 4**). Therefore, the method was abandoned.

Table 4. Peak areas corresponding to 25 µg of IAA and IBA under different conditions. No extraction = peak areas of pure standards directly derivatized; Methanol = peak areas obtained after extraction with methanol; Ethanol = peak areas obtained after extraction with ethanol; Acetone = peak areas obtained after extraction with acetone.

	No extraction	Methanol	Ethanol	Acetone
IAA	132,220,988	2,473,481	10,524,371	35,233,685
IBA	122,302,715	1,357,488	747,414	5,596,701

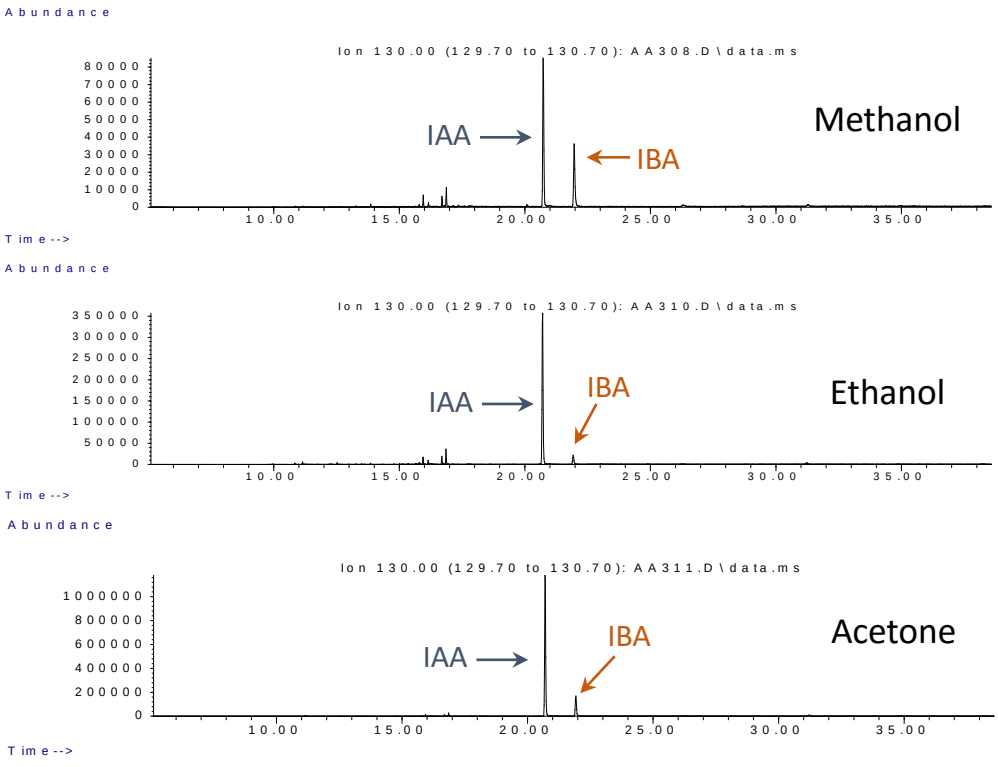


Figure 22. Extracted ion chromatograms (m/z 130) of the organic phases resulting from LLE of a 'Cobrançosa' sample extracted with methanol, ethanol and acetone.

Silylation

The main drawback of the derivatization method used until this point (methylation by methanolysis) was a very long reaction time, incompatible with high-throughput analysis of high numbers of samples. Methods like microwave-assisted derivatization (MAD) can be used to overcome this issue. MAD is based on the absorption of microwave energy by a material (whether a solvent or reagent), heating it and making it more reactive (Kouremenos *et al*. 2010), a process known as "dielectric heating." Heating by microwave is rapid and efficient, thus, one of the main advantages of MAD is that it can greatly reduce derivatization time when compared with convection methods (Poole, 2013). Ideally, a laboratory-designed microwave oven should be used to carry out chemical reactions, however this kind of equipment is expensive and not affordable by many labs. However, most MAD applications so far use domestic or ordinary microwave ovens (Poole, 2013), which may not provide optimal conditions for a chemical reaction to occur because accurate temperature and pressure cannot be set. Nevertheless, the results obtained so far have been impressive and promising, making MAD by domestic microwave oven a viable and practical alternative.

Silylation is the second most used reaction for auxin derivatization (discussed in Chapters II and III). Preliminary experiments performed with TMS-BSA reagent showed satisfactory results regarding auxin silylation in the microwave (data not shown). However, because TMS-BSA is a reagent with relatively low reactivity (Poole 2013) and produces a white residue, subsequent experiments were performed with BSTFA, a more reactive, cleaner reagent.

Optimization of microwave conditions started by determining optimum reaction power, by examining the derivatives produced when the microwave was set at 50 to 100% power level, corresponding to 350 to 700W (**Figure 23**).

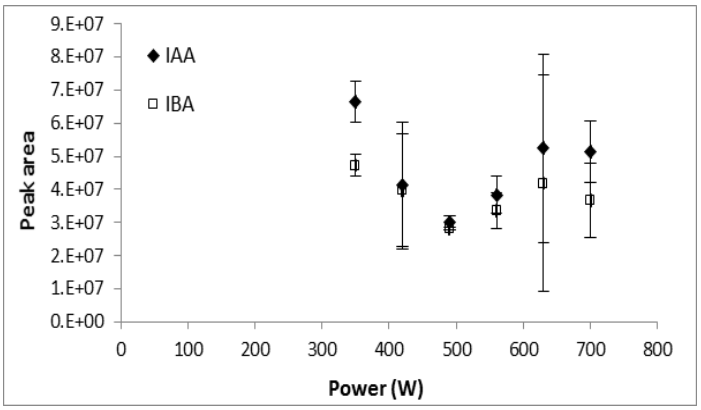


Figure 23. Effect of power level on chromatographic response of IAA and IBA.

The results were very disparate, and although the highest error was associated with 630 W (90% of the total capacity of the microwave), only two replicates were included in the experiment and in one of the replicates the highest peak areas among power levels were obtained for both IAA and IBA. Therefore, 630 W were selected as reaction power and used in subsequent experiments.

To minimize the volume of reagents used and to concentrate the analytes, GC vials containing removable inserts were used initially. However, because of the high temperature and pressure inside the vials, volume losses and inconsistent results were often experienced. While trying to overcome this problem, different types of GC vials were tested for the reaction: vials with removable insert, vials with fused insert and conical vials (**Figure 24**).

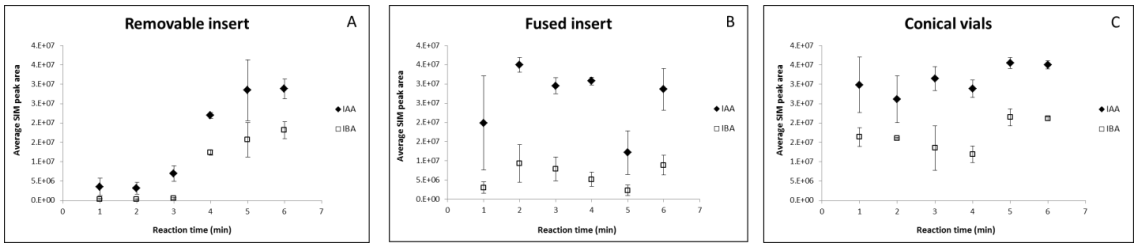


Figure 24. Comparative results of different GC vials used in derivatization. (A) Amber vials with removable insert; (B) Amber vials with fused insert; (C) Conical amber vials.

Figure 24a shows the effect of reaction time on SIM peak area while using vials with removable insert. The results show an increase in peak area over time, although at higher reaction times the reproducibility between replicates decreased. This was attributed to a higher temperature inside the vial, which intensifies the evaporation of volatile compounds, leading to a higher volume loss. Nevertheless, because removable inserts were being used, the volume deposited in the bottom of the vial was manually collected with a glass syringe in such cases.

Volume loss through the top of the insert was a recurring problem, observed also when using vials with a fused insert (**Figure 24b**). However, in this case, because the insert was not removable, it was impossible to collect the volume deposited at the bottom of the vial which led to lower peak areas and higher errors considering the volume lost through the top of the insert may never be the same between replicates of the same experiment.

Also, when using vials with insert (fused or removable), the reaction conditions are not very reproducible possibly due to the vial structure itself. The presence of an empty space between the insert (containing the analytes) and the outside walls of the vial hampers the dispersion of microwaves, leading to an unequal distribution of heat inside the vial.

Indeed, the best results in term of peak area and reproducibility were obtained with conical vials (**Figure 24c**). The increase in peak area over time observed with removable inserts (**Figure 24a**) was not visible in this case since the peak areas were higher at all reaction times with this type of vials. Although at lower reaction time there is a considerable difference between replicates, at higher reaction times the reproducibility increases significantly.

Optimization of reaction time is described in Chapter III. The results obtained with MAD were very satisfactory, especially because changing the derivatization procedure to silylation by MAD allowed dramatically decreasing reaction time from 4h to 5 min, making this protocol much more compatible with high-throughput analysis than methylation by methanolysis.

The last step in optimization of microwave conditions was to test the effect of microwave pre-heating. IAA and IBA standards (1 µg) were derivatized with and without pre-heating the microwave for 3 min at 630 W and the results were compared (**Figure 25**). Although smaller peak areas were observed without pre-heating, the differences were not significant ($p > 0.05$) and this step was omitted in subsequent experiments to prevent overheating.

