

Universidade de Évora - Instituto de Investigação e Formação Avançada
Universidade do Algarve - Faculdade de Ciências e Tecnologia

Programa de Doutoramento em Ciências Agrárias e Ambientais

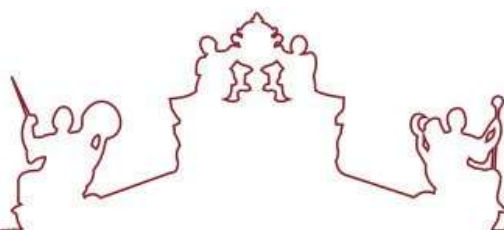
Tese de Doutoramento

New Insights into Somatic Embryogenesis Efficiency in *Olea europaea* L.

Rita Nobre Pires

Orientador(es) | Augusto António Peixe
Hélia Cristina Cardoso

Évora 2026



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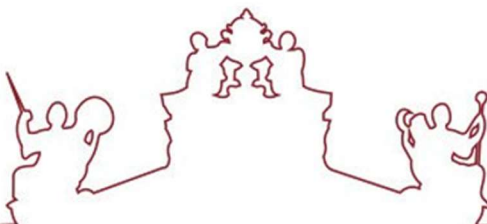
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A tese de doutoramento foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor do Instituto de Investigação e Formação Avançada:

Presidente | Luís Leopoldo Silva (Universidade de Évora)

Vogais | Andreia Cristina Silva Viegas Mata Figueiredo (Universidade de Lisboa)
Hélia Cristina Cardoso (Universidade de Évora) (Orientador)
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Tiago dos Santos Lourenço (Universidade de Nova de Lisboa)

Évora 2026

*O que dá o verdadeiro sentido ao encontro é a busca, e é preciso andar muito para se
alcançar o que está perto*

José Saramago

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A realização desta tese contou com o contributo, apoio e incentivo de várias pessoas que, de diferentes formas, marcaram este percurso. A todas elas deixo o meu sincero agradecimento.

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Novos contributos para o estudo da eficiência da embriogénese somática em
Olea europaea L.

RESUMO

A embriogénese somática (ES) apresenta-se como uma estratégia alternativa para a propagação clonal de oliveira, com particular interesse em génotipos caracterizados por elevada recalcitrância ao desenvolvimento de raízes adventícias quando propagados pelos métodos convencionais. No entanto, a elevada recalcitrância dos tecidos adultos à indução de estruturas embriogénicas continua a restringir a aplicação desta tecnologia. Assim, o estudo dos mecanismos moleculares associados à resposta embriogénica assume particular relevância para o desenvolvimento de sistemas de propagação alternativos aplicáveis à olivicultura nacional. A ES constitui uma resposta morfogénica induzida por estímulos exógenos que envolve uma reprogramação celular ao nível molecular, refletida em alterações bioquímicas e fisiológicas que culminam na diferenciação de estruturas embriogénicas. Neste processo, a interação entre os explantes e o meio de cultura é determinante, incluindo o contacto com biomoléculas secretadas pelo material vegetal. Este intercâmbio de moléculas, nomeadamente proteínas e metabolitos, entre os espaços intra e extracelulares poderá participar na modulação da resposta embriogénica. O presente estudo teve como objetivo identificar mecanismos envolvidos na eficiência da ES em oliveira através da caracterização do proteoma de linhas embriogénicas de alta (HE) e baixa (LE) eficiência e correspondente secretoma, por LC-MS/MS, complementada pela análise do exometaboloma por ¹H RMN. A capacidade das biomoléculas secretadas para induzir a resposta embriogénica foi avaliada através da aplicação de meio de cultura (CM) de linhas HE a linhas LE e material adulto. Os resultados revelaram uma maior abundância de proteínas associadas ao transporte vesicular, sinalização e reorganização celular em linhas HE, que apresentaram, no perfil extracelular, proteínas relacionadas com a regulação redox e a dinâmica da parede celular, bem como uma utilização mais eficiente do carbono. A aplicação do CM permitiu validar o papel das moléculas secretadas na resposta embriogénica ao promover um aumento da proliferação celular e a formação de estruturas embriogénicas em material recalcitrante.

Palavras-chave: Oliveira; embriogénese somática; culturas berço; secretoma; recalcitrância

ABSTRACT

Somatic embryogenesis (SE) has emerged as an alternative strategy for the clonal propagation of olive trees, particularly for genotypes characterised by high recalcitrance to adventitious root development when propagated using conventional methods. However, the high recalcitrance of adult tissues to the induction of embryogenic structures continues to limit the application of this technology. Therefore, understanding the molecular mechanisms associated with the embryogenic response is of particular importance for the development of alternative propagation systems applicable to the national olive industry. SE is a morphogenic response induced by exogenous stimuli that involves cellular reprogramming at the molecular level, reflected in biochemical and physiological changes that culminate in the differentiation of embryogenic structures. During this process, the interaction between explants and the culture medium is crucial, including exposure to biomolecules secreted by plant tissues. This exchange of molecules, particularly proteins and metabolites, between intracellular and extracellular compartments may contribute to the modulation of the embryogenic response. The present study aimed to identify mechanisms involved in SE efficiency in olive through the characterisation of the proteome of high-efficiency (HE) and low-efficiency (LE) embryogenic lines and their corresponding secretome by LC–MS/MS, complemented by exometabolome analysis using ¹H NMR. The ability of secreted biomolecules to induce the embryogenic response was assessed by applying conditioned medium (CM) from HE lines to LE lines and adult material. The results revealed a higher abundance of proteins associated with vesicular transport, signalling, and cellular reorganisation in HE lines, which also exhibited, at the extracellular level, proteins related to redox regulation and cell wall dynamics, together with more efficient carbon utilisation. Application of the CM validated the role of secreted molecules in the embryogenic response by promoting increased cell proliferation and the formation of embryogenic structures in recalcitrant material.

Keywords: Olive; somatic embryogenesis; nurse cultures; secretome; recalcitrance

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ABBREVIATIONS, SYMBOLS AND UNITS

ABBREVIATIONS

ABA	Absciscic acid
ACN	Acetonitrile
AMY	Amylase
APX	Ascorbate peroxidase
CAN	Acetonitrile
AGPs	Arabinogalactan proteins
APF	Apoplastic fluid
APS	Ammonium persulfate
ATP	Adenosine triphosphate
ATPB	Adenosine triphosphate synthase subunit beta
BA	6-benzyladenine
BAK	Brassinosteroid insensitive-associated kinase
BIP	Binding immunoglobulin protein
BRI	Brassinosteroid insensitive
BSA	Bovine serum albumin
CAA	Chloroacetamide
CE	Capillary electrophoresis
CE-MS	Capillary electrophoresis-mass spectrometry
CM	Conditioned medium
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DAPs	Differentially expressed proteins
DIR	Dirigent
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECO	Embryogenesis cyclic olive
EPS	Electrophoresis power supply
ER	Endoplasmic reticulum
ESI	Electrospray ionization
EVs	Extracellular vesicles
EXPO	Exocyst-positive organelle
FA	Formic acid

FASP	Filter-aided sample preparation
FDR	False discovery rate
FER	Feronia
FLA	Fasciclin-like arabinogalactan
GA	Gibberellic acid
GC	Gas chromatography
GC-MS	Gas Chromatography–Mass Spectrometry
GLN	Glutamine
GLPs	Germin-like proteins
GO	Gene Ontology
GPI-Aps	Glycosylphosphatidylinositol-anchored proteins
GTPase	Guanosine triphosphatase
HCD	Higher-energy collisional dissociation
HCl	Hydrochloric acid
HE	High efficient
HPLC	High-performance liquid chromatography
HSPs	Heat shock proteins
IBA	Indole-3-Butyric Acid~
IT	Injection time
iTRAQ	isobaric Tags for relative and absolute quantification
KEGG	Kyoto encyclopedia of genes and genomes
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LE	Low efficient
LDH	Lactate dehydrogenase
LFQ	Label-free quantitative
LTPs	Lipid transfer proteins
MAD	Median absolute deviation
MCC	Maximal clique centrality
MDH	Malate dehydrogenase
MIRO	Mitochondrial Rho GTPase
miRNA	Micro ribonucleic acid
MNA	1-methylnicotinamide
MS	Mass spectrometry
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide, oxidized

NADH	Nicotinamide adenine dinucleotide, reduced
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NMR	Nuclear magnetic resonance
OMc	Olive medium culture
OT-2	Opentrons OT-2
PEN	Penetration
PRXIIF	Peroxiredoxin-IIF
PMS	Phenazine methosulfate
PMSF	Phenylmethanesulfonyl fluoride
POD	Peroxidase
PSMs	Peptide spectral matches
PVP	Polyvinylpyrrolidone
RAPD	Random amplified polymorphic deoxyribonucleic acid-
REDOX	Reduction–oxidation reaction
Rho	Ras homologous
RLKs	Receptor-like kinases
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
SE	Somatic embryogenesis
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNAs	Small interfering ribonucleic acids
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SP3	Single-Pot Solid-Phase-enhanced Sample Preparation
sRNAs	Small non-coding ribonucleic acids
SSR	Simple sequence repeat
S-TRAP	Suspension Trapping
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl) phosphine
TEAB	Triethylammonium bicarbonate
TEMED	Tetramethylethylenediamine
TET	Tetraspanins
TMT	Tandem mass tag
TOM	Topological overlap matrix
TPX	Phioredoxin peroxidase

TSP	Trimethylsilylpropanoic acid
UHPLC	Ultra-high-performance liquid chromatography
UPLC	Ultra performance liquid chromatography
UPS	Unconventional protein secretion
UV	Ultraviolet
WGCNA	Weighted gene co-expression network analysis
1D	One dimensional
2DE	Two-dimensional polyacrylamide electrophoresis
2DE-DIGE	Two-dimensional differential in-gel electrophoresis
2iP	6-dimethylallylamino-Purine
2,4D	2,4-dichlorophenoxyacetic acid

SYMBOLS AND UNITS

°C	Celsius degrees
µg	Microgram
µL	Microlitre
µmol	Micromole
cm	Centimetres
g	Gram
g	Esth's gravitational acceleration
h	hour
Hz	Hertz
kDa	Kilodalton
L	Litre
m	Meters
M	Molar
mg	Milligrams
MHz	Megahertz
min	Minutes
mL	Millilitres
mm	Millimeters
mM	Millimolar
ppm	Parts per million
s	Second
v	volume

CHAPTER I

Introduction

I. 1. INTRODUCTION

Somatic embryogenesis (SE) has been employed for the mass propagation of several agronomically important plant species, offering advantages over microcuttings and semi-hardwood cuttings, including high rates of direct regeneration, efficient embryo conversion, and the production of healthy plants. Nevertheless, SE has not yet been routinely applied to the propagation of olive (*Olea europaea* subsp. *europaea*) because of the recalcitrant behaviour that characterises most woody plant species. In olive, most SE protocols rely on juvenile plant material derived from zygotic embryos as initial explant, such as radicles and cotyledons. However, because zygotic embryos originate from open pollination, each embryo represents a unique genotype distinct from that of the donor plant, resulting in genetic variability and thereby limiting their use for clonal propagation.

The development of SE protocols based on adult material, currently the only reliable means of preserving genetic identity, has proved particularly challenging, mainly due to the recalcitrance of adult tissues used as initial explants. This difficulty explains the limited number of olive cultivars successfully established through this approach to date. Successful examples include the wild olive (*O. europaea* subsp. *sylvestris*), using petioles from mature leaves, the cvs. ‘Canino’ and ‘Moraiolo’ (Rugini and Caricato, 1995), and ‘Chetoui’ (Trabelsi et al., 2011), using foliar tissues from adult plants, as well as the cvs. ‘Dahbia’ and ‘Picual’ (Mazri et al., 2013; Toufik et al., 2014), using leaf segments from *in vitro*-cultured plantlets.

Different approaches have been undertaken to overcome the recalcitrant response of SE in adult tissues. The role of various factors influencing the embryogenic response has been investigated, with primary consideration given to the type of explant used for the establishment of *in vitro* cultures, its developmental stage and physiological status, growth conditions (particularly photoperiod and temperature), and the chemical composition of the culture medium (including the type and concentration of plant growth regulators) (Cardoso et al., 2010, 2019a, b; Loyola-Vargas, 2016). Studies in this field have predominantly focused on the plant material, particularly the explant source, genotype, and physiological status, as key determinants of embryogenic response. Nevertheless, an important factor reported to influence the SE response, yet not consistently afforded adequate attention and, to date, remaining unexplored in studies involving olive, is the release of organic bioactive molecules by explants into the culture medium (Quintana-Escobar et al., 2025). Previous studies developed in different plant species already demonstrate that molecules secreted into the extracellular space, which, in *in vitro* culture systems, corresponds to the culture medium, modulate SE efficiency, acting as inhibitors or stimulators of the embryogenic response. The use of so-called “cell nurse cultures” to promote somatic embryo formation in recalcitrant tissues has been employed for several decades (Eigel and Koop, 1989; Westcott, 1994; Komai et al., 2006; Hargreaves et al., 2017). In *Cichorium* species, the

use of conditioned culture medium has also been reported as an effective strategy to enhance SE and improve the response of explants (Couillerot et al., 2012).

Although originally established empirically, based on the assumption that “nurse cultures” modify the extracellular environment through the release of growth-promoting biomolecules essential for SE induction (Eigel and Koop, 1989), several studies have since focused on elucidating the composition of the culture medium through the identification of secreted molecules. Polysaccharides, amino acids, growth regulators, vitamins, proteins, lipophilic molecules, and secondary metabolites produced and secreted by plant tissues have been shown to play crucial roles in the induction and regulation of SE (Nic-Can et al., 2015).

Among the secreted biomolecules, extracellular proteins, collectively referred to as the secretome, perform essential functions in the differentiation and morphogenesis of somatic cells. Proteins such as arabinogalactan proteins, heat shock proteins, and peroxidases, which are involved in multiple aspects of plant growth and development, have been directly correlated with SE efficiency (Encina et al., 2023; Pernis et al., 2023). In addition, numerous other proteins have been proposed as biomarkers of embryogenic potential (Ben-Amar et al., 2021).

In parallel, the metabolite composition of the culture medium, including both the compounds originally present in the medium and the metabolites secreted by plant tissues, significantly influences SE, either enhancing or inhibiting embryogenic induction. In *Coffea canephora*, a species highly competent in SE, an inhibitory effect on somatic embryo formation was observed when caffeine and chlorogenic acid from *C. arabica*, a recalcitrant species, were applied to the culture medium (Nic-Can et al., 2015). Overall, the integrated characterisation of the secretome and metabolome enables a comprehensive understanding of the extracellular and intracellular factors influencing embryogenic induction, highlighting mechanisms that can be exploited to optimise *in vitro* propagation protocols.

I. 2. THESIS OBJECTIVES AND OUTLINE

The main aim of this work is to exploit the integration of proteomic and metabolomic approaches to contribute to a deeper understanding of the mechanisms regulating SE in *O. europaea*, while promoting the development of an alternative vegetative propagation protocol to conventional methods. To achieve this, embryogenic lines with contrasting efficiencies were established using zygotic embryos as initial explants, and the composition of the culture medium of embryogenic lines exhibiting that contracting behaviour was characterised to identify extracellular signals associated with embryogenic competence. This thesis is organised into ten chapters.

Chapter I presents the general framework of the thesis, introducing SE in plants and the role of the extracellular environment in plant tissue culture systems, thereby establishing the conceptual basis for the experimental work developed throughout the thesis. Attention is given to the processes involved in embryogenic induction and competence acquisition, as well as to the role of extracellular signals and intercellular communication in the regulation of SE.

Chapter II presents a comprehensive review of the molecules released into the culture medium during SE, with particular emphasis on secreted proteins and metabolites implicated in embryogenic competence. Even not explored in the present thesis, special attention is given to the emerging role of extracellular vesicles as mediators of targeted intercellular communication during early embryogenic reprogramming, which could be explored in future perspectives. The chapter discusses the molecular cargo transported by EVs—including stress-related and development-associated proteins—and examines how extracellular signals interface with intracellular regulatory pathways to coordinate cellular responses to SE stimuli.

Chapter III focuses on the establishment and evaluation of the embryogenic potential of zygotic embryo-derived explants from the cv. ‘Galega Vulgar’ and cv. ‘Arbequina’. Embryogenic lines were classified as high-efficiency (HE) and low-efficiency (LE) based on somatic embryo production, regenerative capacity, and incidence of tissue necrosis, establishing a robust experimental system for subsequent analyses.

Chapter IV describes the development of a protocol for the isolation of extracellular biomolecules from CM from a HE embryogenic line, including the removal of cellular contaminants by filtration, with purity assessed using a cytosolic marker to verify the absence of intracellular contamination. Protein and metabolite fractions were subsequently separated and concentrated by lyophilisation, enabling downstream proteomic and metabolomic analyses.

Chapter V applies the established methodology to conditioned medium (CM) obtained from three previously selected HE and three LE embryogenic lines. The secretome and exometabolome were characterised through high-throughput platforms, namely liquid chromatography–tandem mass spectrometry (LC–MS/MS) and proton nuclear magnetic resonance (^1H NMR), respectively, enabling the comparative analysis of differentially abundant components and their associated metabolic pathways in relation to embryogenic competence.

Chapter VI evaluated the applicability of the CM using a “nurse culture” approach to assess its influence on LE embryogenic lines. Additionally, its potential to promote morphogenic responses was tested during the induction of adult explants of the Portuguese cv. ‘Galega Vulgar’.

Chapter VII describes the LC–MS/MS analysis of embryogenic *calli* from both HE and LE embryogenic lines, using two alternative sample digestion methods. This approach was designed to explore the proteomic methodology of interest to support the integrated study of embryogenic tissues and their corresponding secretome, enabling the assessment of intracellular–extracellular molecular dynamics associated with SE. The comparative analysis of the digestion strategies further allowed the evaluation of workflow performance and its impact on the detection of embryogenesis-related proteins.

Chapter VIII presents a complementary gene- and protein-level analysis based on selected proteomic findings. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to evaluate the relative expression of genes corresponding to differentially expressed proteins, while western blotting was used to confirm protein abundance. Complementary gene- and protein-level analyses were performed to extend the proteomic perspective on the molecular regulation underlying SE, integrating intracellular and extracellular processes. Emphasis was placed on peroxidases, including the characterisation of this enzyme family in olive. Appropriate reference genes were selected to ensure robust normalisation and reliable comparison between embryogenic lines with contrasting efficiency.

Chapter IX presents the general conclusion, synthesising the major findings from the preceding chapters and their contribution to the understanding of SE. This chapter highlights the overall significance of integrating proteomic and metabolomic approaches to elucidate the molecular mechanisms underlying embryogenic competence in olive.

Chapter X outlines the future perspectives of this work, discussing potential follow-up studies and applications arising from the findings, as well as possible directions for further research in SE and its molecular regulation.

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CHAPTER II

Secreted molecules as modulators of somatic embryogenesis efficiency - an overview

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II. 1. ABSTRACT

Somatic embryogenesis (SE) is a biological process in which somatic cells undergo molecular reprogramming, leading to embryo formation. SE is considered the most efficient plant propagation system, supporting breeding programs that use genetic engineering/editing and omics-based research. However, its success depends on a complex interaction between internal and external factors, including biomolecules released by explants into the culture medium. The use of nurse cell cultures, established decades ago, exemplifies this process. Nevertheless, few studies have characterised the secreted molecules involved or investigated their role in SE efficiency. This review compiles current knowledge on molecules released during SE, focusing on secreted proteins and metabolites. In addition, the involvement of extracellular vesicles (EVs) as modulators of SE response is hypothesised, emphasising their capacity to mediate targeted communication during the early stages of embryogenic reprogramming. EVs transport regulatory biomolecules and may participate in coordinating the cellular responses triggered by SE-inducing stimuli, as supported by the recurrent identification of EVs-associated proteins linked to stress perception and developmental signalling. Understanding how these extracellular factors interface with intracellular pathways could advance efforts to optimise SE and broaden its biotechnological applications.

II. 2. INTRODUCTION

Somatic embryogenesis (SE) is a morphogenic process driven by complex reprogramming mechanisms that enable cell dedifferentiation, reacquisition of meristematic competence, and the *de novo* differentiation of embryogenic structures (Aguilar-Hernández & Loyola-Vargas, 2018). Regardless of its occurrence under natural conditions, SE has been gaining high practical interest for the large-scale *in vitro* propagation of elite cultivars. It gains particular relevance for clonal propagation of woody plant genotypes that are recalcitrant to conventional methods, such as the semi-hardwood cuttings and the microcuttings (Egertsdotter et al., 2019). It also represents the most promising *in vitro* regeneration system to support molecular breeding programs, owing to its high efficiency in regenerating whole plants from single cells (Bogdanović et al., 2021).

According to the theory of plant cell totipotency, any living plant cell that maintains its integrity can undergo a complex reprogramming process involving molecular, biochemical, and physiological changes, ultimately leading to the development of a new plant (Nic-Can et al., 2015). Despite this broad definition of totipotency, it is well known that not all explants respond similarly to embryogenic stimulus, even when taken from the same plant (Fehér, 2019). The developmental and physiological status of the explant tissue are key factors that define the cell's ability to respond efficiently to SE stimuli. This is particularly evident in woody species, where SE efficiency declines with tissue ageing

(Cardoso et al., 2010; Nic-Can & Loyola-Vargas, 2016; Hazubska-Przybył et al., 2020). Explant age has thus been recognised as a major limiting factor in the success of regeneration systems involving de novo morphogenesis (Nhut et al., 2023; Zhang et al., 2025). Protocols based on SE induction, established for woody plant species, report mainly the use of tissues taken from zygotic embryos (Corredoira et al., 2013; Kim, 2015; Pires et al., 2020; Pires et al., 2025a) or tissues involved in the development of micro or macrogametophytes (Cardoso et al., 2019; Cardoso et al., 2010). In comparison with older tissues, juvenile tissues contain a higher number of meristematic cells contributing to efficient cell response upon the SE stimulus (Yang & Zhang, 2010).

Moreover, several events control this morphogenic process, which is initiated by an SE stimulus perceived by plant cells as a stressful condition (Arnholdt-Schmitt et al., 2016). After stimulus, plant cells immediately activate several signalling cascades that lead to changes in gene expression patterns responsible for the molecular reprogramming events, essential for the reacquisition of cell meristematic characteristics and expression of totipotency (Elhiti et al., 2022). According to Leljak-Levanic et al. (2011), most of the genes expressed during SE encode proteins belonging to four different categories: a) transcription factors; b) proteins acting in the cell cycle; c) enzymes involved in the biosynthesis of growth regulators, most notably auxins; and d) proteins involved in signalling pathways. More recently, epigenetic mechanisms acting as regulators of gene expression have also been highlighted, with particular emphasis on DNA methylation (Cordeiro et al., 2023; Peng et al., 2025) and miRNA (Ferraz et al., 2023; Cordeiro et al., 2025).

Although extensive research has elucidated the molecular mechanisms underlying SE and its efficiency, most studies have focused on plant tissue, and only a few have considered the role of the extracellular environment. At the tissue level, the apoplast – comprising the continuum external to the plasma membrane, including cell walls and intercellular spaces – plays a fundamental role in maintaining cellular integrity and function (Wang et al., 2018). The apoplastic fluid (APF), which circulates within this compartment (Tanveer et al., 2014), is a key mediator of multiple physiological processes, including the passive transport of water, ions, and nutrients, thereby facilitating mass flow across tissues, as well as plant defence responses and intercellular signalling involved in stress adaptation (Nielsen & Thordal-Christensen, 2013; Karimi et al., 2025). Establishing a parallel between the whole-plant system and *in vitro* liquid cell culture systems, the culture medium can be considered a substitute for the APF, functioning as a surrogate extracellular environment. In this context, the culture medium accumulates biomolecules secreted by cells, including a diverse array of proteins, metabolites, and nucleic acids (such as miRNAs and siRNAs) released through different secretory mechanisms, as well as extracellular vesicles (EVs), which transport these and other bioactive molecules involved in intercellular communication (Woith et al., 2021). This culture medium containing secreted biomolecules

is referred to by several authors as conditioned medium (CM) (Umehara et al., 2005; Nic-Can et al., 2015).

The role of CM in modulating SE efficiency has been recognised since the 1980s, when nurse culture systems were introduced to promote the formation of somatic embryos in recalcitrant tissues (Eigel & Koop, 1989). Several studies have aimed to identify the medium components responsible for modulating SE responses and promoting growth, mostly focusing on proteomics and metabolomics approaches. Extracellular proteins, collectively referred to as the secretome, have been reported to regulate several biological and physiological processes, including signalling, cell proliferation, responses to stress conditions, and cell–cell interactions (Tanveer et al., 2014), which therefore could function as promoters or modulators of SE (Duchow et al., 2016). Likewise, several extracellular metabolites have been identified as positive or negative regulators of SE responses (Nic-Can et al., 2015). Regarding the EVs, besides their identification in liquid cell cultures, their potential involvement in morphogenic processes in plants remains largely unexplored. In mammals, EVs have been shown to play crucial roles in physiological processes and cell-to-cell communication, and they can even induce secondary embryonic development by regulating organogenesis (Krause et al., 2018; Mulzer et al., 2025). Consequently, vesiculomics, the comprehensive study of EVs, including their biogenesis, molecular composition, functions and roles in cell-to-cell communication (Clift et al., 2024; Meng et al., 2024), has emerged as a rapidly expanding field in animal research (Mazzarella et al., 2024). Similar to animal systems, plant-derived vesicles can transport small non-coding RNAs (sRNAs), including small interfering RNAs (siRNAs) and microRNAs (miRNAs), suggesting a regulatory role in gene expression and potential involvement in cell proliferation and differentiation (Cui et al., 2020; Rito et al., 2025). In the context of SE, it can be hypothesised that these EVs may carry key biomolecules that mediate molecular reprogramming, thereby modulating cellular embryogenic competence.

Understanding these secreted factors may contribute to the development of improved plant propagation protocols and open new perspectives for biotechnological applications. This review provides an overview of the current knowledge regarding proteins and metabolites involved in SE that have been identified in the extracellular environment and may act as SE modulators, some of which may be secreted via EVs-mediated pathways. In addition, the hypothesis that EVs are secreted by *in vitro* embryogenic calli (EC) and act as modulators of SE efficiency through the transport of bioactive molecules will be explored.

II. 3. MECHANISMS FOR SECRETION OF CELLULAR BIOMOLECULES

Secretion is central to plant cellular function, enabling the delivery of biomolecules to the extracellular space and supporting intercellular communication, defence, and developmental plasticity (Ten et al., 2025). These pathways mediate the exchange of proteins and metabolites required for cell

wall remodelling, signal transmission, and the establishment of a supportive extracellular environment for embryogenic development.

While the proteome represents the complete set of proteins expressed in a particular cell, tissue, or organ at a given time and under specific conditions (Garrels, 2001), the secretome, in turn, comprises the subset of these proteins that are actively secreted into the extracellular matrix via defined secretory mechanisms (Tanveer et al., 2014). Protein secretion occurs through both the conventional endoplasmic reticulum (ER)–Golgi pathway and the unconventional protein secretion (UPS) pathways, mechanisms recently reviewed by Wang et al. (2018) (Figure II.1). In the classical pathway, proteins are transported from the ER to the Golgi apparatus, passing through the trans-Golgi network, which sorts them into vesicles directed to the plasma membrane, the cell surface, or intracellular compartments such as endosomes and lysosomes (Wang et al., 2018; Ford et al., 2021). These vesicles subsequently travel through cytoskeletal filaments before fusing with the plasma membrane to release their contents outside the cell. While the conventional pathway predominates, the UPS pathway also contributes to protein export and may be involved in shaping the extracellular environment associated with developmental transitions (Wang et al., 2018; Cui et al., 2020).

The UPS pathway encompasses various mechanisms by which plant cells export biomolecules independently of the classical ER–Golgi route (Wang et al., 2018) (Figure II.1). These include (i) non-vesicular transport, in which molecules are directly translocated across the plasma membrane via specific transporters or channels; (ii) vesicle-mediated secretion involving intracellular organelles; and (iii) the release of EVs. Plant EVs are lipid-bound structures secreted into the extracellular environment under both physiological and stress conditions (Doyle & Wang, 2019). They carry diverse cargo, including proteins, lipids, carbohydrates, metabolites, and nucleic acids such as small regulatory RNAs, either within their lumen or on their surface, highlighting their role in intercellular communication and long-distance plant defence (Rutter & Innes, 2017; Cui et al., 2020; Zheng et al., 2022). In addition, EVs-associated cargo has been linked to developmental reprogramming processes, suggesting a potential role in modulating the extracellular environment during cellular transitions. Among the proteins commonly enriched in these EVs are tetraspanins (e.g., TET8) (Cui et al., 2020), the secretory syntaxin PEN1 (Liu et al., 2022), and heat shock protein 70 (HSP70) (Ngcala et al., 2020), all associated with EVs biogenesis or stress-related cargo. Proteomic analyses of plant EVs have revealed a diverse set of proteins involved in stress responses, signalling, and cellular remodelling (Chaya et al., 2024), many of which overlap with those reported in the secretome associated with developmental reprogramming processes. In this context, proteins linked to SE-related pathways may also be exported via EVs, suggesting a potential role for these vesicles in shaping the extracellular environment required for embryogenic competence. In addition to proteins, EVs transport diverse metabolites and small RNAs implicated in signalling and developmental regulation (Xiao et al., 2026), further supporting their

involvement in modulating the extracellular environment during such transitions. These aspects will be further explored in the following sections.

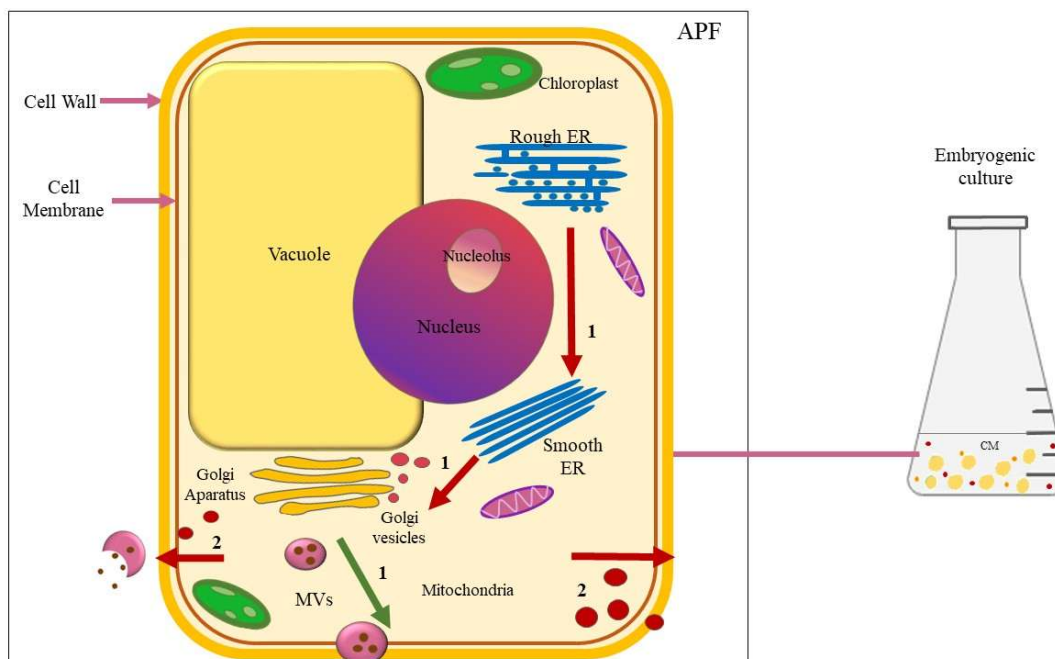


Figure II.1. Overview of extracellular proteins secretion pathways by embryogenic cultures into culture medium, following a similar procedure described for apoplastic fluid (APF). 1) Conventional secretion pathway through the endoplasmic reticulum (ER) system, Golgi apparatus and by secretory vesicles, which constitute the exocytosis pathway; 2) Non-canonical or UPS secretion pathway through direct transfer across the plasma membrane; 2a) secretion from endosomal/multivesicular body-related components; 2b) release within plasma membrane-derived extracellular vesicles (EVs) (2c).