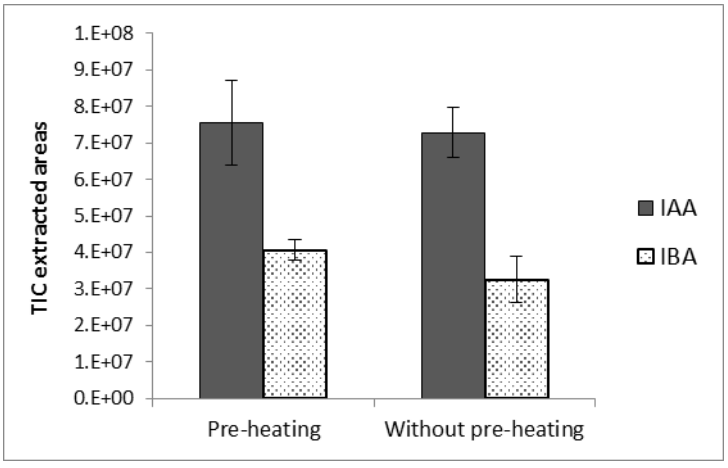


Figure 25. Effect of microwave pre-heating on chromatographic response.

It should be mentioned, however, that the main disadvantage associated with silylation by MAD is the production of multiple derivatives per analyte derivatized. Unlike methylation through methanolysis which produces only one derivative per auxin, silylation resulted always in two derivatives per auxin, corresponding to the mono- and di-silylated forms of IAA and IBA (**Figure 26**). Nevertheless, the decrease in reaction time achieved with MAD is sufficiently high to compensate for this shortcoming.

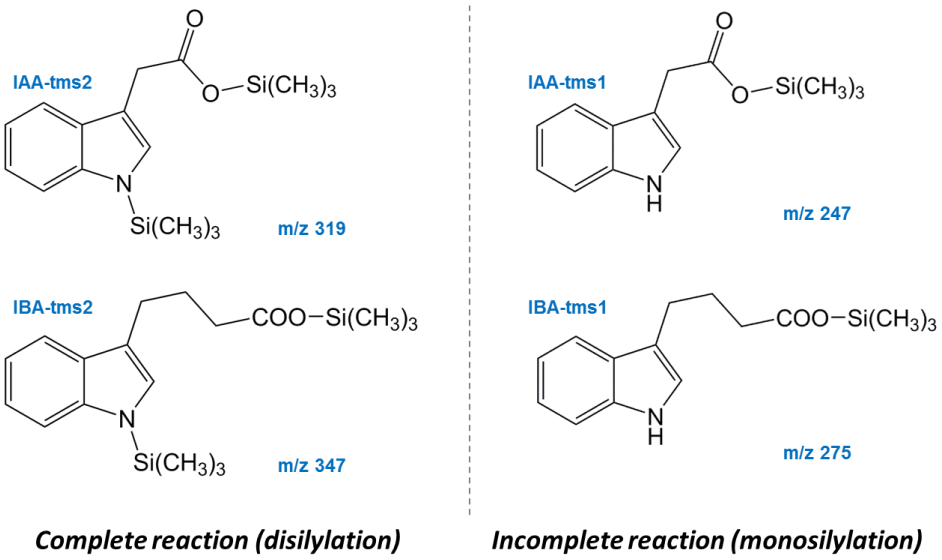


Figure 25.Derivatives produced during auxin silylation with BSTFA by MAD.

To assure the best conditions had been chosen, derivatization was also performed at lower power (350 W) for longer reaction times (6, 8 and 10 min), and in this case lower reproducibility was found (**Figure 26**). Furthermore, increasing reaction time considerably increased the temperature of the microwave forcing it to overheat which ultimately reduces its lifespan.

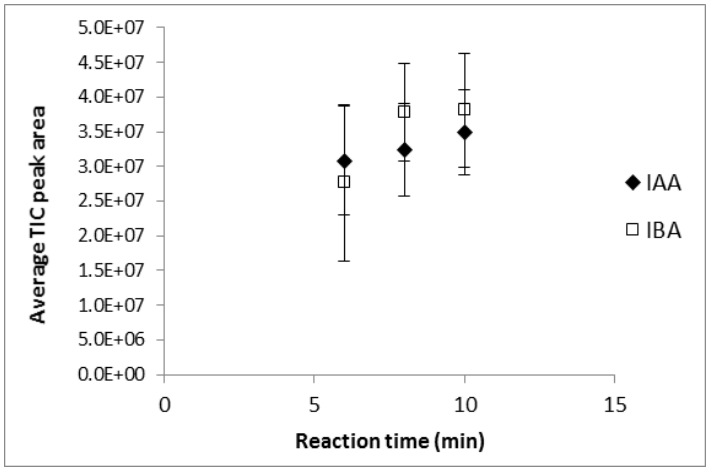


Figure 26. Effect of longer reaction times at lower power on derivatization.

Having a derivatization strategy optimized, and considering that a reliable, robust method hadn't still been found until this point, a sample of olive semi-hardwood cuttings was subjected to *accelerated solvent extraction* (ASE 150 Accelerated Solvent Extractor, Thermo Scientific Dionex) and derivatized in the microwave under optimized conditions. The resulting extract was very dark and cloudy, indicative of a highly complex matrix, which couldn't be cleared by filtration. The sample was never analyzed by GC/MS because it solidified after derivatization. This approach was not pursued because it would involve several steps of sample purification and, in parallel experiments, dispersive liquid-liquid microextraction (DLLME) had also been applied to olive samples and the results obtained by this method were much more satisfactory.

Therefore, the last approach to auxin quantification in olive samples was DLLME. This technique proved to be faster than any other method investigated in this work; it is simple in operation, requires low volumes of solvents and yields a fairly purified sample for analysis by GC/MS.

Based upon the work of Lu *et al*. (2010), who had applied DLLME to auxin extraction from plant tissues, the conditions optimized by these authors were applied to several olive samples and consistent results were observed, although some improvements were needed to increase recovery. Indeed, these authors were successful in extracting auxins from a unicellular algae (*Chlorella vulgaris*), but not from an evergreen shrub (*Duranta repens*). Based on this information, and because auxin extraction from olive tissues was not very efficient, all DLLME conditions were optimized, from volume of solvents to pH and ionic strength. These experiments are described in detail in Chapter III.

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Appendix II

CHANGES IN OXIDATIVE ENZYME ACTIVITIES DURING ADVENTITIOUS ROOT FORMATION OF OLIVE SEMI-HARDWOOD CUTTINGS

Throughout this work, the possible biochemical mechanisms controlling olive adventitious root formation were studied using *in vitro*-cultured microshoots as plant sampling material. However, as mentioned previously in Chapters I and IV, semi-hardwood cuttings are still the major source of plant material for olive vegetative propagation. Therefore, one of the goals of this work is also to study adventitious root formation in olive semi-hardwood cuttings. To do so, and similarly to the studies performed in Chapter IV with microcuttings, two cultivars with contrasting rooting performance were compared. 'Galega vulgar' and 'Cobrançosa' were chosen as difficult-to-root and easy-to-root cultivars, respectively.

To investigate the different rooting behaviors, temporal changes in enzyme activities and auxin levels were measured throughout the rooting period in semi-hardwood cuttings of both cultivars, and the results from such studies are presented herein.

Materials and Methods

Plant material, rooting procedure, and culture conditions are described in detail in Chapter IV.

Sample collection

During rooting, ten segments from the basal portion (approx. 1 cm from the base) of the cuttings were collected in triplicate at 4, 8, 24, 48, 96, 144, 192, 240, 336, 432, 528, 624, and 720 h after auxin treatment. In addition, at the time of collection of mother plants (1 hour before treatment) and before auxin treatment (0 hours after treatment), ten segments were collected in triplicate and used as control samples. All samples were flash frozen in liquid nitrogen and stored at –80°C for subsequent analyses.

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

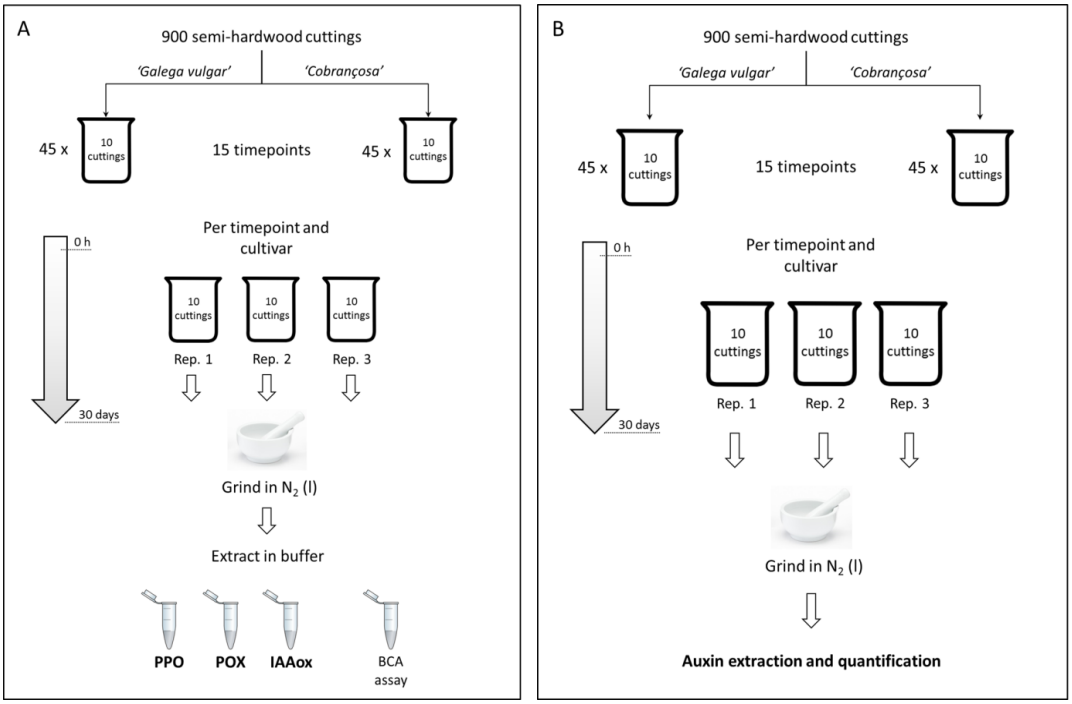

Figure 1. Schematic representation of the experimental design used for sample collection. (A) Enzyme analysis; (B) Auxin analysis.