Plant EVs are generally classified into three main types based on their biogenesis, size, and cargo composition (Chukhchin et al., 2020; Joshi et al., 2020) (Figure II.2). Exocyst-positive organelles (EXPOs) are double-membrane compartments, distinct from endosomes and autophagosomes, that mediate the exocytosis of cytosolic material to the cell wall and thus represent vesicle-mediated secretion mechanisms. that may contribute to EVs-mediated delivery (Lin et al., 2015). Microvesicles (or ectosomes) are formed by outward budding of the plasma membrane, whereas exosomes (30–150 nm in diameter) are generated within multivesicular bodies (MVBs) and released upon their fusion with the plasma membrane (Liu et al., 2025). Although plant UPS and EVs pathways remain poorly understood, at least three distinct vesicle-mediated secretion mechanisms have been described: (1) EXPO-mediated secretion, (2) outward or inward budding of vesicles, including EVs, from the plasma or endosomal membrane, and (3) fusion of MVBs with the plasma membrane (Cui et al., 2020). EXPO enables the direct transport of cytosolic proteins to the cell wall or extracellular environment (Ding et al., 2014), with the exocyst complex mediating the fusion of post-Golgi vesicles to the plasma membrane

(Wang et al., 2018). Exocytosis is integrated with the endomembrane trafficking network, including endocytosis, endosome dynamics, and autophagy (Žárský, 2022), ultimately delivering luminal contents to the apoplast through a two-step process: fusion of the outer membrane with the plasma membrane, followed by degradation of the inner membrane (Ding et al., 2014). Autophagosomes then fuse with the lysosome or vacuole to complete cargo processing (Lin et al., 2015).

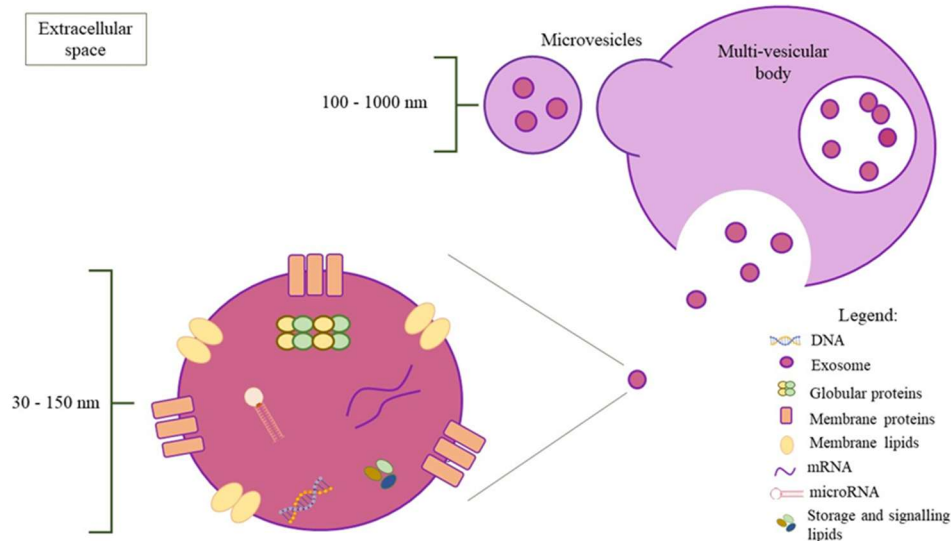


Figure II.2. Exosome and microvesicle biogenesis. Extracellular vesicles (EVs) shown include exosomes and microvesicles (MVs). Exosomes are generated within multivesicular bodies (MVBs), which fuse with the plasma membrane to release their intraluminal vesicles.

The UPS also mediates the extracellular transport of diverse small molecules, particularly secondary metabolites. These may be secreted passively, by diffusion or osmosis when intracellular concentrations are high, or actively via energy-dependent mechanisms. Mauseth (2001) described three types of active secretion: (i) holocrine, involving cell rupture and disintegration; (ii) granulocrine, in which products are stored in vesicles or transported by ATP-dependent processes; and (iii) eccrine, where transporters mediate molecular export across the plasma membrane, also ATP-dependent. Lipids and hydrophobic secondary metabolites are often enriched in exosomes, reflecting their endosomal origin and lipid-rich membranes, which facilitate stable delivery into the extracellular environment and potentially contribute to the conditions required for embryogenic competence.

II. 4. INVOLVEMENT OF SECRETED PROTEINS IN SOMATIC EMBRYOGENESIS

Secreted proteins, collectively referred to as the secretome, mediate key processes such as cell–cell communication, cell wall remodelling, and stress signalling, all of which are essential for the

induction and progression of SE. Advances in proteomic technologies have enabled the large-scale identification of secreted proteins from the CM of embryogenic cultures. This chapter reviews the methodological approaches used in secretome profiling, highlighting the importance of extraction and sample preparation strategies as key determinants of the subset of proteins ultimately identified. It also examines the analytical methodologies employed, including gel-based and gel-free proteomics, as well as the functional roles of identified secreted proteins in the context of SE.

II. 4.1. Protein Extraction and Sample Preparation

Secretome analysis in plant systems presents distinct technical challenges compared to total proteome studies, as it focuses on proteins released into the extracellular space or culture medium, often at low abundance and within complex biological matrices. Within this context, extracellular proteins include both secreted proteins and cell wall-associated proteins, which may range from loosely bound components that freely diffuse into the culture medium to more tightly associated proteins embedded in the cell wall polysaccharide matrix or APF. Consequently, their recovery depends strongly on the extraction strategy employed, which may differentially target loosely or tightly bound protein fractions (Ghahremani et al., 2016). Unlike whole-tissue proteomics, where protein extraction is performed directly from biological material, secretome studies rely on the efficient recovery of extracellular proteins while minimising interference from residual medium components and non-secreted proteins. Low protein abundance, contamination from co-isolated components, and sample loss during preparation can further limit reproducibility and sensitivity in mass spectrometry-based workflows (Mattamana et al., 2025).

The protocols described for the recovery of extracellular proteins from culture medium include centrifugal filtration (Charmont et al., 2005; Pires et al., 2025b), tangential flow ultrafiltration (Basu et al., 2006), and freeze-drying (Oh et al., 2005; Tran & Plaxton, 2008). In some cases, an additional concentration step is applied, typically involving solvent precipitation using acetone or methanol (Kaffarnik et al., 2009; Pires et al., 2025b). Additional strategies to improve protein yield and stability have also been reported. For instance, the inclusion of polyvinylpolypyrrolidone (PVPP), which contributes to the removal of phenolic compounds and reduces non-specific interactions that can lead to protein aggregation and precipitation, has been shown to enhance protein recovery and expand proteome coverage (Charmont et al., 2005).

In addition, the release of intracellular proteins into the extracellular environment, due to cell damage or lysis during sample handling, represents a well-recognised limitation in secretome studies. This can compromise the specificity of the extracellular proteome, requiring careful experimental design and data interpretation to distinguish truly secreted proteins from potential contaminants. To assess

sample purity, the presence of intracellular marker enzymes, such as malate dehydrogenase (Loureiro et al., 2011; Figueiredo et al., 2021; Pires et al., 2025b), aldolase (Ebner et al., 2016), and glucose-6-phosphate dehydrogenase (Oh et al., 2005), is commonly evaluated using immunoblotting or enzymatic activity assays. In suspension cultures, direct microscopic observation has also been employed to assess cell viability and detect dead or damaged cells that may contribute to secretome contamination (Alexandersson et al., 2013). Overall, careful optimisation of protein extraction and sample preparation is essential to ensure reliable secretome characterisation and to minimise factors that may affect downstream proteomic data analyses.

The recovery of extracellular proteins can be achieved through two main approaches: collection from CM in liquid culture systems or extraction of APF from intact plant tissues. CM-based extraction is a non-destructive strategy in which proteins secreted by cultured cells or tissues accumulate naturally in the surrounding medium, allowing repeated sampling and generally providing sufficient material for downstream analyses (Abbineni et al., 2022). Nevertheless, CM may also contain intracellular proteins released from damaged or senescent cells, particularly in long-term cultures, making cell viability assessment essential (Park et al., 2021). In contrast, APF extraction provides more direct access to the native extracellular space and proteins associated with the cell wall. This approach typically involves vacuum infiltration followed by centrifugation or other mechanical procedures to recover APF contents (Chen et al., 2025). However, because tissue manipulation is required, APF extraction is inherently more invasive and more prone to contamination by cytoplasmic proteins released during processing. Consequently, the reliability of both CM and APF-based secretome analyses strongly depends on careful sample preparation and rigorous quality control procedures to minimise intracellular contamination and ensure accurate characterisation of extracellular proteins.

II. 4.2. Methods used for Identification of Secreted Proteins – the Secretome Profile

The first analysis in this research field was conducted using classical proteomic methods, including gel-based techniques such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing, and two-dimensional polyacrylamide electrophoresis (2DE) (reviewed by Tchorbadjieva, 2006). Nonetheless, gel-free proteomics methods such as Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have emerged in recent years, demonstrating high applicability on secretome characterisation related to SE (Pernis et al., 2023; Pires et al., 2025b).

II. 4.2.1. Gel-based proteomics techniques

Gel-based proteomics techniques have been widely used to analyse protein expression profiles and characterise the secretome associated with SE. SDS-PAGE is a commonly applied method for separating complex protein mixtures according to their molecular mass, allowing the resolution of proteins ranging approximately from 5 to 250 kDa (Wijethunga & Emenike, 2024). Several studies on SE have employed SDS-PAGE to detect extracellular proteins (see Table II.1). These analyses revealed distinct protein profiles among the studied species, with many proteins identified as extracellular or associated with the cell wall. Interestingly, some extracellular proteins were not detected in cell wall extracts, highlighting the complexity and specificity of protein localisation within plant cell walls. However, owing to the intrinsic limitations of SDS-PAGE in resolving complex protein mixtures and enabling definitive protein identification, it is commonly complemented by additional techniques, such as 2DE and Western blotting, allowing more comprehensive protein separation and characterisation.

Among gel-based methods, 2DE remains one of the most widely used techniques for protein separation (see Table II.1). It is considered a powerful tool for the high-resolution separation and fractionation of complex protein mixtures from biological samples (Magdeldin et al., 2014) and has been extensively applied in SE studies. Nevertheless, several technical limitations have been reported, including issues related to reproducibility, limited detection of low-abundance proteins, and difficulties in analysing highly hydrophobic or extremely large or small proteins (Speda et al., 2017). To overcome variability between gels, the two-dimensional differential in-gel electrophoresis (2DE-DIGE) approach has been developed and applied. However, the requirement for specialised equipment, particularly laser scanners for gel image acquisition, remains a limitation for its broader application (Aguilar-Hernández & Loyola-Vargas, 2018).

Table II.21. Analytical techniques for protein identification from conditioned medium (CM) of embryogenic cultures across different plant species.

Protein group	Species	Inoculated material	Proteomic technique	References
Arabinogalactan	<i>Persea americana</i>	EC	HPLC	Encina et al., 2024
	<i>Daucus carota</i>	Cell culture	Crossed electrophoresis	Kreuger and van Holst, 1993
	<i>Gossypium hirsutum</i>	EC	SDS-PAGE; HPLC	Poon et al., 2012
	<i>Pelargonium sidoides</i>	EC	Size exclusion chromatography	Duchow et al., 2016
	<i>Pinus caribaea</i>	EC and NEC	SDS-PAGE, Western blot	Domon et al., 2000
	<i>Sorghum bicolor</i>	Shoots from <i>calli</i> culture	iTRAQ, LC-MS/MS	Ngcala et al., 2020
	<i>Pinus caribaea</i>	EC and NEC	SDS-PAGE, Western blot	Domon et al., 2000
Chitinases	<i>Dactylis glomerata</i>	Proembryogenic masses	SDS-PAGE, 2DE, Isoelectric focusing	Tchorbadjieva & Pantchev, 2006
	<i>Cucurbita pepo</i>	EC	Immunoblotting	Ben-Amar et al., 2010
	<i>Pinus nigra</i>	EC	SDS-PAGE, Electrophoresis	Fraterová et al., 2013
Esterases	<i>Arabidopsis thaliana</i>	EC	SDS-PAGE; MALDI-TOF	Yugay et al., 2023
	<i>Dactylis glomerata</i>	EC and NEC	SDS-PAGE	Tchorbadjieva & Odjakova, 2001
	<i>Marchantia polymorpha</i>	Cell culture	SDS-PAGE	Izumi et al., 1995
Germin-like proteins	<i>Olea europaea</i>	EC	LC-MS/MS	Pires et al., 2025b
	<i>Saccharum spp.</i>	EC and NEC	nanoUPLC/-HDMSE	Heringer et al., 2015
	<i>Pinus caribaea</i>	Cell lines	SDS-PAGE, Western blot	Domon et al., 1995
Lipid transfer proteins	<i>Daucus carota</i>	Cell culture	SDS-PAGE; RNA Gel Blot	Sterk et al., 1991
	<i>Hordeum vulgare</i>	Cell culture	Immunoblotting; SDS-PAGE	Mundy and Rogers, 1986
	<i>Vitis spp.</i>	Cell culture	SDS-PAGE	Coutos-Thevenot et al. 1992
		Cell culture	SDS-PAGE; HPLC	Coutos-Thevenot et al. 1993

Abbreviations: EC (Embryogenic *calli*; NEC (Non embryogenic *calli*); HPLC (High-performance liquid chromatography); iTRAQ (Isobaric tags for relative and absolute quantification); LC (Liquid chromatography); MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight); MS/MS (Tandem mass spectrometry); SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

II. 4.2.2. Gel-free proteomics techniques

Gel-free proteomics techniques, such as LC–MS/MS-based approaches (including label-free quantification and isobaric labelling strategies such as iTRAQ or Tandem Mass Tag, TMT), offer high sensitivity and accuracy with multiplexing capabilities that allow simultaneous identification and quantification of proteins from multiple samples in a single run. By minimising variation across MS runs, these approaches further improve analytical precision (Tian et al., 2019). However, these approaches also present certain limitations. Labelling-based methods, such as iTRAQ and TMT, while enabling the simultaneous comparison of multiple samples (up to 10), are relatively costly and may be affected by issues such as ratio compression, which can compromise quantitative accuracy. Label-free approaches, while more flexible and cost-effective, are more susceptible to run-to-run variability and missing values. In contrast, labelling-based strategies rely on the incorporation of distinct isotopic or isobaric mass tags, such as iTRAQ, TMT, N,N-dimethyl leucine, deuterium isobaric amine-reactive tags, 10-plex isobaric tags, and sulfoxide-based reagents, to enable multiplexed quantitative analysis (Chen et al., 2021). After labelling, the samples are pooled and analysed by MS, enabling the precise detection of dynamic changes in differential protein expression between samples.