Extraction of oxidative enzymes

Extraction of oxidative enzymes and enzyme activity measurements were performed as described previously. A detailed description of this experimental procedure is provided in Chapter IV.

Auxin quantification by gas chromatography/mass spectrometry (GC/MS)

Auxin quantification was also performed as described previously. A detailed description of this experimental procedure is provided in Chapter IV.

Statistical analysis

Temporal changes in enzyme activities and auxin levels were analyzed by one-way ANOVA followed by post-hoc Tukey HSD test. Significant differences were considered at $p < 0.05$. Differences between cultivars or rooting trials 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).

Results and discussion

To obtain statistically significant data, and because rooting performance may be affected by season (Therios, 2009), two rooting trials were performed in different seasons. While 'Cobrançosa' cuttings displayed a homogenously good rooting performance in both trials, 'Galega vulgar' showed a typically low rooting rate during winter but an exceptionally high rooting rate during fall (**Table 1**). Because this abnormal behavior was not expected and yet is very interesting, instead of being considered as oddity, the results from this trial were rather compared with those from winter. Therefore, and similarly to the studies performed with microshoots described in Chapter IV, the activity of PPO, POX and IAAox was evaluated during adventitious root formation of semi-hardwood cuttings of each cultivar, in both rooting trials.

Table 1. Rooting performance of 'Galega vulgar' and 'Cobrançosa' semi-hardwood cuttings in two different rooting trials.

Trial	Rooting performance at 60 days after IBA treatment (%)	
	'Galega vulgar'	'Cobrançosa'
Winter	4	60
Fall	62	56

Changes in activities of oxidative enzymes

During winter, when 'Galega vulgar' cuttings presented a very low rooting performance (**Table 1**), the trend in PPO activity was very similar between cultivars, increasing significantly during the rooting period (**Figure 2A**), as described by Yilmaz *et al.* (2003). In turn, POX activity also increased in both cultivars, but while in 'Galega vulgar' the observed increase was constant until 720 h, in 'Cobrançosa' a peak of activity was observed at 624 h, with activity levels decreasing at 720 h to a level similar to that of 432 h (**Figure 2B**). Similar changes had been reported by Caboni *et al.* (1997) who described a peak in POX activity of an easy-to-root cultivar of *Prunus dulcis* but not in a difficult-to-root one.

On the contrary, and in agreement with results from Mato and Vieitez (1986), IAAox activity decreased significantly during rooting in 'Galega vulgar', which is demonstrated by an increase in the residual IAA amount detected by HPTLC. However, in the case of 'Cobrançosa', IAAox activity decreased significantly until 528 h, described a minimum at 624 h, and increased again significantly at 720 h (**Figure 2C**). These results are in agreement with Rout (2006), who described a decline in IAAox activity during induction and initiation and an increase during expression.

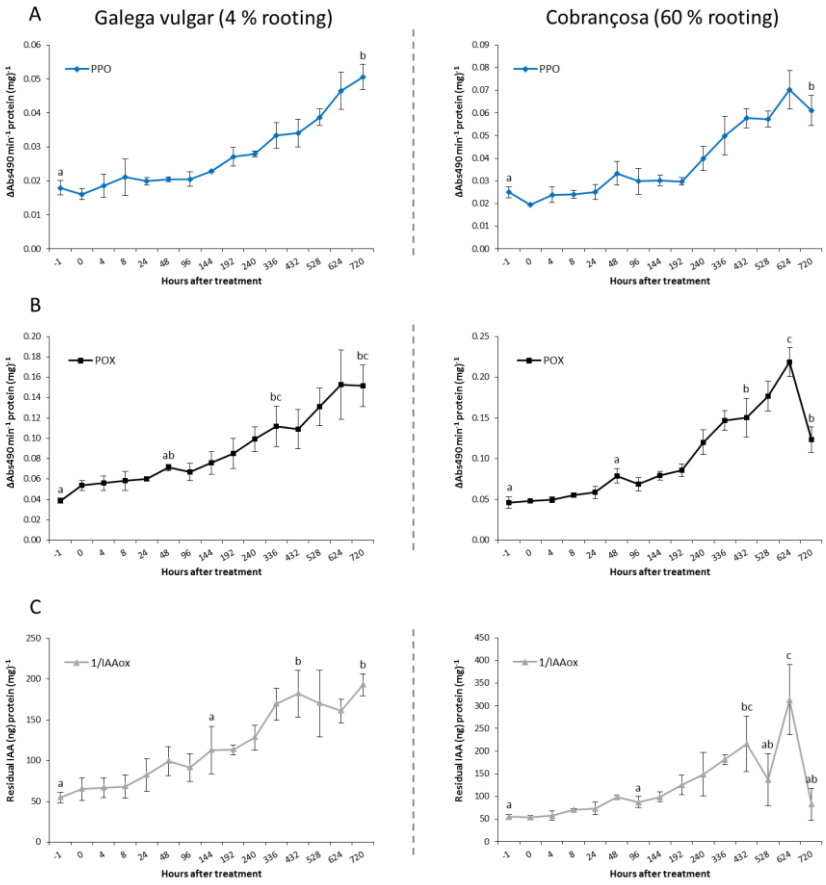

Figure 2. Changes in enzyme activity during adventitious root formation in olive semi-hardwood cuttings during winter. Activity levels of (A) PPO, (B) POX and (C) IAAox were measured on semi-hardwood cuttings of the cultivar 'Galega vulgar' (left) and 'Cobrançosa' (right). Different lower-case letters correspond to statistically significant differences ($p < 0.05$).

Differences were also found between cultivars regarding each enzyme activity (**Figure 3**). 'Cobrançosa' cuttings had significantly higher PPO activity levels throughout the rooting period (**Figure 3A**), and a similar trend was observed for POX activity, although significant differences were only observed after 336 h (**Figure 3B**). These results are in agreement with Van Hoof and Gaspar (1976), who described a higher POX activity in

easy-to-root cuttings of asparagus, but contradict Ludwig-Muller (2003) who found higher POX activity in difficult-to-root shoots of *Grevillea petrophioides* and *Protea* hybrid 'Pink Ice'. In contrast, differences in IAAox activity were only observed in the end of the rooting trial. 'Cobrançosa' cuttings had significantly lower IAAox activity than 'Galega vulgar' at 624 h but at 720 h the inverse behavior was observed (**Figure 3C**).

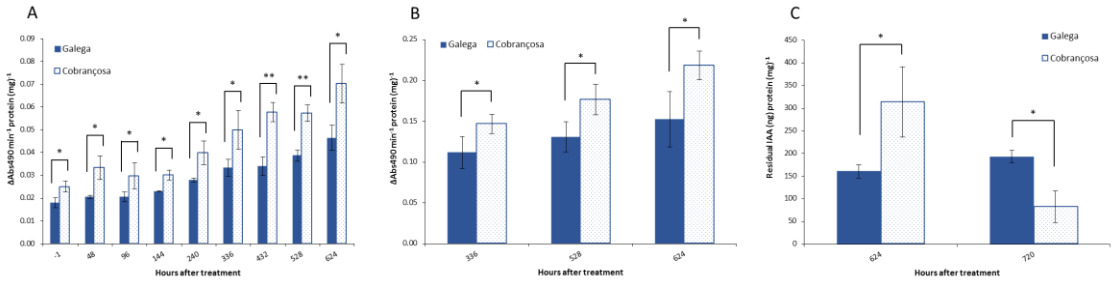

Figure 3. Effect of cultivar on individual enzyme activities during winter. (A) PPO activity, (B) POX activity, (C) IAAox activity. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

During fall, when 'Galega vulgar' showed an abnormally high rooting percentage (**Table 1**), PPO activity of both cultivars described a similar trend as observed during winter (**Figure 2A**), increasing significantly during the observed period (**Figure 4A**). Hence, no relationship between PPO activity and rooting ability could be found in our results, as previously described by Yilmaz *et al.* (2003).

POX activity also increased during root formation in both cultivars, but while in 'Cobrançosa' an almost linear increase was observed, in 'Galega vulgar' POX activity increased in a straight manner until 336 h, decreasing to a transient minimum at 528 h when it reached levels similar to those at 336 h, and increased again significantly until 720 h (**Figure 4B**). However, the biggest differences were observed in IAAox activity. While in 'Cobrançosa' IAAox activity decreased progressively during root formation, in 'Galega vulgar' IAAox activity decreased gradually until 432 h, reaching a minimum at this point, and then increased steeply until 624 h. At this point and until 720 h, IAAox activity decreased again, reaching levels similar to those at 336 h (**Figure 4C**).

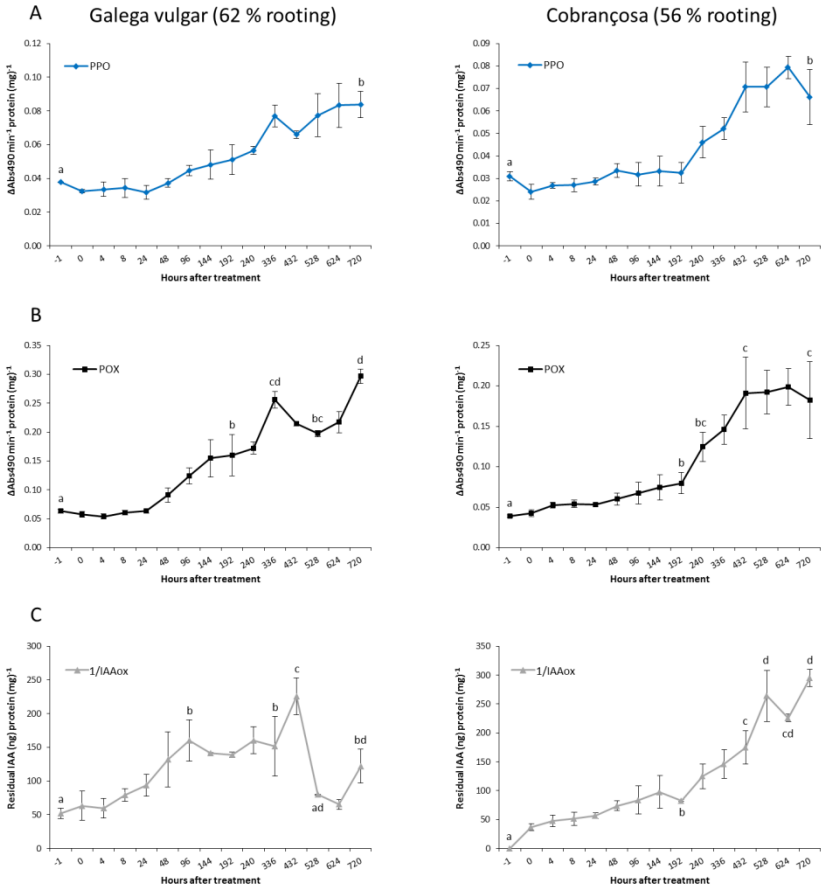

Figure 4. Changes in enzyme activity during adventitious root formation in olive semi-hardwood cuttings during fall. Activity levels of (A) PPO, (B) POX and (C) IAAox were measured on semi-hardwood cuttings of the cultivar 'Galega vulgar' (left) and 'Cobrançosa' (right). Different lower-case letters correspond to statistically significant differences ($p < 0.05$).

The results obtained during fall were fairly different than those obtained during winter, both in terms of rooting performance (**Table 1**), as well as in terms of enzyme activity. In contrast with results observed during winter (**Figure 3**), during fall significantly higher PPO and POX activity were observed in 'Galega vulgar' cuttings throughout the observational period (**Figures 5A and 5B**). In turn, IAAox activity was lower in 'Galega vulgar' until 192 h, and after this point 'Cobrançosa' cuttings had significantly higher IAAox activity (**Figure 5C**).

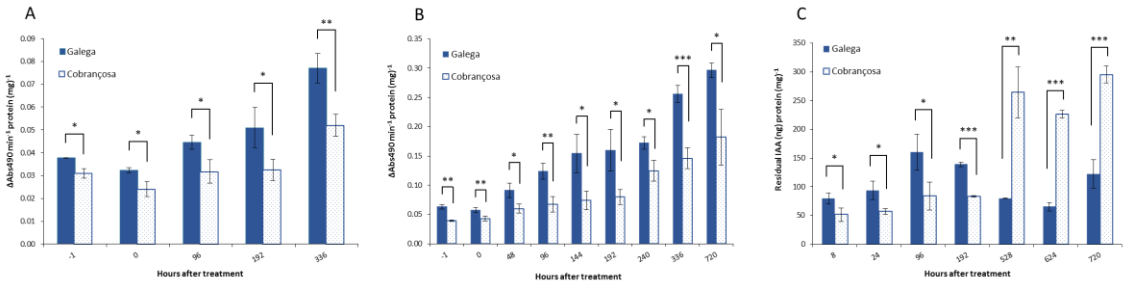

Figure 5. Effect of cultivar on individual enzyme activities during fall. (A) PPO activity; (B) POX activity; (C) IAAox activity. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Differences in the activity of each enzyme, for the same cultivar, were also found between rooting trials. In 'Cobrançosa' few differences were observed, especially regarding PPO and POX activities, a predictable result considering that the rooting performance of this cultivar did not differ greatly between rooting trials. Control cuttings taken during the winter had higher PPO activity than the ones taken during the fall (**Figure 6A**). Regarding POX activity, cuttings taken during winter had higher POX activity than those taken during the fall at 48 h after treatment (**Figure 6B**).

On the other hand, in 'Galega vulgar' enzyme activities were predominantly higher during the fall, when this cultivar displayed a higher rooting percentage. PPO activity was higher throughout root formation in cuttings taken during the fall (**Figure 6E**), and similar results were found in POX activity (**Figure 6F**). Only during the initial phase (0 – 24 h) no differences between seasons were found regarding POX activity. IAAox activity of 'Cobrançosa' cuttings during the fall was lower than during winter regarding induction and initiation, but in expression phase cuttings taken during winter had lower IAAox activity than those taken during the fall (**Figure 6C**). In 'Galega vulgar', no differences between seasons were found during induction phase. During initiation phase IAAox activity was lower in cuttings taken during winter, when rooting percentage was low, but this trend was inverted during expression phase (528 – 720 h) where cuttings taken during the fall (when rooting percentage was high) had significantly lower IAAox activity (**Figure 6F**).

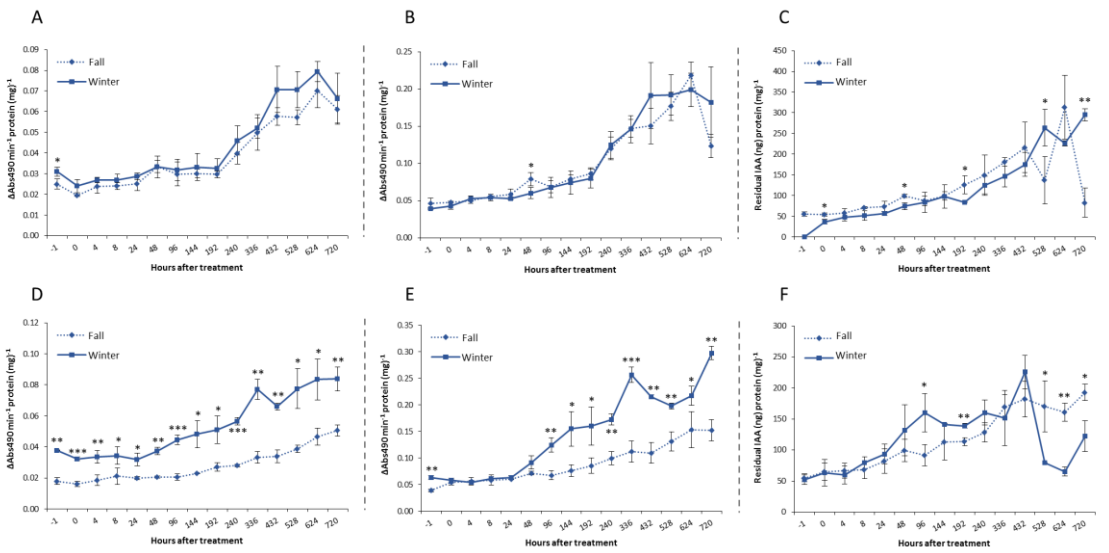

Figure 6. Effect of season on individual enzyme activities. (A) PPO activity of 'Cobrançosa' cuttings; (B) POX activity of 'Cobrançosa' cuttings; (C) IAAox activity of 'Cobrançosa' cuttings; (D) PPO activity of 'Galega vulgar' cuttings; (E) POX activity of 'Galega vulgar' cuttings; (F) IAAox activity of 'Galega vulgar' cuttings. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Although some of the results are coherent with the literature, it is hard to draw conclusions from the available data. Changes in enzyme activity were only found in the later rooting period, which had been referred by several authors (Upadhyaya *et al.* 1986, Gaspar *et al*. 1992), suggesting a possible role of oxidative enzymes in the late stages of root formation. However, several contradictions were found in the results, preventing valid conclusions. For instance, although a higher POX activity of 'Galega vulgar' cuttings during the fall could explain the differences in rooting performance (Van Hoof and Gaspar, 1976), the changes in POX activity of 'Cobrançosa' cuttings during what is considered the expression phase do not support this hypothesis. Also, some authors suggest that a peak of POX activity precede or accompanies root formation (Ludwig-Muller, 2003), which we could only observe in 'Cobrançosa' cuttings during the fall. Furthermore, if an increase in IAAox activity during expression phase is associated with rooting (Rout, 2006), the significantly different IAAox activity of 'Cobrançosa' cuttings in the two rooting trials doesn't correlate with its consistently good rooting performance.