In a study by Ngcala et al. (2020), iTRAQ was applied to analyse *Sorghum bicolor* cell suspension cultures upon heat stress, specifically examining secreted proteins enriched in the CM. This approach enabled the identification of 65 secreted proteins. Notably, 84% of these proteins possessed a predicted signal peptide, suggesting targeting through the classical secretory pathway. Nevertheless, to date, the application of LC-MS/MS-based techniques to protein expression profiling and secretome characterisation in SE has remained limited (Table II.1).

II. 4.3. The Involvement of Secreted Proteins in Somatic Embryogenesis

Several proteins secreted through conventional pathways into the extracellular space – contributing to the CM composition – have been identified as key modulators of SE, acting as either inducers or inhibitors of signalling molecules. This section focuses on classically secreted proteins, typically exported via the ER-Golgi secretory route, although some may also follow UPS pathways. Notable examples include Arabinogalactan Proteins (AGPs), Chitinases, and Germin-Like Proteins (GLPs), which have been consistently reported as extracellular markers of SE, as summarised in Table II.1. AGPs constitute a large, diverse family of hydroxyproline-rich glycoproteins characterised by type II arabinogalactans linked to hydroxyproline residues and an N-terminal secretion signal (Leszczuk et al., 2023). Their structural variants include classical AGPs, arabinogalactan peptides, lysine-rich, and chimeric AGPs, which actively participate in extracellular matrix remodelling, signalling, and induction of SE and further development of somatic embryos (Pérez-Pérez et al., 2019) (see Table II.1). Kreuger

and Van Holst (1993) demonstrated that AGPs in CM enhance embryogenic efficiency in *Daucus carota* by reprogramming non-embryogenic cells and restoring embryogenic competence. Later studies confirmed that exogenous AGP supplementation stimulates somatic embryo differentiation and proliferation. In these studies, AGPs were applied directly within the *in vitro* culture system, typically after callus induction (Poon et al., 2012), and during the proliferation and embryogenic expression (Encina et al., 2023) phases. Specifically, AGPs extracted from embryogenic tissues were incorporated into the culture medium, enhancing the embryogenic competence and development of established callus cultures. This supports their use as SE inducers, especially in recalcitrant genotypes.

Esterases, hydrolytic enzymes that catalyse the decomposition of the ester bond, serve as specific biomarkers of embryogenic cells. In plant cell cultures, such as *Marchantia polymorpha*, the hydrolysis of acetates into the corresponding alcohols occurs rapidly, presumably due to the secretion of esterases into the culture medium (Izumi et al., 1995). Supporting this, esterase-like proteins were first identified in *Dactylis glomerata* embryogenic suspension cultures, where a 36 kDa acidic esterase was secreted during early stages of SE and associated with initial morphogenic processes (Tchorbadjieva & Odjakova, 2001).

Lipid Transfer Proteins (LTPs) are small, ubiquitous proteins that play essential roles in lipid trafficking and intercellular communication in plants. Initially, LTPs have been found associated with cellular membranes, including those of mitochondria and the ER. However, certain LTP isoforms are targeted to cytosolic compartments. These proteins are synthesised as precursors containing N-terminal extensions with sequence features characteristic of signal peptides, indicating subcellular targeting (Kader, 1996). Early studies in *Hordeum vulgare* identified an LTP, initially annotated as a putative amylase/protease inhibitor, which was later shown to be secreted into the aleurone cell culture medium (Mundy & Rogers, 1986). In *D. carota*, an LTP was detected among extracellular proteins in embryogenic cell cultures and in CM, and its encoding gene was shown to be expressed in embryogenic cultures, including in somatic embryos (Stern et al., 1991). Similar findings were reported in *Vitis* spp., where 9-kDa LTPs were secreted into the culture medium, suggesting a role in membrane dynamics and signalling (Coutos-Thevenot et al., 1992). Notably, overexpression of VvLTP1 in *Vitis* spp. was associated with the maintenance of embryogenic potential (Maes et al., 1997). Alongside LTPs, chitinases (endo-type chitinases, EC 3.2.1.14) are glycoside hydrolases that decompose β -1,4-N-acetyl-D-glucosamine linkages in chitin (Zieliński et al., 2021). They likely cleave N-acetyl-D-glucosamine residues within AGPs, contributing to extracellular matrix remodelling (Van Hengel et al., 2002). Both endo-chitinases and β -1,3-D-glucanases are constitutively expressed at low levels and show increased accumulation in embryogenic tissues, particularly during the acquisition of embryogenic competence and subsequent embryo development. (Domon et al., 2000).

Alongside the action of LTPs in cell wall dynamics, GLPs, members of the cupin superfamily, are apoplastic glycoproteins implicated in modulating the extracellular environment, notably through manganese binding and hydrogen peroxide (H₂O₂) production. The activity of GLPs in the extracellular matrix modulates cell wall properties through oxidative cross-linking, crucial for morphogenetic transitions during SE (Mathieu et al., 2006). Initially identified in *Pinus caribaea* var. *hondurensis*, GLPs such as GP III, GP103, and GP94 have been proposed as molecular markers for SE (Domon et al., 1995). Their expression is enriched at early SE stages in species like *Larix* spp. and *Saccharum* spp., implicating them in embryogenic identity establishment (Heringer et al., 2018). Recent secretome analyses of *Olea europaea* embryogenic cultures identified GLPs from subfamilies 1 and 2, including GLP2a, in extracellular protein fractions (Pires et al., 2025b), reinforcing their role as extracellular regulators of embryogenic competence.

Recent advancements in SE regulation have highlighted the role of novel extracellular proteins as potential markers. These emerging proteins offer new insights into the molecular mechanisms underlying SE and could enhance the understanding and efficiency of plant regeneration processes. Alpha-amylase (α -amylase) refers to a family of enzymes characterised by the presence of a predicted secretory signal peptide that cleaves internal alpha 1-4 glycosidic bands of starch and other polysaccharides to produce several products such as glucose and maltose (Avwioroko et al., 2018). In plants, these enzymes catalyse starch and glycogen hydrolysis, playing a key role in starch degradation in cereal grain endosperms, where they are synthesised in secretory tissues and released into the endosperm (Dobrenel et al., 2013). Studies on SE in *Medicago sativa* (Kępczyńska & Zielińska, 2006) and *Musa* spp. (Kumaravel et al., 2020) have demonstrated that α -amylase expression is regulated by endogenous gibberellic acid (GA) secreted within the embryo. GA synthesised in the embryo is transported to surrounding tissues, where it enhances α -amylase secretion, promoting starch hydrolysis and improving germination by facilitating starch breakdown, supplying the required energy for the germination process (Kumaravel et al., 2020). Although several studies on SE in plant tissues have reported the presence of α -amylase, few have identified this enzyme in the CM. An example is the work on *D. glomerata* suspension cultures, where the α -amylase (DgAMY1) was identified (Rakleova et al., 2012). The authors reported increased α -amylase activity associated with embryogenic competence and microcluster formation, suggesting that α -amylase activity may serve as a physiological marker of the early phases of SE, including embryogenic competence and early differentiation. In *P. nigra* (Pernis et al., 2023), higher levels of α -amylase were also reported in the CM of highly embryogenic cultures, leading to increased enzymatic activity and enabling the distinction of cell lines based on their embryogenic capacity. Similarly, Pires et al. (2025b) identified *OeAMY1* and *OeAMY2* in the secretome of high-efficiency embryogenic *O. europaea* cultures. The presence of this protein in the CM, where the essential carbon source for *in vitro* culture is found, highlights the crucial role of Amy in the SE process.

Another group of extracellular proteins implicated in the regulation of SE are the peroxidases (PODs). PODs are heme-containing enzymes classified as oxidoreductases, involved in plant stress responses. Among them, class III PODs (EC:1.11.1.7) are particularly notable as secreted enzymes involved in a wide range of physiological and developmental functions. In *P. nigra*, Pernis et al. (2023) reported the accumulation of POD isoforms in the secretome of highly EC cell lines. Similarly, Ngwenya et al. (2024) in *S. bicolor* and Pires et al. (2025b) in *O. europaea* also reported the identification of PODs in CM of high-efficiency embryogenic lines compared with low-efficiency lines. These studies confirm the potential of extracellular POD enzymes as biomarkers of high embryogenic capacity.

Complementing the oxidative activity of PODs in lignification, Dirigent (DIR) proteins have been identified as mediators of regio- and stereoselective coupling of phenoxy radicals during lignan biosynthesis. Subsequent studies demonstrated that recombinant DIR proteins confer strict regio- and stereospecificity on monolignol free-radical coupling, highlighting their essential role in lignification (Burlat et al., 2001), as evidenced by their expression in developing xylem and other lignified tissues (Pei et al., 2023). Beyond lignan and lignin formation, DIR proteins are increasingly recognised as key regulators of plant stress responses and cell wall remodelling (Paniagua et al., 2017). In the context of SE, DIRs have been consistently identified in the secretomes from embryogenic cultures of *P. nigra* (Pernis et al., 2023), *S. bicolor* (Ngwenya et al., 2024), and *O. europaea* (Pires et al., 2025), suggesting a conserved pattern of secretion across species. DIRs are proposed to contribute to extracellular matrix formation and cell wall reinforcement (Gasper et al., 2016; Pernis et al., 2023), potentially modulating the structural and biochemical environment that facilitates totipotency acquisition. Although the precise functions of DIRs in embryogenic induction remain unclear, their recurrent presence in SE-related secretomes and association with cell wall dynamics highlight their potential role in regulating the SE process.

II. 4.4. Synthesis: Functional Integration of Secreted Proteins in Somatic Embryogenesis

The identification of extracellular proteins across different species and culture systems highlights common functional patterns that help shape the extracellular environment during SE. Rather than acting independently, these proteins likely operate within coordinated networks that regulate cell wall remodelling, signalling, and redox regulation throughout embryogenic development.

Among the most recurrent proteins detected in embryogenic secretomes are those associated with cell wall modification and extracellular matrix organisation, including AGPs, chitinases, GLPs, and DIRs. These proteins consistently appear in embryogenic secretomes across phylogenetically diverse species, suggesting conserved mechanisms for establishing a permissive extracellular

environment. AGPs function as both structural components and signalling molecules, with their glycosylation patterns potentially encoding developmental information (Poon et al., 2012; Chen et al., 2025). Chitinases and GLPs contribute to polysaccharide processing (Zieliński et al., 2021) and oxidative cross-linking (Mathieu et al., 2006), respectively, while DIRs mediate stereospecific coupling of phenolic compounds during lignin formation (Pernis et al., 2023). Together, these proteins orchestrate cell wall loosening and reinforcement in spatially and temporally defined patterns that accommodate cellular dedifferentiation while maintaining tissue integrity. The recurrent identification of α -amylase, PODs, and LTPs in embryogenic CM suggests that extracellular metabolic adjustments accompany cellular reprogramming. α -Amylase activity reflects carbohydrate mobilisation supporting the energetic demands of embryogenic transitions (Pernis et al., 2023; Pires et al., 2025b), while PODs participate in reactive oxygen species (ROS)-mediated signalling and cell wall modification (Chen et al., 2026). LTPs facilitate lipid trafficking and membrane remodelling, processes essential for the extensive cellular reorganisation characteristic of SE (Potocka et al., 2012). The coordinated secretion of these proteins may establish metabolic gradients and redox conditions that promote embryogenic competence while protecting cells from oxidative damage.

Current evidence suggests that the extracellular environment during SE functions as an active regulatory compartment rather than a passive reservoir of secreted molecules. The convergence of cell wall-modifying enzymes, signalling molecules, and metabolic regulators in embryogenic secretomes indicates that successful SE depends on establishing a precisely orchestrated extracellular milieu. Future research should prioritise functional validation of these proteins through gain- and loss-of-function approaches and explore their synergistic interactions to develop evidence-based strategies for enhancing embryogenic efficiency in recalcitrant genotypes.

II. 5. EXTRACELLULAR METABOLOME – THE EXOMETABOLOME

Secondary metabolism in plants produces a wide range of bioactive compounds derived from primary metabolic pathways. These metabolites play essential roles in plant development and stress response, with profiles shaped by genotype, developmental stage, and environmental or experimental conditions (Mo et al., 2009). The secreted metabolome, or exometabolome, comprises metabolites released into the extracellular space, reflecting active secretion or passive diffusion (Kosina et al., 2018).

Due to limited studies on secreted metabolites during SE, this chapter reviews detection methods and highlights key metabolite families potentially relevant to SE, either via active secretion or exogenous supplementation in culture systems.

II .5.1. Methods for Characterising the Extracellular Metabolome

Several analytical techniques have been employed to identify and quantify metabolites, with the choice of method depending on factors such as metabolite concentration, chemical class, physicochemical properties, and the nature of the biological matrix. Among the most used approaches in metabolomics are nuclear magnetic resonance (NMR) spectroscopy and chromatographic methods like gas chromatography (GC) and LC, often coupled to detectors such as MS, ultraviolet (UV), or electrochemical sensors (Seger et al., 2013).

NMR spectroscopy is a non-destructive and highly reproducible technique that enables the simultaneous identification and quantification of multiple small molecules through the detection of specific nuclear responses under magnetic fields (Seger et al., 2013); however, its relatively low sensitivity compared to MS-based approaches may limit the detection of low-abundance compounds. In SE studies, this approach has yielded valuable insights into the metabolic adaptations of embryogenic tissues. For instance, Dowlatabadi et al. (2009) used one-dimensional (1D) ^1H NMR to analyse the CM of somatic embryo cells of *Picea glauca*, identifying both consumed and secreted metabolites in two different culture medium. Mahmud et al. (2014) applied ^1D ^1H and two-dimensional (2D ^1H ^{13}C) NMR to investigate the relationship between nutrient availability and metabolic adaptation in embryogenic and non-embryogenic calli of *S. officinarum* during SE. More recently, Pires et al. (2025b) utilised ^1H NMR to profile secreted metabolites in EC of *O. europaea*, identifying compounds in the CM that provided key insights into metabolic pathways maintaining embryogenic competence. In *Coffea* spp., Nic-Can et al. (2015) compared the CM metabolite profiles of *C. arabica* (a recalcitrant SE-responsive species) and *C. canephora* (highly responsive to SE) using GC-MS and ultra-performance LC coupled with electrospray ionisation tandem MS (UPLC-ESI-MS/MS).

The integration of advanced analytical techniques such as NMR, GC-MS, and LC-MS has significantly enhanced the profiling and quantification of metabolites involved in SE, offering comprehensive insights into the extracellular metabolome and the biochemical pathways regulating this complex developmental process. Despite the availability of these highly sensitive and versatile methods, the most impactful findings to date, regarding metabolite secretion and modulation of embryogenic competence, have predominantly emerged from GC-MS and LC-MS analyses of CM. However, LC-MS approaches may be affected by matrix effects and ion suppression, which can influence quantification accuracy (Zhu et al., 2026). Nonetheless, the application of these techniques directly to extracellular metabolome studies remains relatively limited, highlighting a valuable opportunity for future research to deepen our understanding of the metabolite dynamics underlying SE.

II. 5.2. The Involvement of Extracellular Metabolites as Modulators of Somatic Embryogenesis Response

A deeper understanding of extracellular metabolites is essential, given their crucial role as modulators of the SE process. This chapter explores major classes of extracellular metabolites, such as amino acids, carbohydrates, phenolic compounds, plant growth regulators, and redox-related molecules, highlighting their specific roles and impact during SE development.

Evidence for the active secretion of amino acids by embryogenic cells into the culture medium remains limited, as secretome studies have predominantly identified other classes of metabolites, with most research focusing on their intracellular roles or exogenous supplementation (Pullman et al., 2015; Daniel et al., 2018). Nevertheless, the presence of amino acids in the extracellular environment may contribute to nitrogen availability and metabolic signalling, given their well-established roles in plant metabolism. In particular, pathways related to amino acid and nucleotide metabolism have been linked to embryo development and the acquisition of embryogenic competence (Guo et al., 2019). Phenolic compounds have been shown to play significant roles in regulating SE, often acting as either promoters or inhibitors depending on their concentration and the plant species involved (Umehara et al., 2005, 2007). Nic-Can et al. (2015) identified several metabolites in the CM of embryogenic cultures of the low-competence species *C. arabica*, including caffeine, chlorogenic acid, hydroxybenzoic acid, and trans-cinnamic acid. Among these, caffeine, although present at the lowest concentration, had the strongest inhibitory effect on SE when applied to *C. canephora* cultures during somatic embryo proliferation. Chlorogenic acid also showed a notable negative impact, indicating that certain phenolic compounds abundant in *C. arabica* CM may suppress embryogenic potential in more competent genotypes. Although most studies have focused on the effects of exogenous application, these findings indicate that phenolic compounds present in the extracellular environment can modulate embryogenic responses. In addition, phenylpropanoid metabolism has been associated with stress responses and cell wall reinforcement, processes that may indirectly support embryo development in *Persea americana* (Olivares-García et al., 2020). Moreover, phenylpropanoid metabolism contributes to the synthesis of lignin and flavonoid precursors, which reinforce the cell wall and enhance stress tolerance, thereby supporting somatic embryo development. Overall, these results highlight the role of phenolic compounds as extracellular signals in the regulation of SE, while emphasising the need for further studies addressing their secretion and function in the culture medium.

Plant growth regulators are naturally biosynthesised compounds that regulate various physiological processes in plants. They are broadly classified into growth promoters, including auxins, gibberellins, and cytokinins, and growth inhibitors, such as abscisic acid (ABA), jasmonates, and ethylene (Kępczyńska & Kępczyński, 2023). In SE, plant growth regulators are key components of the culture medium that control the entire process. Additionally, during the induction of embryogenic

competence, the presence of endogenous auxins in combination with cytokinins is necessary for the SE process (Shmakov & Konstantinov, 2020). In a study on *P. glauca* (Dowlatabadi et al., 2009), the metabolome of embryogenic culture medium was analysed, revealing changes in secreted and consumed metabolites associated with cellular organisation. The addition of 2,4-dichlorophenoxyacetic acid (2,4-D) and N⁶-benzyladenine (BA), combined with the absence of ABA, inhibited cellular organisation and apical meristem formation, correlating with specific shifts in amino acids and other metabolic products in the medium. Polyamines, often considered non-classical growth regulators, were investigated by Vuosku et al. (2012) in the CM of *P. sylvestris* embryogenic cell cultures during early growth. The authors observed low levels of secreted polyamines, with putrescine notably accumulating while spermidine declined. This shift was linked to increased putrescine production, likely via the arginine decarboxylase pathway, and reduced spermidine and spermine synthesis.

Regarding the role of carbohydrates, they ensure a steady supply when added to the culture medium, preserving cell osmotic potential, and serving both as energy and carbon sources for various developmental processes (Yaseen et al., 2013). Concerning SE, carbohydrates play a significant role in inducing embryogenic competence, acting as a signalling molecule and regulator of gene expression (Dobrenel et al., 2013). In a study by Pires et al. (2025b) in *O. europaea* CM, the sucrose supplied to the culture medium was hydrolysed into glucose and fructose, while the total concentration of these monosaccharides remained equivalent to the initial sucrose level. This pattern reflects concurrent sucrose hydrolysis in the culture medium, likely mediated by extracellular invertase activity, and uptake of glucose and fructose by the cultured tissues, resulting in a relatively stable concentration of soluble sugars despite ongoing carbon utilisation. The availability and metabolism of these simple sugars are closely associated with the activity of carbohydrate-modifying enzymes involved in central carbon metabolism. During glycolysis, glyceraldehyde 3-phosphate dehydrogenase reduces NAD⁺ to NADH, crucial for energy production. To sustain glycolysis and glucose flux, lactate dehydrogenase (LDH) oxidises NADH back to NAD⁺ (Lees et al., 2020), maintaining redox balance essential for energy, cellular homeostasis, and SE induction. This role has been reported in embryogenic tissues of *Brassica napus* (Ashihara et al., 2008) and *Gossypium* spp. (Ge et al., 2025). In the same study, Pires et al. (2025b) reported that a highly embryogenic cell line secreted lactate, 1-methylnicotinamide (MNA), 2,3-butanediol, acetaldehyde, and ethanol. Lactate secretion reflects LDH activity regenerating NAD⁺ under low-oxygen conditions. Extracellular lactate and MNA indicate active NAD⁺ metabolism, essential for cellular homeostasis (Zhang et al., 2025). Metabolites like NADH offer valuable insights into SE molecular regulation. NAD⁺ and its precursors maintain cellular metabolism and redox balance, linking glucose flux and NAD⁺ regeneration to embryogenic competence as potential metabolic markers.

II.6. THE ROLE OF EXTRACELLULAR VESICLES IN SOMATIC EMBRYOGENESIS

In animals, EVs are involved in early development, where they mediate intercellular communication by transferring proteins, lipids, and regulatory RNAs. EVs contribute to embryo-maternal signalling, embryo development and viability, and occur in biological fluids such as semen, amniotic fluid and blood (Krause et al., 2018). Recent studies further show that embryonic signals can modulate EVs biogenesis and secretion pathways in endometrial cells, highlighting their functional relevance at the embryo–maternal interface (Guzewska et al., 2023). EVs have also been investigated in embryo culture systems, particularly in CM. However, only a limited number of studies have addressed this topic, highlighting both promising biological roles and significant gaps in current knowledge (Xue et al., 2024). Together, these findings illustrate the importance of EVs-mediated communication during animal embryogenesis.

In plants, EVs similarly transport proteins and small RNAs with signalling potential, but direct evidence for their involvement in morphogenesis or SE remains lacking. A major limitation is that most studies detecting secreted proteins during embryogenic induction rely on apoplastic or culture-medium proteomics, which do not distinguish between conventional secretion and EVs-associated release. Nevertheless, proteins such as annexins, tetraspanins (TETs), and heat shock proteins (HSPs) have been frequently identified in EVs-enriched fractions across plant systems (Cui et al., 2020), which could lead us to hypothesise that EVs may play active roles in cellular signalling, membrane dynamics, and cellular reprogramming, thereby contributing to the modulation of the SE response. Table II.2 summarises the main proteins identified in these unconventional secretion pathways, together with their sources, detection methods and proposed functions. The main achievements regarding the methods used for EVs isolation in plants, along with their potential involvement in SE, are presented below.

Table II.2. Overview of non-classically secreted and EV-associated proteins identified in plants.

EVs	Specie	System	Analysis	Function	Reference
Annexins	<i>Arabidopsis thaliana</i>	<i>In planta</i>	SDS-PAGE; Immunoblot	Growth, development, and responses to environmental stresses	Koch et al., 2024
		<i>In planta</i>	Immunoblot		Rutter & Innes, 2017
	<i>Solanum lycopersicum</i>	<i>In planta</i>	SDS-PAGE; LC-MS/MS		Palma et al., 2020
Glycosylphosphatidyl inositol-anchored proteins	<i>Arabidopsis thaliana</i>	<i>In planta</i>	RT-qPCR	Signal transduction; Lipid transport; cell wall remodelling; stress response	Xiong et al., 2022
		<i>In vitro</i>	RT-qPCR		Li et al., 2015
	<i>Oriza sativa</i>	<i>In vitro</i>	RT-qPCR		Xu et al., 2022
	<i>Triticum aestivum</i>	<i>In planta</i>	RT-qPCR		Koudri et al., 2018
Fasciclin-like arabinogalactan proteins	<i>Arabidopsis thaliana</i>	<i>In planta</i>	SDS-PAGE; Immunoblot; Protein blots	Cell wall formation; cell expansion; signalling; stress responses	Ma et al., 2023
		<i>In vitro</i>	MALDI-TOF MS; RNA Gel-Blot		Johnson et al., 2003
	<i>Areca catechu</i>	<i>In vitro</i>	RT-qPCR; Fluorescence microscopy		Li et al., 2023
	<i>Olea europaea</i>	<i>In vitro</i>	SDS-PAGE; LC-MS/MS		Pires et al., 2025b
Feronia	<i>Arabidopsis thaliana</i>	<i>In vitro</i>	RT-qPCR; Immunoblot	Plant growth and reproduction; morphogenesis; hormone signalling; immunity; stress responses	Wang et al., 2024
		<i>In planta</i>	RT-qPCR; Immunoblot; Confocal microscopy		Lin et al., 2022
		<i>In planta</i>	RT-qPCR; Western blot		Wang et al., 2020
	<i>Oriza sativa</i>	<i>In vitro</i>	NMR; Microscopy		Wong et al., 2007
Heat shock proteins	<i>Solanum lycopersicum</i>	<i>In planta</i>	SDS-PAGE; HPLC	Response to stress; cellular survival; cell signalling; immunity	Alcântara et al., 2025
	<i>Olea europaea</i>	<i>In vitro</i>	SDS-PAGE; LC-MS/MS		Pires et al., 2025b
	<i>Sorghum bicolor</i>	<i>In vitro</i>	iTRAQ, LC-MS/MS		Ngwenya et al., 2024 Ngcala et al., 2020
Receptor-like kinases	<i>Olea europaea</i>	<i>In vitro</i>	SDS-PAGE; LC-MS/MS	Response to abiotic stress; phytohormone signaling; immunity; plant development and reproduction	Pires et al., 2025b
	<i>Triticum aestivum</i>	<i>In planta</i>	RT-qPCR		Yan et al., 2023
	<i>Arabidopsis thaliana</i>	<i>In vitro</i>	RT-qPCR; Western blot		Wang et al., 2020
		<i>In planta</i>	RT-qPCR		Li et al., 2019

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	<i>Gossypium spp.</i>	<i>In planta</i>	RT-qPCR		Sun et al., 2018
		<i>In planta</i>	Confocal microscopy		Liu et al., 2022
Penetration 1	<i>Arabidopsis thaliana</i>	<i>In planta</i>	LC-MS/MS	Targeted secretion in response to pathogens; growth and development	Johansson et al., 2014
		<i>In planta</i>	SDS-PAGE; Immunoblot		Assaad et al., 2004
Tetraspanin	<i>Arabidopsis thaliana</i>	<i>In planta</i>	Immunoblot; qRT-PCR	Marker of EV subpopulations; stress-responsive secretion; plant immunity	Koch et al., 2025
	<i>Solanum betaceum</i>	<i>In vitro</i>	RT-qPCR		César, 2023
	<i>Salvia dominica</i>	<i>In vitro</i>	SDS-PAGE; Western blot		Boccia et al., 2022

Abbreviations: HPLC (High-performance liquid chromatography); iTRAQ (Isobaric tags for relative and absolute quantification); LC (Liquid chromatography); MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight); MS/MS (Tandem mass spectrometry); qRT-PCR (Quantitative real-time reverse transcription PCR); SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

II. 6.1. Extraction and Purification Methods of Extracellular Vesicles

Efficient recovery of plant EVs is essential to preserve their structural integrity, stability and biological activity. While for the isolation of EVs in mammalian systems several methods have been developed, as a consequence of the extensive investigation that has been carried out, those from plants remain understudied, largely due to a lack of standardised protocols (Maricchiolo et al., 2026). Nevertheless, with the growing interest in plant EVs research, several approaches have been proposed to improve the specificity and efficiency of extraction. Compared to animals, the isolation of plant-derived vesicles requires additional considerations due to the complex composition of plant matrices, such as the plant cell wall. In plants, EVs extraction generally relies on juice, flesh, roots, seeds, dried tissues, or CM (Li et al., 2026). Because plant EVs are predominantly located in the apoplastic space, the isolation represents the most critical step in their extraction. Consequently, the choice of extraction method depends on the specific goals of the study—including the desired purity, yield and the type of sample analysed (Ye et al., 2025).

The methods used for plant EVs isolation are largely based on these mammalian protocols, with the additional step of collecting APF. In cell culture systems, EVs are commonly isolated from CM, where they accumulate as a result of cellular secretion (Zhang et al., 2020). Because plant EVs accumulate either in the apoplast or in CM, the isolation of a clean extracellular medium is critical for downstream analyses (Rutter & Innes, 2017; Huang et al., 2022). Current isolation methods generally rely on vesicle size, density, or specific surface markers, with the most commonly used approaches including differential ultracentrifugation and ultrafiltration, which exploit size- and density-dependent separation principles (Nueraihemaiti et al., 2025).

Differential ultracentrifugation is the most widely used method for isolating EVs from cell culture supernatants and biological fluids. In plant systems, once APF has been collected, vesicles are typically concentrated through sequential centrifugation steps in which progressively higher centrifugal forces remove contaminants of decreasing size, culminating in ultracentrifugation to sediment small vesicles (Huang et al., 2025). Although this approach is relatively straightforward and does not require specialised reagents or costly consumables, the mechanical stress generated during centrifugation and handling may compromise vesicle integrity (Doyle & Wang, 2019). In addition, the performance of this technique is influenced by several experimental parameters, including rotor configuration, centrifugal force, duration and temperature, as well as sample-related factors such as viscosity and dilution, which can ultimately affect separation efficiency and vesicle recovery. Despite its widespread use, differences in centrifugation parameters and APF collection procedures mean that a universally standardised protocol for plant EVs isolation is still lacking (Huang et al., 2021). To increase vesicle purity, differential ultracentrifugation is frequently coupled with complementary purification methods such as density gradient ultracentrifugation, ultrafiltration or size-exclusion chromatography (Li et al., 2026).

Density gradient ultracentrifugation separates vesicles according to their density using sucrose or iodixanol gradients, improving sample purity and vesicle integrity, although it is relatively time-consuming and requires specialised equipment (Maricchiolo et al., 2026). Ultrafiltration relies on membranes with defined pore sizes to separate particles according to size, including nanomembranes ranging from approximately 20 to 200 nm (Doyle & Wang, 2019) or membranes with specific molecular weight cut-offs that enable the selective capture of vesicles from cell culture supernatants or biological fluids (Maricchiolo et al., 2026); however, membrane obstruction and reduced vesicle recovery have been reported as major limitations (Palencia et al., 2025).

The isolation of plant EVs systems remains a technically challenging task due to the need to preserve vesicle integrity while achieving sufficient purity and yield. Differential ultracentrifugation and ultrafiltration are among the most widely used methods, each with distinct advantages and limitations, and their combination is often employed to optimise recovery. The choice of approach must consider the specific characteristics of the biological matrix, the target vesicle population, and the intended downstream applications. Continued methodological refinement will be essential to fully exploit the potential of EVs in both basic research and biotechnological applications, particularly focused on SE.

II. 6.2. Extracellular Vesicles as Potential Mediators of Somatic Embryogenesis Signalling

EVs, as previously introduced, comprise a heterogeneous population of vesicles with distinct biogenesis pathways, including endosome-derived and plasma membrane-derived vesicles. However, in SE, the specific EV subtypes involved remain poorly characterised, and most evidence derives from EVs-enriched extracellular fractions rather than from purified vesicle populations.

Among the proteins identified in EVs-enriched fractions or extracellular secretomes of embryogenic cultures, only a subset has also been independently associated with SE. Fasciclin-like arabinogalactan proteins (FLAs), members of the AGP family, are among the most consistently reported extracellular proteins linked to embryogenic competence. FLAs are associated with membrane microdomains involved in vesicle trafficking and EVs biogenesis (Kouidri et al., 2018). In *O. europaea*, several FLAs were identified in the extracellular secretome of embryogenic cultures and assigned to apoplast- and vesicle-related GO categories (Pires et al., 2025b). Independent studies in embryogenic tissues further support the involvement of AGPs/FLAs in SE. In *Areca catechu*, genes encoding for FLA7-1 and FLA7-2 were co-expressed with genes related to hormone signalling and cell wall remodelling during SE induction (Li et al., 2023).