The high variability of the results could be attributed to the nature of the plant material. While microshoots cultured *in vitro* are genetically identical clones of the same mother plant, semi-hardwood cuttings are associated with an inherently higher genetic variability. Semi-hardwood cuttings are taken from mother plants which, depending on

their location, may be exposed to different climatic conditions, or may have differential access to soil nutrients. Alternatively, some mother plants could be involved in mychorrhizal associations, which would give them a physiological advantage over plants that are not involved in this type of symbiotic relation.

Sampling of semi-hardwood cuttings is also a possible source of variability. Because not all cuttings are collected from the same mother plants, and because the same mother plant is used to produce several cuttings, samples may not be completely homogenous, resulting in a higher contribution of a specific mother plant to a specific sample. Furthermore, in cuttings taken during winter, sample collection is hampered by the higher water content of the cuttings, resulting in the collection of tissues outside of the root formation zone. Not only can this procedure introduce variability, but it can also "dilute" the results because enzyme activities could be measured in tissues that may not have been related with root formation.

Moreover, considering the high diversity of information found in the literature (reviewed in Chapter I), and considering that the length of adventitious rooting phases in olive was determined in *in vitro*-cultured tissues (Macedo *et al.* 2013), we cannot affirm that in semi-hardwood tissues the length of the different rooting stages will be the same. In fact, rooting trials using semi-hardwood cuttings usually take longer than 30 days (720 h) because this type of material typically responds later to root-inducing treatments. Therefore, we cannot affirm that the changes observed after 528 h after treatment correspond to the expression phase. It could be possible that the changes observed during the chosen observational period correspond to a lag phase and that only after 720 h after treatment the actual changes in enzyme activity would be visible.

Changes in auxin levels

Temporal changes in free IAA and IBA levels are shown in **Figure 7** and **Figure 8**. During winter, 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 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 (**Figure 7A**). In contrast, 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 (**Figure 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 (**Figure 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 (**Figure 7D**).

During fall, IAA levels increased progressively in both cultivars during the first 8 h. While in 'Cobrançosa' cuttings IAA amounts started decreasing at this point until 96 h (**Figure 7G**), in 'Galega vulgar' IAA levels kept increasing until 24 h and only then decreased to a transient minimum at 96 h (**Figure 7E**). After 96 h, 'Cobrançosa' IAA levels decreased to non-quantifiable amounts at 336 h and 528 h but no other significant changes were observed until the end of the rooting period (**Figure 7G**). In 'Galega vulgar' a non-significant increase was observed at 144 h and IAA levels also decreased to non-quantifiable amounts at 432 – 624 h.

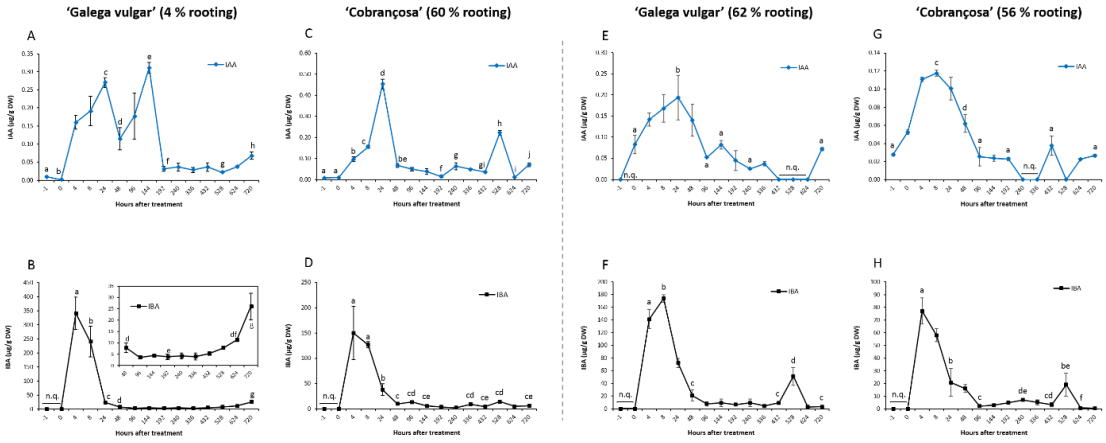

Figure 7. Changes in free IAA and IBA levels during rooting of semi-hardwood cuttings in winter (left) and fall (right). (A) and (E) IAA levels of 'Galega vulgar' cuttings; (B) and (F) IBA levels of 'Galega vulgar' cuttings; (C) and (G) IAA levels of 'Cobrançosa' cuttings; (D) and (H) IBA levels of 'Cobrançosa' cuttings. Different lower-case letters correspond to statistically significant differences ($p < 0.05$). n.q. = not quantified.

When comparing the two rooting experiments, several differences in auxin levels were found between cultivars. During winter, 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 (**Figure 8A**). By contrast, during the fall, the trend in IAA levels was reversed and 'Galega vulgar' cuttings had consistently higher IAA levels than 'Cobrançosa' cuttings (**Figure 8C**). Even though a peak was still observed during initiation (144 h), it was transient, non-significant and significantly lower than that observed when rooting rates were low. Furthermore, IAA levels during induction phase were significantly

higher than those during initiation phase and the evolution of IAA levels in both cultivars was much more similar in this trial.

On the other hand, IBA levels were higher in 'Galega vulgar' cuttings during induction phase (4 – 8 h) in both seasons. While this trend persisted during initiation and expression phases in fall (**Figure 8D**), during winter '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 (**Figure 8B**).

These results suggest that the contrasting rooting behavior of 'Galega vulgar' cuttings could be related with the metabolism of free IAA. High IAA levels during initiation are likely inhibitory of root formation, as observed during winter and as suggested by De Klerk *et al.* (1995), while the absence of an IAA peak during initiation leads to an improved rooting performance. Hence, this latter relationship was observed not only in 'Galega vulgar' cuttings during fall, but also in 'Cobrançosa' cuttings in both seasons.

On the contrary, a clear relationship between IBA levels and rooting performance couldn't be established from these results. Despite the different behavior of 'Galega vulgar' cuttings in the two studied seasons, the corresponding IBA levels were always higher than those in 'Cobrançosa' cuttings. Interestingly, however, the maximum concentrations of both IAA and IBA found during the fall were almost 50% lower than those during the winter. This could imply that higher concentrations of free auxins are associated with lower rooting rates, at least in the case of 'Galega vulgar'. Moreover, at 528 h, a statistically significant peak of IBA was found in both cultivars, although the significance of this peak is not yet fully understood.

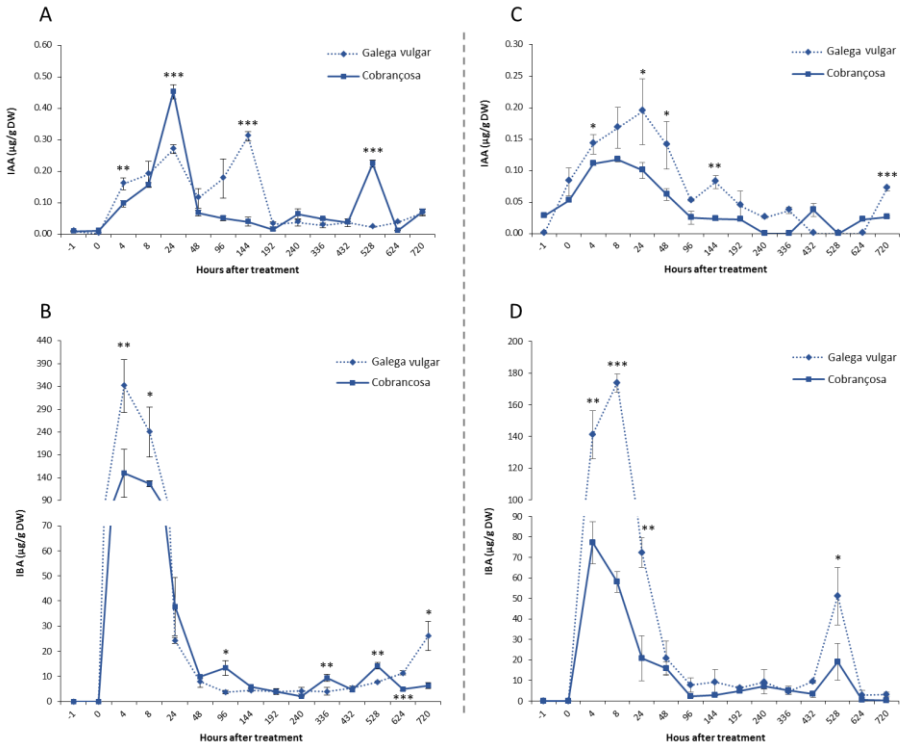

Figure 8. Changes in auxin levels between olive cultivars in two different seasons. (A) Free IAA levels recorded during winter; (B) Free IBA levels recorded during winter; (C) Free IAA levels recorded during fall; (D) Free IBA levels recorded during fall. (** $p < 0.01$; *** $p < 0.001$)

Conclusions

Differences in rooting performance seem to be related with auxin metabolism, rather than with enzyme activity. High levels of free IAA during the initiation phase appear to be an impediment for root formation, while increased IBA levels during expression may be desirable. A relationship between rooting ability and enzymatic activity couldn't be established, as the results obtained were highly discrepant. The reason for the lack of consistency in the obtained results is likely related with the nature of the plant material, which, as a result of its intrinsic characteristics, introduces a high level of variability. Hence, studies involving semi-hardwood cuttings should ideally be complemented with parallel studies using *in vitro*-cultured microshoots, which are associated with a higher genetic homogeneity and thus provide a more consistent response to rooting treatments.

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Appendix III

ADVENTITIOUS ROOT FORMATION IN OLIVE (*Olea europaea* L.) MICROSHOOTS: ANATOMICAL EVALUATION AND ASSOCIATED BIOCHEMICAL CHANGES IN PEROXIDASE AND POLYPHENOL OXIDASE ACTIVITIES

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Adventitious root formation in olive (*Olea europaea* L.) microshoots: anatomical evaluation and associated biochemical changes in peroxidase and polyphenol oxidase activities

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SUMMARY
Trials were performed using *in vitro*-cultured microshoots of the olive (*Olea europaea* L.) cultivar 'Galega vulgar', as initial explants, to identify histological events and modifications in peroxidase and polyphenol oxidase activities during adventitious root formation. Explant bases were submitted to a 10 s quick-dip treatment to promote rooting, using a sterile solution of 14.7 mM indole-3-butyric acid (IBA). Samples for histology and quantification of enzyme activities were collected at pre-established periods from 0 to 720 h. The first signs of modifications in stem cell morphology were observed 96 h after explant inoculation on olive culture medium (OM), with some cortical cells showing a dense cytoplasm and a large central nucleus, with visible nucleoli. The first mitotic events were observed after 144 h and evolved *via* two different pathways: non-specific cell division, leading to callus formation; and organised cell division, leading to the formation of root meristemoids. After 456 h, the first organised root primordia became visible. No root formation was achieved without earlier callus development, and 89% of root primordia originated from tissues other than cambial/phloem tissue. Peroxidase and polyphenol oxidase activities were recorded throughout the whole rooting process. The first significant modification in enzyme activity, with a drop from 0.19 to 0.14 ΔA_{490} units min^{-1} 50 mg^{-1} of explant material, was observed for peroxidase within the first 4 h after IBA treatment. Subsequent changes in both enzyme activities could be correlated with different phases of the adventitious rooting process.

Considerable progress has been made in the last 20 – 30 years towards understanding rooting by characterising it as an evolutionary process consisting of a successive series of interdependent phases (i.e., induction, initiation, and expression), each having specific physiological and environmental requirements (Moncousin *et al*., 1988; Gaspar *et al*., 1992; Rout *et al*., 2000).

Adventitious roots originate *via* the redifferentiation of several cell types such as those from sub-epidermal tissues, the cortex, cambium, secondary phloem, pericycle, or vascular bundles. In olive, the capacity to develop adventitious roots has proved to be extremely variable among cultivars (Salama *et al*., 1987; El-Said *et al*., 1990; Fouad *et al*., 1990). Differences in the anatomical structure of cuttings were proposed to explain this dependence on genotype, with several authors stating that the presence of a continuous ring of sclerenchyma, between the phloem and the cortex, may act as a mechanical barrier to root emergence (Salama *et al*., 1987; Qrunfleh *et al*., 1994). Nevertheless, other reports have provided evidence that the difficulty in rooting olive cuttings could not be correlated with the anatomical structure of the cutting, and that genetic, biochemical, or physiological causes, rather than anatomical ones, could be related to the incapacity of several olive cultivars to form adventitious roots (Bakr *et al*., 1977; Fabbri, 1980).

Several studies on adventitious root formation have highlighted the important role that oxidative enzymes such as peroxidases (POX) and polyphenol oxidases (PPO) play in this process (Moncousin and Gaspar, 1983; Berthon *et al*., 1989; Gaspar *et al*., 1992; Rival *et al*., 1997; Rout *et al*., 1999; Cheniany *et al*., 2010; Fu *et al*., 2011).

Plant peroxidases (POX; E.C. 1.11.1.7) are haem-containing enzymes that catalyse the oxidation of a diverse group of organic compounds. Studies on adventitious root formation have shown that POX isoenzymes play a fundamental role in the rooting of cuttings, with changes in POX activity often being used as a biochemical marker for the rooting process (Gaspar *et al*., 1992; Metaxas *et al*., 2004; Syros *et al*., 2004; Hatzilazarou *et al*., 2006).

Typically, the minimum POX activity appears at the root induction phase, while a subsequent increase, reaching a peak of activity, marks the end of root initiation and the start of the root development phase (Gaspar *et al*., 1992). Chao *et al*. (2001) reported that a

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decrease in POX activity corresponded to a rise in endogenous indole-3-acetic acid (IAA) levels in indole-3-butyric acid (IBA)-treated tissue, during the induction of roots from soybean hypocotyls. This result was recently confirmed by Cho *et al*. (2011), who reported a significant reduction in POX activity following the application of IBA during the induction of adventitious roots in *Cinnamomum kanehirae*.

Polyphenol oxidase (PPO; E.C. 1.14.18.1) is a copper-containing enzyme located in the thylakoids of plastids which catalyses the oxidation of phenolic compounds into quinones. PPO also seems to play a key role in rhizogenesis (Gonzalez *et al*., 1991; Gaspar *et al*., 1997), where it is involved in regulating the synthesis of the phenolic precursors needed for lignin biosynthesis during root differentiation (Haissig, 1986). Moreover, PPO can also catalyse the formation of IAA-phenol complexes, classified by some authors as "rooting cofactors", that can promote the occurrence and development of adventitious roots (Haissig, 1974; Bhattacharya, 1988; Balakrishnamurthy and Rao, 1988).

Despite extensive research over the past 20 – 30 years aimed at achieving a better understanding of adventitious rooting, the process is far from being resolved, especially in recalcitrant genotypes. The present study aimed to provide updated information on anatomical events, and on the activities of POX and PPO enzymes during *in vitro* adventitious root formation on explants of the difficult-to-root olive (*Olea europaea* L.) cultivar 'Galega vulgar'.

MATERIALS AND METHODS

Plant material, rooting procedure, and culture conditions

Microshoots of a single clone of the olive (*Olea europaea* L.) cultivar 'Galega vulgar', already established *in vitro* according to the protocol of Peixe *et al*. (2007), were used in all these experiments.

Explants with four-to-five nodes were prepared from *in vitro*-cultured microshoots, and all leaves, except for the upper four, were removed. To induce rooting, explant bases (approx. 1.0 cm) were submitted to a 10 s quick-dip treatment in a sterile solution of 14.7 mM IBA. The explants were then inoculated, *in vitro*, in 500 ml glass flasks containing 75 ml semi-solid olive culture medium (OM; Rugini, 1984), 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 (all supplied by Merck-Portugal, Lisboa, Portugal). The pH of the medium was adjusted to 5.8 prior to sterilisation in an autoclave (20 min at 121ºC). All cultures were kept in a growth chamber at day/night temperatures of 24ºC/21ºC (± 1ºC), 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.

Histology

During rooting, ten samples from the basal portion (approx. 1 cm from the explant base) of *in vitro*-cultured explants were collected at 0, 4, 8, 24, 48, 96, 144, 192, 240, 336, 432, 528, 624, and 720 h after auxin treatment. All samples were fixed in 3.0 ml of 1:1:8 (v/v/v) formaldehyde:acetic acid:70% (v/v) ethanol (FAA). Each sample was placed, individually, in a small plastic tube (10 ml) and kept uncovered in a vacuum chamber for 1 h. The tubes were then closed and the samples were left in the fixative for 2 d at 4ºC. After fixation, samples were washed twice in 70% (v/v) ethanol, dehydrated through a graded series of ethanol and increasing butanol solutions (Table I), cleared in xylene, and embedded in paraffin according to the procedure of Johansen (1940).

Low melting-point (56ºC) paraffin (Jung-Histowax, Cambridge Instruments, Nussloch, Germany) was used, and paraffin blocks were prepared using Leuckart's bars.

Thick (10 – 15 µm), serial transverse sections were cut on a MicroTec-Cut 4055 rotary microtome (MicroTec Laborgeräte GmbH, Walldorf, Germany), attached to microscope slides covered with a thin film of Haupt's adhesive, and air dried overnight at room temperature. Sections were stained with 0.6% (w/v) Safranin O + 2% (w/v) Orange G and observed under an Olympus CK-40 inverted optical microscope (Olympus-Portugal, Lisboa, Portugal) equipped with a 50 Watt mercury arc-lamp fluorescent unit, with a green light filter cube (U-MWG; 510 – 550 nm excitation filter, 590 nm emission filter, and 570 nm dichromatic mirror). Using this filter combination, lignin and Safranin O-stained cells and organelles should present a light red fluorescence.

Measurement of peroxidase and polyphenol oxidase activities

Each sample (ten *in vitro*-cultured explant bases) was collected at the same time-points after auxin treatment as used for the histological observations. The experiment was repeated three-times on three parallel sub-cultures, resulting in a total of 30 samples collected at each time-point. All samples were frozen immediately in liquid nitrogen and stored at –80ºC for subsequent enzyme assays.

The collected material (ten explants per sample per time-point) was ground and homogenised in a mortar with liquid nitrogen. Approx. 50 mg of explant material was introduced into a 1.5 ml microtube for extraction. Samples were extracted with 1.0 ml of extraction buffer containing 50 mM sodium acetate (Merck-Portugal), 2.0 mM ethylenediamine-tetra-acetic acid (EDTA; VWR-Portugal, Carnaxide, Portugal), 1.0 mM magnesium chloride (VWR-Portugal) and 1.0 mM phenyl-methylsulfonyl fluoride (PMSF; AppliChem, Darmstadt, Germany) at pH 5.5. Each extract was mixed for 15 s and centrifuged (10,000 × *g*) at 4ºC for 20 min. The supernatant was transferred to a fresh 1.5 ml microtube and stored at –20ºC for enzyme activity assays.