Heat shock proteins (HSP70 and HSP90) and the ER chaperone BINDING IMMUNOGLOBULIN PROTEIN (BiP) are recurrent components of plant EV proteomes and

extracellular fractions (Pobre et al., 2019). In embryogenic systems, proteomic analyses based on iTRAQ and LC-MS/MS identified HSPs among secreted proteins recovered from the culture medium of *S. bicolor* embryogenic suspension cultures subjected to stress conditions (Ngcala et al., 2020; Ngwenya et al., 2024). Similarly, LC-MS/MS analysis of the CM from *O. europaea* embryogenic cultures also detected stress-related chaperones in the extracellular secretome (Pires et al., 2025b). These observations support the association of stress-responsive extracellular proteins with embryogenic cultures and cellular reprogramming during SE.

Tetraspanins, particularly TET8, are recognised markers of plant EVs due to their enrichment in apoplastic vesicles and association with vesicle trafficking (Rutter and Innes, 2017; Koch et al., 2025). In *Solanum betaceum*, embryogenic calli showed a higher abundance of TET8-positive vesicles compared with non-embryogenic calli (César, 2023), indicating that changes in vesicle dynamics may be associated with the acquisition of SE. Although the functional role of TET8 during SE remains unclear, these observations provide preliminary evidence linking canonical plant EV markers with embryogenic cultures.

Collectively, the available evidence suggests that some proteins identified in EV-enriched extracellular fractions from embryogenic cultures are also recurrently associated with SE in independent experimental systems. Nevertheless, the available studies consistently associate these proteins with biological processes known to be central to embryogenic transition, including stress responses, extracellular signalling, cell wall remodelling, and developmental reprogramming. Specifically, proteins linked to the acquisition of SE, proteostasis, and receptor-mediated signalling have been frequently found in both extracellular fractions and embryogenic tissues, indicating that EV-associated cargoes may play a role in the development and maintenance of the embryogenic state. Although the functional roles of specific EV populations during SE remain largely unresolved, current findings support the hypothesis that extracellular vesicle-mediated communication participates in the cellular reprogramming events underlying SE.

II. 7. CONCLUSIONS

Diverse strategies have been explored to overcome the recalcitrance observed in SE. While many factors have been considered, an important aspect that is often overlooked –but crucial to the SE response is the release of organic bioactive molecules by explants into the culture medium. During *in vitro* culture, a broad spectrum of molecules is actively released by explants. Their accumulation in the extracellular environment reflects dynamic metabolic exchanges between the explant and the culture medium, suggesting that the culture medium itself becomes a biologically active compartment rather than a passive support system. These molecules may significantly influence SE efficiency, given their

involvement in essential biological processes and their potential roles in cellular communication. The current review highlights the biomolecules secreted during SE and emphasises their essential role in regulating the embryogenic process, acting as key components of the extracellular environment that mediate communication between explants and the culture medium and influence critical processes. Looking toward future perspectives, understanding how these extracellular signals operate and how they interact with intracellular pathways offers promising possibilities for improving SE efficiency. However, several research gaps remain. A major challenge lies in the standardisation of methodologies used in SE studies, including the preparation of conditioned medium, as well as the analytical approaches used in proteomic and metabolomic studies, all of which can significantly influence the comparability and interpretation of results. Additionally, extracellular vesicles represent a further methodological frontier, requiring specific consideration for their isolation and characterisation from plant tissues. Distinguishing passive leakage from active secretion in SE cultures also remains a critical limitation for correctly interpreting secretome data. Furthermore, integrating multi-omics approaches will be essential for identifying functional biomarkers associated with embryogenic competence and for establishing mechanistic models linking secreted factors with intracellular reprogramming. Altogether, these perspectives open new insights for understanding SE and provide a foundation for developing innovative strategies to overcome current challenges and broaden the applications of this important biological process.

II. 8. REFERENCES

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CHAPTER III

In Vitro Establishment and Maintenance of Culture Lines with Differentiated Somatic Embryogenesis Capacity in Olive (*Olea europaea* L.)

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III. 1. ABSTRACT

Somatic embryogenesis is a powerful biotechnological tool for large-scale clonal plant propagation. However, most woody species exhibit a recalcitrant response. *Olea europaea* L., a highly valuable tree crop, is among these recalcitrant species. Unravelling the molecular mechanisms underlying SE efficiency in *O. europaea* is, therefore, essential. Access to embryogenic lines with contrasting capacities for somatic embryo differentiation is a key requirement for such studies. Mature fruits of olive cultivars ‘Galega Vulgar’ and ‘Arbequina’ were collected from open-pollinated orchard-grown plants, and various explants taken from zygotic embryos were used to establish SE. A differentiated response was observed both within each cultivar and between cultivars, with cv. ‘Arbequina’ showing the highest embryogenic induction, particularly when radicles were used. Secondary SE was successfully established for both high- and low-efficiency lines, providing valuable material for future molecular studies. Somatic embryo conversion into plants, a key indicator of embryo quality, was successfully achieved in both cultivars. Flow cytometry analysis revealed a high degree of chromosomal stability. This study presents a reliable procedure to obtain and maintain distinct embryogenic responses in *O. europaea*, identifying lines with both high and low embryogenic efficiency that can serve as model systems for future molecular investigations.

III. 2. INTRODUCTION

Somatic embryogenesis (SE) is an *in vitro* technique in which asexual embryos arise from cells of a tissue or an organ, enabling the regeneration of a complete plant (Méndez-Hernández et al., 2019). SE presents several applications, including the large-scale propagation of elite genotypes, the production of synthetic seeds for germplasm preservation, and *in vitro* plant regeneration to support breeding programs (Sánchez-Romero et al., 2021). The capacity to switch the developmental pathway of cells from vegetative to embryogenic, thereby generating somatic embryos, is restricted to a limited subset of cells and is affected by several endogenous and exogenous factors (Nic-Can et al., 2016; Pais et al., 2019). Plant genotype is considered one of the most important factors (Niskanen et al., 2005). Even within the same species, cultivars and even individual plants within a cultivar can show considerable variation in their ability to initiate and develop somatic embryos. *Vitis vinifera* (Cardoso et al., 2010), *Cocos nucifera* (Udyawar et al., 2016), *Theobroma cacao* (Daouda et al., 2019), and *Physocarpus opulifolius* (Jagiello-Kubiec et al., 2021) are examples where a strong effect of genotype has been reported, highlighting the need to establish a protocol for each new cultivar within a species.

Understanding the sequential stages of SE is essential, as the success of an SE protocol relies on the ability of the initial explants to progress through the phases of induction, expression, maturation, and germination (Guo et al., 2024). The induction phase, commonly characterised as a stress-related

response (Arnholdt-Schmitt et al., 2016) involves the perception and activation of a complex signalling network, which, in turn, modulates the expression of target genes (Frederico et al., 2009; van Der Knaap & Verrijzer, 2016). Besides genotype dependency, factors such as explant type, hormone balance, and culture conditions strongly influence the efficiency of this phase (Bollati et al., 2025). In olive (*Olea europaea* L.), SE is neither routinely nor widely applied, largely because of its strong genotype dependence (Sánchez-Romero, 2021), and its frequent recalcitrant behaviour in response to *in vitro* protocols (Rugini & Caricato, 1995). This limitation is particularly evident when attempting to induce SE using adult tissues as the initial explant, although successful cases have been reported in a few cultivars such as ‘Canino’ and ‘Moraiolo’ (Rugini & Caricato, 1995), ‘Chetoui’ (Trabelsi et al., 2011), ‘Dahbia’ (Mazri et al., 2013), ‘Picual’ (Toufik et al., 2014), and ‘Moroccan Picholine’ (Oulbi et al., 2018).

The expression and maturation phases are characterised by embryo differentiation and key morphological changes (Fehér, 2003; Corredoira et al., 2015; Zhang et al., 2021), including cell elongation, dehydration, and the accumulation of storage compounds such as carbohydrates, lipids, and proteins (Merkle et al., 2005; Savane et al., 2023). Therefore, physiological maturation is essential for transition to germination, the final phase in which somatic embryos develop into complete plantlets (Vidales-Fernández et al., 2003; Merkle et al., 2005). Low germination rates remain a major constraint for large-scale SE propagation in olive. Key factors affecting embryo germination include the composition of the culture medium (Benzekri & Sánchez-Romero, 2012; Titouh et al., 2022), the use of osmotic agents (Cerezo et al., 2011), environmental growth conditions (Afreen et al., 2005; Bradaï et al., 2016; Mazri et al., 2020; Pires et al., 2020; Cavallaro et al., 2022), and the presence of abnormal embryos (Rugini et al., 2020). The differentiation of abnormal embryos and the appearance of somaclonal variations have been attributed to multiple factors, including genetic stability and the occurrence of epigenetic events (Krishna et al., 2016; Escobedo-Garcia-Medrano et al., 2018; Bustami, 2023; Duta-Cornescu et al., 2023), with a strong dependence on the genotype and the duration of culture maintenance (Bradaï et al., 2016a). Studies conducted on the olive cv. ‘Picual’ were verified using flow cytometry and molecular markers to establish the link between somaclonal variations and long-term cultures (Bradaï et al., 2016a, b).

In secondary SE, the initiation of new embryos from primary somatic embryos (Bogdanović et al., 2021) allows for the long-term maintenance and proliferation of embryogenic cultures (Sánchez-Romero, 2021; Bogdanović et al., 2021). Although morphogenic capacity may decline as a result of somaclonal variation, culture ageing, or repeated subculturing (Krishna et al., 2016; Bustami, 2023; Duta-Cornescu et al., 2023), secondary SE effectively supports high-frequency plant regeneration in species such as *Vitis* spp. (Carra et al., 2024), *Rosa hybrida* (Duan et al., 2024), *Quercus* spp. (Martínez et al., 2015; Ben Ali et al., 2023), and *Centaureum erythraea* (Bogdanović et al., 2021). By ensuring

consistent proliferation, uniform cultures, and sustained embryogenic competence, secondary SE represents a reliable strategy for the long-term maintenance of productive clonal lines (Corredoira et al., 2015; Bogdanović et al., 2021). Although a gelled medium is used in the majority of studies on secondary SE in olive (Cerezo et al., 2011, 2019; Mazri et al., 2020; Pires et al., 2020), a liquid culture may provide a more favourable environment to regulate cluster interaction, nutrient uptake, and cell proliferation (Ben-Amar, 2021). Moreover, this system enables the identification and establishment of embryogenic lines with distinct capacities by differentiating morphogenic clusters in suspension cultures based on their morphology and gene expression patterns (Quiroz-Figueroa et al., 2001; Elmeer et al., 2013).

In the study presented here, an efficient method is described to establish *O. europaea* embryogenic lines with different embryogenic capacities through secondary SE in a liquid culture medium using the Portuguese olive cv. ‘Galega Vulgar’ and the Spanish cv. ‘Arbequina’. The germination ability of these long-term cultures, as well as the genetic stability of embryos and plantlets, was also investigated. This approach enables the selection of both high- and low-efficiency (HE and LE, respectively) embryogenic lines, providing a platform for future research into the molecular mechanisms regulating SE efficiency in olive.

III. 3. MATERIALS AND METHODS

III. 3.1. Plant Material

Mature olive fruits from the cv. ‘Galega Vulgar’ and cv. ‘Arbequina’ were collected after complete ripening, in November, from 12-year-old plants in open-pollinated orchards located at the Coleção Nacional de Referência de Cultivares de Oliveira—Instituto de Investigação Agrária e Veterinária (Elvas-Portugal). The cv. ‘Galega Vulgar’, a traditional Portuguese cultivar known for its high-quality olive oils, is recognised for its recalcitrance in morphogenetic responses, particularly its low capacity to develop adventitious roots. In contrast, the cv. ‘Arbequina’, a Spanish cultivar that is well adapted to intensive orchard systems, is characterised by high rooting competence (Porfírio et al., 2016). Since adventitious rooting is a morphogenic process responsive to stress, we compared the embryogenic potential of the two genotypes.

The pulp was removed from the fruits, and the seeds were collected, washed, and stored at 4 ± 1 °C for 30 days. Afterwards, the endocarp was then broken using a manual press, and the seeds were placed in sterile water for 42 h in the dark at 24 ± 1 °C. Seeds were disinfected by immersion in 70% ethanol for 30 s, followed by a 20 min treatment with 10% sodium hypochlorite solution containing 0.1% Tween-20 under agitation, and rinsed three times with sterile bi-distilled water. Under aseptic

conditions, radicles and cotyledons (proximal and distal) were excised and used as initial explants, following the procedure previously described by Pires et al. (2020).

Figure III.1 illustrates the entire process from the induction phase through to the analysis of embryogenic competence and the evaluation of conversion performed for each embryogenic line, highlighting the key steps and methodologies employed.

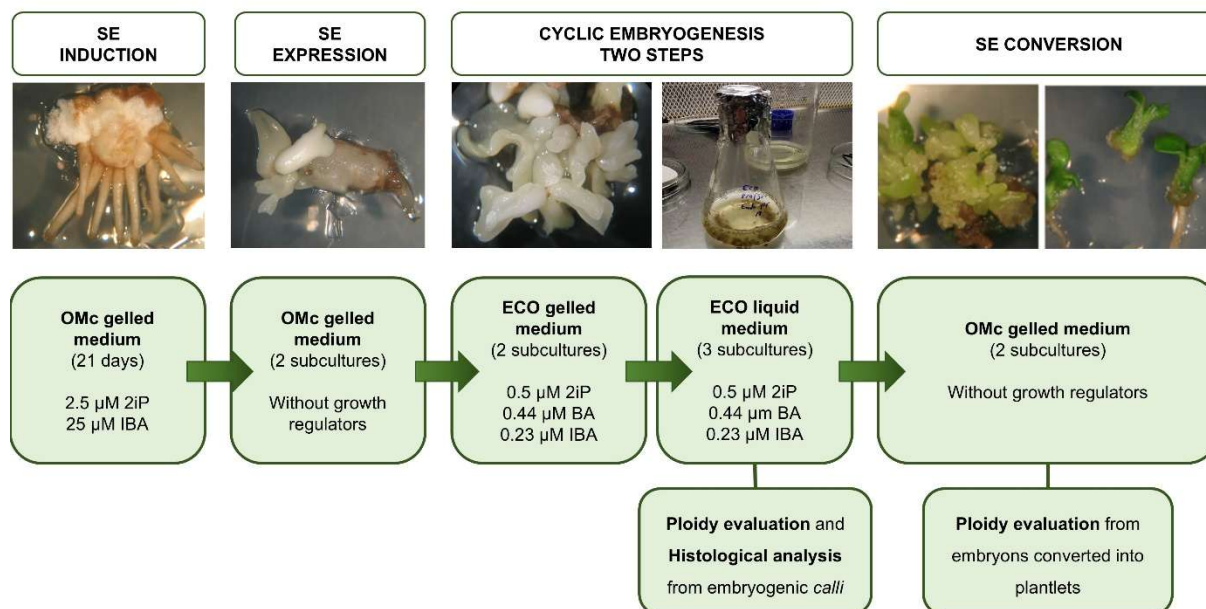


Figure III.1. Schematic representation of the workflow, illustrating the four phases of SE: induction, expression, maturation, and conversion. Additionally, cyclic embryogenesis is presented as a key procedure for the multiplication and maintenance of embryogenic material.

III. 3.2. Establishment and Development of Somatic Embryogenic Cultures

Embryos' radicles and cotyledons, proximal and distal (measuring approximately 0.8 mm and 3.4 mm, respectively, in cv. 'Galega Vulgar', and 0.1 mm and 0.5 mm in cv. 'Arbequina'), were excised from previously disinfected seeds, and inoculated in Petri dishes (7 cm Ø) containing induction OMc culture medium (Orinos & Mitrakos, 1991), supplemented with 2.5 μM 6-dimethylallylamino-purine (2iP), 25 μM indole-3-butyric acid (IBA), and gelled with 6 g L⁻¹ Agar-Powder (VWR, Portugal). The pH was adjusted to 5.8 prior to autoclaving. The three types of explants (radicle, proximal cotyledon, and distal cotyledon) taken from a single seed were inoculated per Petri dish. Explants taken from a total of 134 and 164 seeds were considered for the cv. 'Galega Vulgar' and the cv. 'Arbequina', respectively. Cultures were kept at 25 $\pm$ 1 °C in the dark for 21 days.

After the induction phase, explants were transferred to OMc gelled medium devoid of growth regulators to promote embryo differentiation, henceforth called expression medium. The cultures were

maintained for two months under the same growth conditions as described above, with two subcultures lasting 30 days each.

III. 3.3. Establishment of Cyclic SE in Liquid Medium

To promote cyclic embryogenesis, the embryogenic lines were subcultured into ECO (embryogenesis cyclic olive) gelled medium (Pérez-Barranco et al., 2009a, b) supplemented with 50 mg L⁻¹ myoinositol, 550 mg L⁻¹ l-glutamine, 0.5 µM 2iP, 0.44 µM 6-benzyladenine (BA), 0.23 µM IBA, 20 g L⁻¹ sucrose, and 1 g L⁻¹ casein hydrolysate, and gelled with 2.5 g L⁻¹ Gellan Gum®. The pH was adjusted to 5.8 before autoclaving. Cultures were maintained at 25 ± 1 °C in the dark and subcultured twice, each subculture lasting 30 days. After this period, approximately 200 mg of embryogenic calli was transferred into 100 mL Erlenmeyer flasks containing 50 mL of ECO liquid medium with the same composition. To evaluate the embryogenic capacity of the somatic embryogenic lines, calli were collected by filtration at the end of each subculture period, and the number of differentiated somatic embryos was recorded. This culture approach promoted both the stabilisation and proliferation of embryogenic lines under consistent conditions, enabling the production of sufficient plant material while preserving genetic variability before classification based on SE efficiency. This strategy also facilitates procedures aimed at characterising secreted biomolecules involved in SE efficiency, aligning with the broader objectives of the ongoing research (Pires et al., 2025).

III. 3.4. Conversion of Somatic Embryos

At the end of the third subculture in ECO liquid medium, after approximately 7 months from the beginning of the assay, embryogenic material was collected and transferred to Petri dishes containing OMc gelled expression medium. Embryogenic efficiency was expressed as the number of somatic embryos developed per gram of embryogenic tissue, thirty days after calli establishment. For this purpose, a total of 15 segments, each approximately 200 mg, were collected from the calli of each embryogenic line from both cultivars and inoculated into Petri dishes (9 cm Ø) containing ~20 mL of OMc expression medium. Each calli segment was considered as an independent biological replicate. The cultures were maintained at 25 ± 1 °C under a 16 h photoperiod, with a light intensity of 40–45 µmol m⁻² s⁻¹, for 30 days. The data were subjected to statistical analysis to assess differences in embryogenic potential among lines within each cultivar.

To determine the optimal developmental stage for converting somatic embryos into plants, previously characterised embryos from HE and LE lines were divided into two morphological stages: globular and torpedo. Thirty days after the establishment of embryogenic calli under expression conditions, embryos identified at two developmental stages were isolated and vertically inoculated into

Petri dishes (9 cm Ø) containing fresh OMc gelled expression medium. A total of 488 somatic embryos were established (266 globular and 222 in the torpedo stage). A total of 20 embryos were inoculated per Petri dish, which served as a biological replicate (a minimum of six replicates were considered per embryo developmental stage). The cultures were maintained for 30 days under the same temperature, photoperiod, and light intensity conditions as previously described. At the end of the subculture, the converted somatic embryos were transferred to test tubes containing OMc gelled expression medium supplemented with 1 g L⁻¹ activated charcoal. The embryos were maintained at 26 ± 1 °C, with a 16 h photoperiod and 100 µmol m⁻² s⁻¹ of light intensity, for two months. Obtained plants were further subjected to an acclimation procedure before being transferred to greenhouse conditions.

Plantlets were transferred to polypropylene honeycomb trays containing a mixture of sand, perlite, and peat in the proportion of 1:1:3 (v/v). To prevent dehydration, the trays were kept in stalls with a transparent plastic polyethylene cover. Plants were maintained under controlled conditions in a plant growth chamber with 24 °C/22 °C day/night, 60% humidity, 16 h photoperiod and light intensity of 100 µmol m⁻² s⁻¹. After 15 days, the covers were removed, and plants remained under the same conditions for an additional 15 days. Finally, the plants were transferred to a greenhouse.

III. 3.5. Histological Analysis

To assess differences in tissue microanatomy between HE and LE embryogenic lines, histological analyses were conducted. At the end of the third subculture in ECO liquid medium, three segments each of HE and LE embryogenic calli were randomly sampled and fixed in FAA solution (formaldehyde: acetic acid:70% ethanol, 1:1:8, v/v) for 48 h. The samples were further washed twice with 70% ethanol and subsequently dehydrated in a series of six solutions with increasing concentrations of butanol (10, 20, 35, 55, 75, and 95%), followed by two 30 min washes with xylene. Subsequently, impregnation was carried out by repeatedly submerging the samples in liquid paraffin wax at 58 °C over a total period of 56 h, with the immersion process performed three times in total. To achieve the paraffin wax blocks, the samples were embedded into liquid paraffin (at 58 °C), which was further left to cool in a freezer platform. The blocks were maintained in the freezer until further processing. Sections of 5–10 µm thickness were cut on a rotative microtome (Leica Reichert-Jung 2045, Wetzlar, Germany) and assembled on glass slides. Following slide preparation, the sections were subjected to a staining protocol. Paraffin was removed by two 5 min. washes in xylene, after which the samples were rehydrated in an ethanol series (100%, 95%, and 70%) and placed in distilled water for 5 min. A double staining procedure was then performed according to Sass (1958). Tissues were initially immersed in an aqueous solution of Safranin O (0.01%, w/v) for 1 min to stain chromosomes, nuclei, and lignified cell walls. This was followed by rinsing with distilled water to remove excess stain. The slides were subsequently dehydrated through an ascending ethanol series (70% and 95%), stained with Fast Green FCF (0.5%,

w/v in 95% ethanol) for 5 s to visualise the cytoplasm, and washed thoroughly in absolute ethanol. Finally, the slides were cleared with two 2 min washes in xylene, dried, and mounted with DPX mounting medium for histology (Sigma-Aldrich, Madrid, Spain). Histological observations were made using an automated microscope (Leica DM6000B, Wetzlar, Germany), and images were captured with a camera (Leica CC7000, Wetzlar, Germany).

III. 3.6. Evaluation of Ploidy Variation Through Flow Cytometry

To assess the presence of somaclonal variation associated with ploidy changes, a total of 45 samples were analysed using flow cytometry. These included somatic embryos with normal and abnormal morphology, embryogenic calli with HE and LE, and leaves from plants derived from somatic embryo conversion. Flow cytometry analysis was conducted at Plant Cytometry Services (The Netherlands, Didam) following the protocol of Arumuganathan & Earle (1991). Briefly, approximately 50–100 mg of fresh plant tissue was finely sectioned with a sharp razor blade in an ice-cold DNA buffer to isolate cell nuclei. As an internal standard, fresh tissue from a reference plant was simultaneously sectioned with the olive samples in a DNA buffer (stored at 4 °C) composed of 5 mM HEPES, 10 mM magnesium sulfate heptahydrate, 50 mM potassium chloride, 0.2% Triton X-100, 0.1% dithiothreitol, 1.0% PVP-40, and 2 mg L⁻¹ DAPI, with the pH adjusted to 7.5 (Doležel et al., 2007). The DAPI fluorescent dye selectively complexes with double-stranded DNA to give a product that fluoresces at 465 nm. DAPI has specific DNA-binding properties with a preference for adenine–thymine (AT)-rich sequences. Further, the buffer containing cell constituents and large tissue fragments was passed through a nylon filter of 50 µm mesh size.

The DNA content of the stained nuclei was measured after incubation using a Sysmex CyFlow Ploidy analyser with a UV high-power LED (365). The fluorescence of the stained nuclei, passing through the focus of a light beam from a high-pressure mercury lamp, was measured by a photomultiplier and converted into voltage pulses. These voltage pulses are electronically processed to yield integral and peak signals and can be processed by a computer. A diploid *Allium. schoenoprasum* sample ($2n = 2x = 32$; 2C DNA amount = 33.50 pg) was used as the internal standard. The ratio between the mean fluorescence of G0/G1 nuclei of the olive sample and the internal standard *A. schoenoprasum* was calculated as an indicator of the ploidy level of each sample.

III. 3.7. Statistical Analysis

Statistical analyses were performed using SPSS version 28.0. Normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene’s tests, respectively. Comparisons of mean somatic embryo conversion rates at different developmental stages, between HE and LE embryogenic

lines and between the two cultivars, were conducted using the Student's *t*-test or the nonparametric Mann–Whitney *U* test when parametric assumptions were not met. For callogenesis, rhizogenesis, necrosis, and SE parameters, mean comparisons among different explants within each cultivar were performed using one-way ANOVA (followed by Tukey's HSD test) or the Kruskal–Wallis test when parametric assumptions were not met. The number of somatic embryos from embryogenic lines in both cultivars, across three subcultures in ECO liquid medium and one subculture in OMc gelled expression medium, was analysed using one-way ANOVA (followed by Tukey's HSD test) or the Kruskal–Wallis test when appropriate. Statistical significance was set at $p < 0.05$.

III. 4. RESULTS

III. 4.1. Establishment of Somatic Embryogenesis

The rate of callogenesis and rhizogenesis, as assessed 21 days after explant establishment in the induction medium, indicates calli development in all types of explants (radicles, proximal, and distal cotyledons) in both cultivars. Significant differences were observed in explants from cv. 'Galega Vulgar', with radicles exhibiting the highest callogenesis rate (Table III.1). In contrast, cv. 'Arbequina' showed the highest average callogenesis rate (approximately 70%) and significantly higher rates than cv. 'Galega Vulgar'. No significant differences were observed among the different explant types. The development of adventitious roots (rhizogenesis) was observed in all explants in both cultivars, with cv. 'Arbequina' exhibiting significantly higher rates compared to cv. 'Galega Vulgar'. Despite the overall higher rhizogenesis in cv. 'Arbequina', no significant differences were found among the three explant types within this cultivar. In contrast, in cv. 'Galega Vulgar', the radicle exhibited a significantly higher rhizogenesis rate compared to the proximal and distal regions of cotyledons (see Table III.1).

Table III.1. Callogenesis and rhizogenesis rates (%) obtained during the induction phase, along with necrosis and somatic embryogenesis (SE) rates observed during the expression phase, are presented for all explant types (radicles and proximal and distal cotyledons) in both cultivars. Different lowercase letters indicate statistically significant differences ($p \leq 0.05$) among the three explants (radicle, proximal cotyledon, and distal cotyledon) within a cultivar, while uppercase letters denote significant differences between cultivars for the same explant type. Data are expressed as mean $\pm$ standard error.

Cultivar	Explant	Induction			Expression	
		<i>n</i>	Callogenesis	Rhizogenesis	Necrosis	SE
'Galega Vulgar'	Radicle		48.5 $\pm$ 4.3 Ab	9.7 $\pm$ 2.6 Ab	29.1 $\pm$ 3.9 b	8.2 $\pm$ 2.4
	Proximal	134	27.6 $\pm$ 3.9 Aa	0.8 $\pm$ 0.7 Aa	16.4 $\pm$ 3.2 Aa	2.2 $\pm$ 1.3
	Distal		39.6 $\pm$ 4.2 Aab	0.8 $\pm$ 0.7 Aa	21.6 $\pm$ 3.6 Aab	4.5 $\pm$ 1.8

	Radicle		65.6 ± 3.7 B	30.1 ± 3.6 B	34.4 ± 3.7	9.8 ± 2.3 b
‘Arbequina’	Proximal	163	66.8 ± 3.6 B	21.5 ± 3.2 B	33.1 ± 3.7 B	1.9 ± 1.1 a
	Distal		66.9 ± 3.7 B	22.7 ± 3.3 B	30.1 ± 3.6 B	4.9 ± 1.7 ab

The calli morphology formed at the end of the induction phase showed morphological uniformity across all explants. The obtained calli were predominantly formed on the periphery of the explants at the wound regions, exhibiting a colour ranging from pale yellow to white and friable consistency (Figure III.2).

All the explants that responded to embryogenic induction were transferred into an OMc gelled expression medium to promote embryo differentiation. After 30 days under expression conditions, the embryogenic structures were observed on the surface of the calli and often underneath them, staying in direct contact with the culture medium.

At the expression phase, the percentage of explant necrosis and the percentage of explants that responded positively to SE are presented in Table III.1. In both the proximal and distal cotyledons, the overall rate of necrosis during the expression phase was substantially higher in cv. ‘Arbequina’ than in cv. ‘Galega Vulgar’ (Table III.1). In cv. ‘Galega Vulgar’, significant differences were observed among explants, with radicles showing the highest necrosis rate.

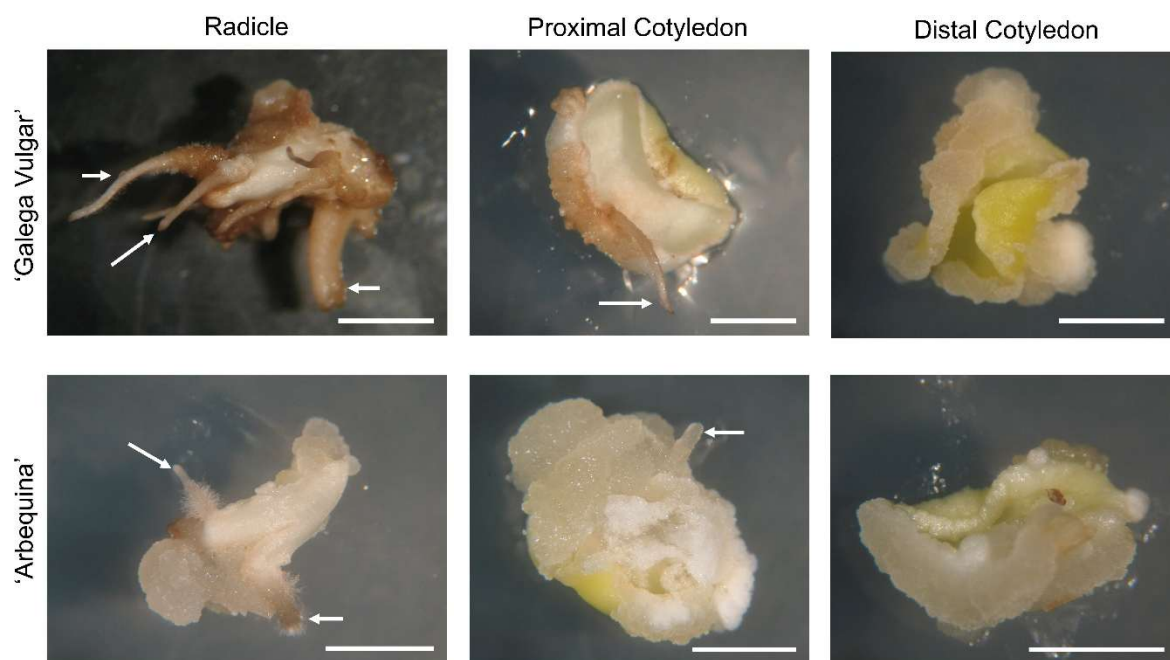


Figure III.2. Aspect of the calli achieved in all types of initial explants after 21 days in induction OMc medium. The presence of adventitious roots is highlighted with white arrows. Bars: 0.5 cm.