To determine PPO activity, 100 µl of the crude explant extract was added to 900 µl of a buffer solution containing 45 mM sodium acetate, 2 mM 3-methyl-2-benzothiazolinone-hydrazone-hydrochloride (MBTH;

TABLE I
Graded series of dehydrating solutions for olive explant samples (values to prepare 100 ml of each solution)

Solution	H_2O (ml)	Ethanol (ml)	Butanol (ml)	Eosin (mg)	Time (h)
I	50	40	10	–	4
II	30	50	20	–	12
III	15	50	35	–	2
IV	–	45	55	25	2
V	–	25	75	25	2
VI	–	5	95	–	12

Merck-Portugal) and 20 mM 4-methylcatechol (Sigma-Aldrich Quimica, S.A., Sintra, Portugal) at pH 5.5. PPO activities were determined by measuring the change in absorbance at 490 nm (ΔA_{490} units min^{-1} 50 mg^{-1} explant material) using a Beckman DU®530 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA).

For POX activity, 100 µl of the crude explant extract was added to 900 µl of 45 mM sodium acetate, 2.0 mM MBTH, 20 mM 4-methylcatechol, and 1.0 mM hydrogen peroxide (Alfa Aesar GmbH, Karlsruhe, Germany) at pH 5.5. POX activities were determined by measuring ΔA_{490} min^{-1} 50 mg^{-1} of explant plant material using a Beckman DU®530 spectrophotometer, as above.

Data analyses

All POX and PPO activity data were submitted to ANOVA. When significant differences occurred within or between treatments, the data were submitted to a *post-hoc* analysis using the Fisher LSD test with significance being recorded at $P \leq 0.05$. Analysis was accomplished using STATISTICA 8.0 software (Stat Soft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Histological observations

The sequence of events leading to the formation of adventitious roots in *in vitro*-cultured explants of the olive cultivar 'Galega vulgar' was recorded. The time-point presented for each histological event corresponded to its first occurrence in the stem samples under observation, because these events were not synchronous in all examined samples.

A transverse section of a stem-base prior to being submitted to IBA treatment is presented in Figure 1A. A collateral vascular bundle forming a ring around the pith, which is a typical feature in dicotyledonous species, can be observed. The cambial zone is represented by a few layers of flat cells between the xylem and the phloem. The epidermis is formed by one or two cell layers, whereas the cortex consists of several layers of large parenchymatous cells.

The changes in stem-base tissues 96 h after IBA treatment can be seen in Figure 1B. Cells distributed at random in both the cortex and sub-epidermal tissues re-acquired the characteristics of meristematic cells with dense cytoplasm, a large centrally-positioned nucleus, and prominent nucleoli.

The first cell divisions were observed 144 h after root induction, and two developmental pathways were observed. The first, following a disorganised pattern of cell divisions, led to the formation of scar calli (Figure 1C, C*). The second involved organised divisions of iso-diametric cells, leading to the development of meristemoid regions. These meristemoids, developing from the upper phloem (Figure 1D, D*) and from the cortex/sub-epidermal region (Figure 1E, E*), were first observed 240 h after IBA root-induction treatment. The more responsive region was the cortex/sub-epidermis, where 89% of all root meristemoids were formed.

The first morphogenetic root zones, resulting from synchronised divisions of meristemoid cells, were observed at 336 h (Figure 1F, F*). Root primordia exhibiting polarisation, due to the presence of a root meristem and a differentiated vascular system connected to that of the stem, were visible 528 h after the root induction treatment (Figure 1G, H).

Using the fluorescence filter combination described above, it was possible to identify stem regions where mitotic activity and lignin deposition were occurring. Figure 2A shows a stem section sampled before root induction. Fluorescent cell walls were observed only in the xylem and in some suberised epidermal regions. No other stem tissues displayed a fluorescent signal, indicating the absence of mitotic activity and/or lignin deposition. A stem section 240 h after inoculation on rooting medium is presented in Figure 2B. Most cell walls exhibit red fluorescence due to the deposition of lignin, while light-red nucleoli can be observed in regions where mitosis is taking place. This image corresponds to the one presented in Figure 1D, indicating the efficiency of observation under florescent light to identify mitotic events during adventitious root formation.

The results presented here allowed us to conclude that, prior to root induction, the stem structure observed in microshoots cultured *in vitro* was basically the same as that described for semi-hardwood olive cuttings (Troncoso *et al*., 1975; El-Nabawy *et al*., 1983; Ayoub and Qrunfleh, 2006). However, we did not observe the sclerenchymal ring reported by these authors in *in vitro*-cultured microshoots, probably due to the softness of the stem tissue used.

The first mitotic events observed in the *in vitro*-cultured explants led to the formation of scar calli. According to Hartmann *et al*. (1997), callus formation prior to rooting normally occurs during indirect root formation and represents a common feature in difficult-to-root explants. In our trials with 'Galega vulgar' olive, these calli arose from cortical cells, which agrees with observations made by Ayoub and Qrunfleh (2006) working with semi-hardwood cuttings of the olive cultivars 'Nabali' and 'Raseei'.

Despite some differences in timing, all other stages of adventitious root formation (e.g., the development of root meristemoids, evolution into morphogenic roots, and the emergence of root primordia) also agreed with the results from similar *in vitro* studies in other temperate fruit species [e.g.. *Malus pumila* 'KSC-3' (Hicks, 1987); *Prunus avium* L. × *P. pseudocerasus* Lind. (Ranjit *et al*., 1988); and *Castanea sativa* L. (Gonçalves *et al*., 1998)].

The major difference between our results and those reported by other authors concerns the stem tissue involved in the formation of a new adventitious root system. In olive cuttings, independent of the rooting ability of the cultivar, most authors have observed adventitious roots arising from the cambial region of the stem [Bakr *et al*. (1977) on cultivar 'Wetaken'; Salama *et al*. (1987) on 'Manzanillo', 'Mission', 'Calamata', and 'Hamed'; and Ayoub and Qrunfleh (2006) on Nabali' and 'Raseei']. However, in this study, we did not observe any adventitious roots arising from the cambial region in 'Galega vulgar' olive. Nevertheless, as stated by Naija *et al*. (2008), the region in which cells become re-activated seems to depend, in part, on physiological gradients of substances entering the shoot from the medium, and on the presence of competent cells to respond to these stimuli.

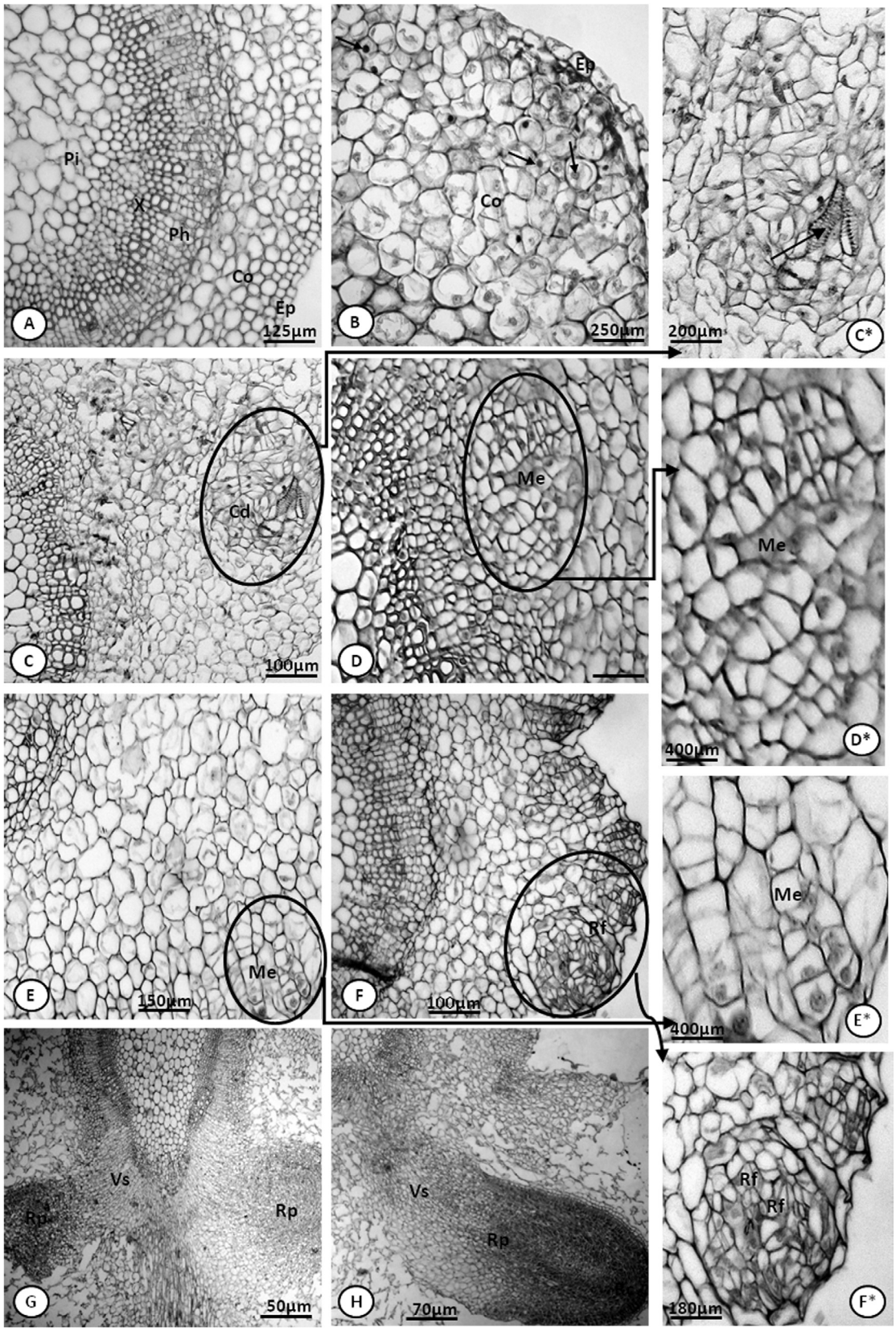

FIG. 1

Sections of the basal stem region, at the site of adventitious root formation, from 0 – 720 h after 14.7 mM IBA root-induction treatment. Panel A, anatomical structure of the stem-base before root-induction treatment, showing a vascular bundle (Pi, pith; Co, cortex; Ep, epidermis; Ph, phloem; X, xylem). Panel B, a transverse section near the stem-base at 96 h. Cells in the cortex re-acquire a meristematic characteristics, with dense cytoplasm, large nuclei, and visible nucleoli (arrows) (Ep, epidermis; Co, cortex). Panel C, first cell divisions (Cd) at 144 h, leading to callus formation. A magnification of the circled region is presented in Panel C*. Xylem tracheids (arrow) are also visible. Panels D and E, stem sections after 240 h on rooting medium showing two meristemoid structures (Me) in the upper phloem in Panel D and in the cortex/sub-epidermal region in Panel E. Magnifications of the circled regions are presented in Panels D* and E*, respectively. Panel F, morphogenic root zones (Rf) developing from sub-epidermal cells 336 h after root-induction treatment. A magnification of the circled region is presented in Panel F*. Panels G and H, root primordia (Rp) at different developmental stages, 528 h after root-induction treatment. The root caps (Rc) and differentiated vascular systems (Vs) can be seen.

Table II
ANOVA of peroxidase and polyphenol oxidase activity data during adventitious rooting of in vitro*-cultured olive stem explants of 'Galega vulgar'*

Effect	F	Effect (df)	Error (df)	*P*
Time	21,482	26	50	≤ 0.001
Replicates	1,653	4	50	0.175
Intercept	3,996,108	2	25	≤ 0.001

Measurements were made at 14 time-points during the rooting process in three successive sub-cultures.

Enzyme activities

POX and PPO activities were evaluated during *in vitro* adventitious root formation on explants of the olive cultivar 'Galega vulgar'. Possible correlations with the different anatomical stages of the rooting process described above were investigated.

No significant differences were observed between the three sub-cultures used as replicates in these assays, whereas significant differences were recorded in both enzyme activities during the rooting process (Table II). Data were submitted to the Fisher LSD test to discriminate confidence intervals at $P \leq 0.05$ and to identify homogeneous groups (Figure 3).

POX activity decreased by 50% during the first 96 h. Within this period, two significant decreases, each of approx. 25%, were detected between 0 – 4 h, and between 48 – 96 h.

The first decrease in POX activity (0 – 4 h) was probably related to the quick-dip treatment in 147 mM IBA. Exogenous IBA, or IBA included in the rooting medium may have the ability to reduce POX activity (Cho *et al*., 2011). The second decline in POX activity (48 – 96 h) resulted in it reaching its lowest level during the whole experiment, and coincided with the first sign of changes in stem cell morphology, as shown in Figure 1B.

It may therefore be assumed that the low POX activity at 96 h correlated with the induction phase of adventitious root formation, as proposed by Gaspar *et al*. (1992; 1994).

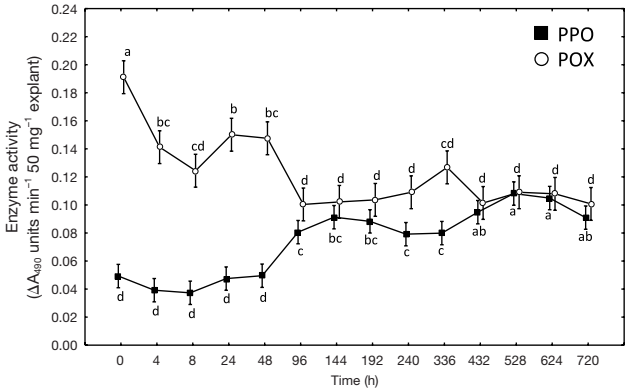

Fig. 3
Changes in peroxidase (POX) and polyphenol oxidase (PPO) activities at different time-points during the development of adventitious roots on *in vitro*-cultured 'Galega vulgar' olive microshoots. Vertical bars denote ± standard errors. Different lower-case letters on the datum points for each enzyme correspond to significant differences at the 95% probability level.

A reverse trend was observed in PPO activity. PPO activity doubled up to 144 h, after a non-significant decrease during the first 8 h. This inverse relationship between POX and PPO activities during the initial phases of adventitious rooting was also observed by Cheniany *et al*. (2010), who concluded that the decrease in PPO activity might cause an accumulation of monophenolic compounds that stimulated POX activity.

A gradual increase in POX activity was observed between 96 – 336 h, with a peak at the end of this period, which differed significantly from the level recorded at 96 h. This increase in POX activity corresponded to the histological changes leading to the formation of root meristemoids (Figure 1D, E) and observation of the first morphogenic root zones (Figure 1F). During the same period, PPO activity remained more-or-less constant, with a non-significant decrease observed between 144 – 336 h.

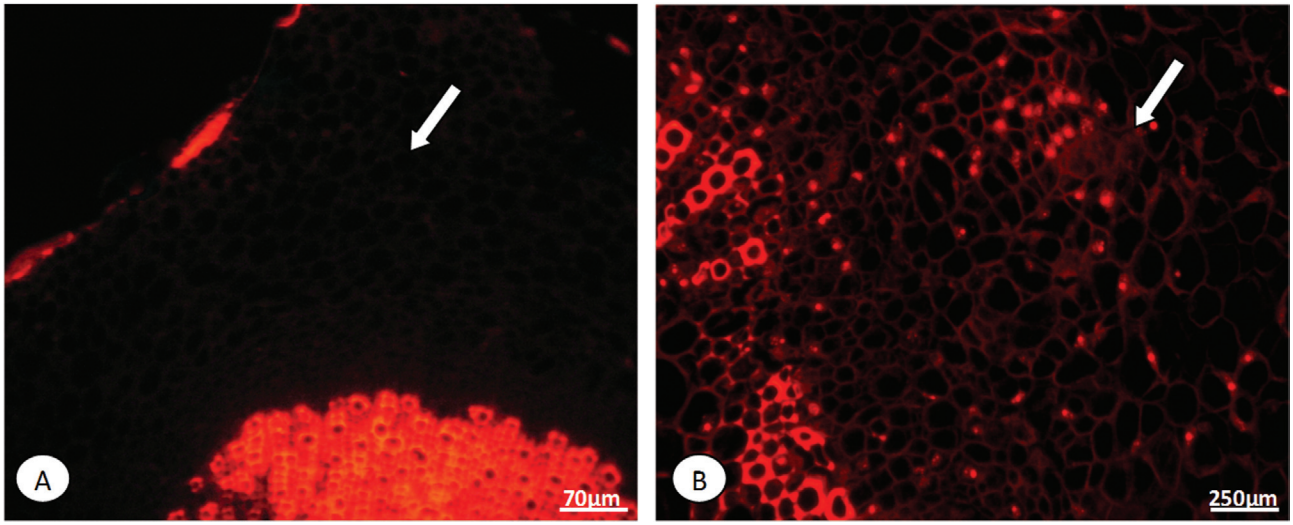

Fig. 2
Sections of the stem-base region at different stages of adventitious root formation observed under fluorescent light. Panel A, a transverse section of the stem-base before root-induction treatment. Arrow indicates the cortex region where no fluorescent signal can be observed due to an absence of mitotic activity and/or lignin deposition. Panel B, a transverse section of a stem-base 240 h after root-induction treatment was applied. A high rate of mitotic events can be observed in the upper-phloem and cortex regions, where cell nuclei and nucleoli, as well as lignin in cell walls, exhibit intense fluorescence (arrow). The image presented in Panel B corresponds to the same image presented in Figure 1D, where it was observed without fluorescent lighting. Scale bars = 70 µm (Panel A) or 250 µm (Panel B).

The increase in POX activity detected between 96 – 336 h may correspond to the initiation phase of rooting, as proposed by Gaspar *et al*. (1992; 1994). Nevertheless, POX activity remained significantly below those values measured before this period, which was not common in other situations where a clear relationship between POX activity and root initiation have been reported (Gaspar *et al*., 1992; Rival *et al*., 1997; Rout *et al*., 2000).

From 336 – 528 h, PPO activity increased significantly, while a significant decrease was observed in POX activity. This behaviour coincided with the intense mitotic activity observed at that time, during the development of newly-formed root meristems (Figure 1G, H).

Both enzyme activities then showed the same declining trend until the end of the period of observation (720 h), corresponding to the phase of root expression which, according to Gaspar *et al*. (1992), was characterised by a gradual drop in POX and PPO activities.

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