The capacity of the explants to respond to the stimulus of SE can also be observed in Table III.1. The radicle exhibited the highest response rate to the SE stimulus when compared to the proximal and distal cotyledons; however, significant differences among explants were only detected in cv. 'Arbequina'. Proximal cotyledons exhibited a significantly lower number of explants developing somatic embryos, although no significant differences were observed between distal cotyledons and radicles.

To promote the proliferation of embryogenic calli, all embryogenic plant material (embryogenic calli and somatic embryos at early developmental phases) was transferred to the ECO gelled medium for two subcultures. At the end of the second subculture, explants were transferred to the ECO liquid culture medium for three additional subcultures.

III. 4.2. Characterisation of the Embryogenic Lines Established Under Liquid Medium

To evaluate the embryogenic potential of the different embryogenic lines established in liquid medium, 200 mg of embryogenic tissue from each line was inoculated in ECO liquid medium and maintained through three consecutive subcultures, followed by a final transfer to OMc expression medium to further evaluate the embryo conversion rate. Each subculture had a duration of 4 weeks, after which the number of somatic embryos formed per gram of calli was recorded (Figure III.3). Initially, eleven embryogenic lines of the cv. 'Galega Vulgar' and twenty lines of cv. 'Arbequina' were established. However, not all lines exhibited sustained viability under liquid culture conditions. Several showed progressive oxidation and loss of morphogenic potential, leading to their exclusion from the experiment. Ultimately, seven embryogenic lines of cv. 'Galega Vulgar' and eleven of cv. 'Arbequina' were processed for subsequent stages of the assay.

Within each embryogenic line, the first subculture in ECO liquid medium produced fewer somatic embryos, with numbers progressively increasing in subsequent subcultures and, in most cases, reaching the highest rates in the third subculture (exceptions were observed in lines A23, A133, and A140). Regarding the transfer to OMc expression medium, not all calli responded with an increase in SE efficiency. While some lines showed an increase in the number of differentiated embryos, others remained stable, and some exhibited a slight decrease (e.g., G78 and G92; A19, A23, A33, A157, and A158). Nevertheless, these changes were only statistically significant in line A113 ($p = 0.042$).

A comparison of somatic embryo production across the embryogenic lines of each cultivar was also conducted within each subculture. During the first subculture in ECO liquid medium, no significant differences between the embryogenic lines in the cv. 'Galega Vulgar' were found ($p = 0.073$); however, line G4 displayed a greater number of somatic embryos. In fact, across the different subcultures, G4

consistently showed the greatest capacity for somatic embryo formation, with significant differences emerging from the first subculture onwards, except in comparison with line G128. Lines G22 and G92 consistently produced the lowest number of somatic embryos (Figure III.3). In cv. ‘Arbequina’, lines A140 and A148 produced the highest number of somatic embryos across all subcultures. In contrast, lines A10, A23, A33, and A158 showed the lowest embryo production, with line A23 consistently exhibiting the lowest values across all subcultures. Similar to what was observed in cv. ‘Galega Vulgar’, some lines (A19, A23, A33, A157, and A158) produced a higher number of somatic embryos during the third subculture in ECO liquid medium, although these differences were not statistically significant.



Figure III.3. Number of somatic embryos per gram of calli of all embryogenic lines in the two cultivars under evaluation (cv. ‘Galega Vulgar’ and cv.

‘Arbequina’) during three subcultures in ECO liquid medium and one subculture in OMc expression medium. Different lowercase letters indicate statistically significant differences ($p \leq 0.05$) between lines, while capital letters represent differences between subcultures. Data are presented as the mean $\pm$ standard error.

Morphological differences in calli structure were consistently observed between high and low-efficiency embryogenic lines in both cultivars. Differences between embryogenic lines are illustrated in Figures III.3 and III.4, which show representative images at the end of the second subculture in ECO gelled medium, after the third subculture in ECO liquid medium, and following transfer to OMc expression gelled medium in both cultivars. In ‘Galega Vulgar’ (Figure III.4), HE lines (G4, G76, G128) developed fragmented calli with abundant globular-stage somatic embryos. In contrast, LE lines (G22, G46, G92) produced large compact calli with markedly fewer embryos.

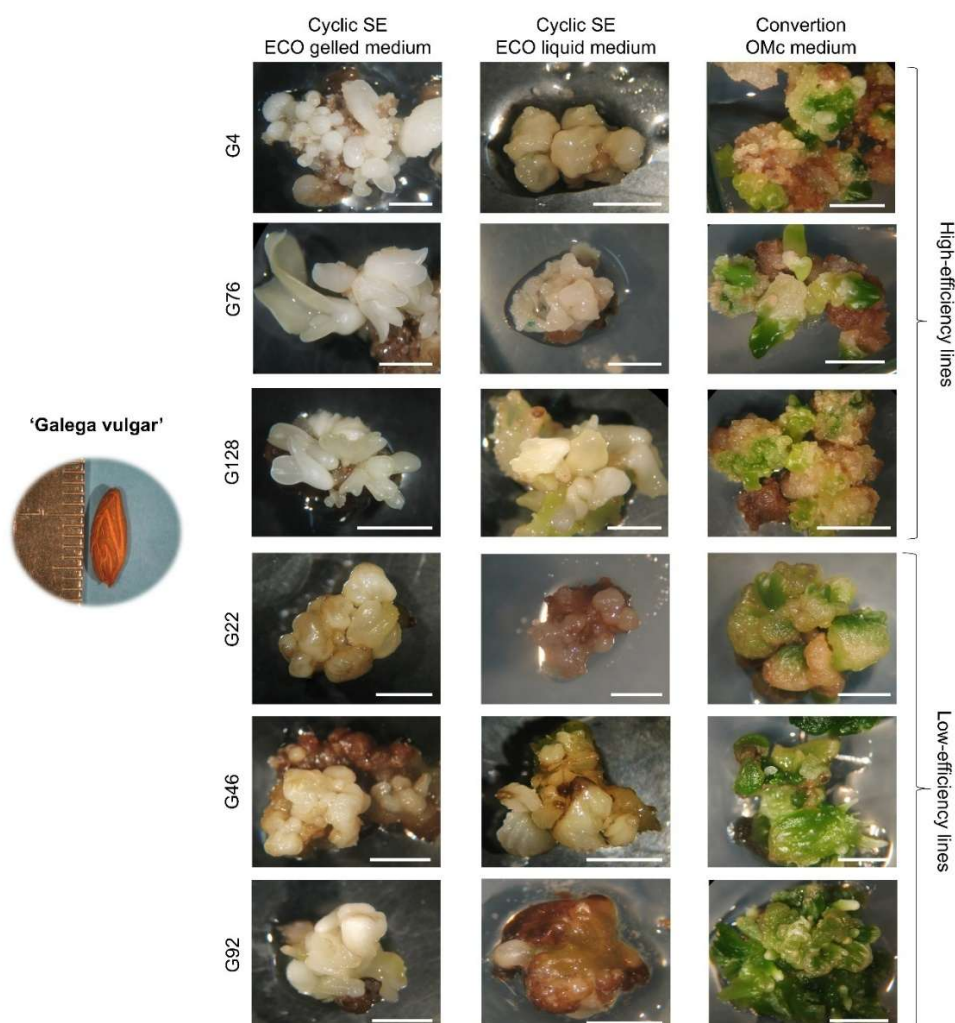
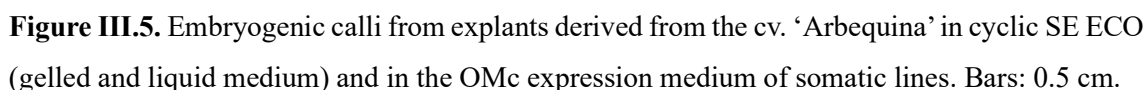


Figure III.4. Embryogenic calli from explants derived from the cv. ‘Galega Vulgar’ in cyclic SE ECO (gelled and liquid medium) and in the OMc expression medium. Bars: 0.5 cm.



The HE and LE embryogenic lines presented in Figures III.4 and III.5 were selected for plant conversion assessment after subculture in OMc expression medium. Classification was based on consistent differences observed throughout the subculture period, including the average number of somatic embryos formed per mg of calli, regenerative capacity of the calli, and incidence of tissue necrosis. HE lines consistently exhibited greater embryo production, enhanced regeneration, and reduced necrosis.

III. 4.3. Conversion of Somatic Embryos

HE and LE embryogenic lines were analysed to determine which were the most suitable for embryo conversion into plants. Following the procedure outlined by Pires et al. (2020), embryos at the globular and torpedo stages were individually collected and inoculated onto OMc expression medium for 30 days. At the end of this period, the number of converted somatic embryos was recorded. The cotyledonary stage was not considered, as most of the somatic embryos observed at this stage exhibited morphological anomalies.

Regarding embryo conversion, somatic embryos from high-efficiency lines yielded results comparable to those from low-efficiency lines (Figure III.6). In cv. ‘Galega Vulgar’, HE lines converted more somatic embryos into plants; however, no significant differences were observed compared to LE lines. Although the torpedo stage resulted in a significantly higher conversion rate of somatic embryos into plants in both cultivars, no significant differences were observed among developmental stages in HE lines of cv. ‘Arbequina’.

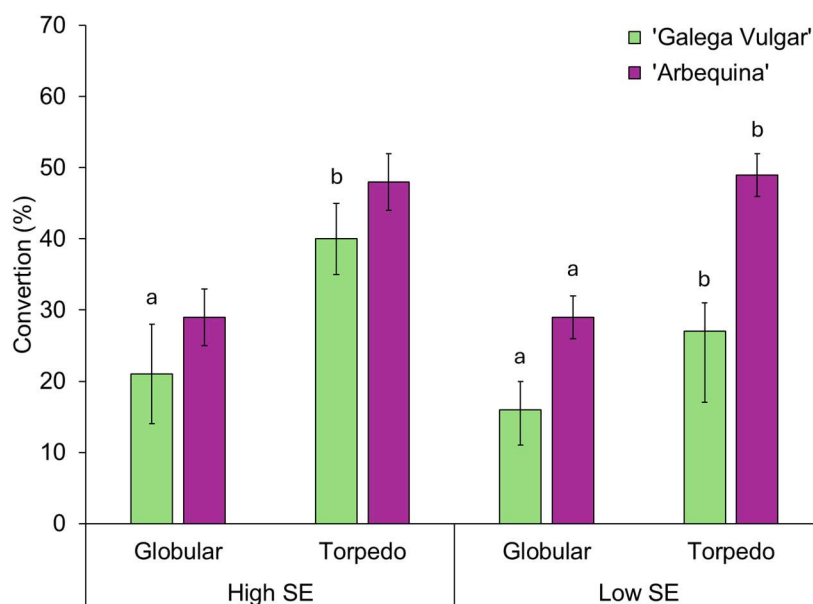


Figure III.6. Conversion rates of somatic embryos collected at different developmental stages from high- and low-efficiency somatic embryogenesis (SE) lines (mean $\pm$ standard error) of both cultivars. Statistically significant differences between stages are denoted by distinct lowercase letters. Statistical significance was set at $p \leq 0.05$.

All somatic embryos, including those exhibiting morphological abnormalities (Figure III.7.A), were transferred to test tubes containing OMc expression medium supplemented with activated charcoal (Figure III.7.B, C). After 4 weeks, leaf samples were collected from plants regenerated from both normal and abnormal somatic embryos to enable flow cytometric analysis for the detection of ploidy level variations. The plants that developed from normal embryos of both cultivars were successfully acclimated under the conditions employed, regardless of the fact that the study did not take the embryos' acclimatisation process into account (Figure III.7.D, E).

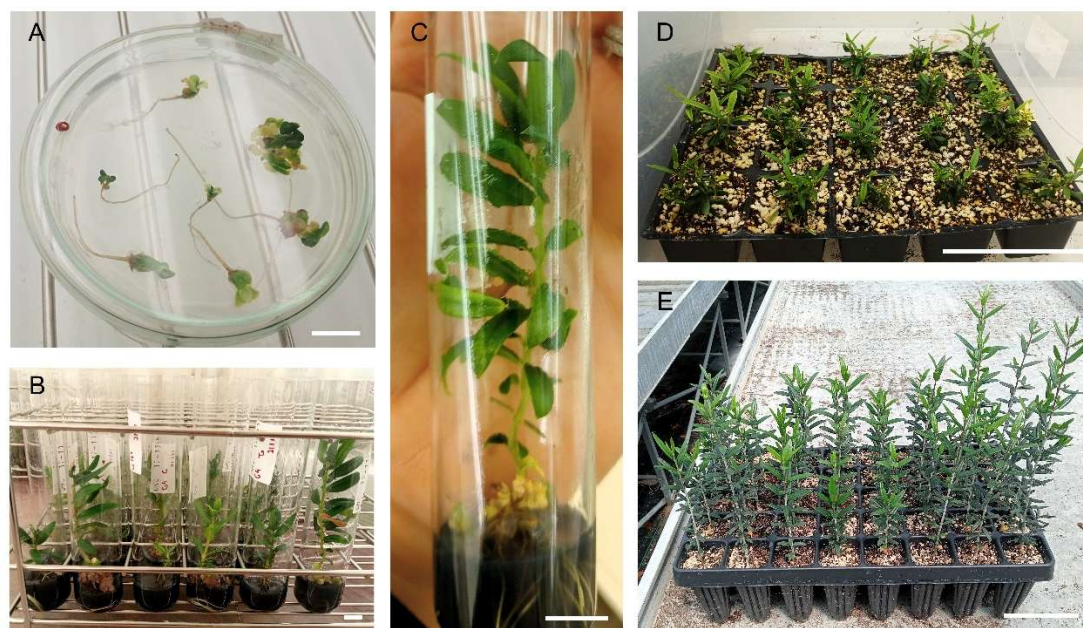


Figure III.7. Converted somatic embryos of cv. ‘Galega Vulgar’ and cv. ‘Arbequina’, and plants at various developmental stages, including acclimatised plants. (A) Somatic embryos at the end of the 30-day conversion period. (B, C) Converted somatic embryos of cv. ‘Galega Vulgar’. (D) Acclimatised plants derived from somatic embryos of cv. ‘Arbequina’. (E) Acclimatised plants derived from somatic embryos of cv. ‘Galega Vulgar’. Bars for (A–C) correspond to 1 cm, and, for (D, E), 10 cm.

III. 4.4. Histological Observations

Histological observations were conducted on HE and LE lines collected at the end of the third subculture in ECO liquid medium (Figure III.8). Clear cytological differences were consistently observed between the two types. In HE lines, embryogenic tissues were mainly composed of isodiametric parenchyma cells, with isolated proembryonal masses originating from the epidermal layer (Figure III. 8. A, C). These cells exhibited dense cytoplasm, a high nucleus-to-cytoplasm ratio, and well-defined cell walls. Frequent periclinal and anticlinal divisions of epidermal cells were also noted (Figure III.8. C). Starch granules were detected in both line types, particularly in epidermal and sub-epidermal layers, but were absent in zones of active cell division (Figure III. 8. C, F, I, L). Embryogenic cells in HE lines formed structured clusters or meristematic zones, rarely observed in LE calli. Additionally, the vascular bundles were seen to be arranged in a concentric arrangement, with phloem situated peripherally and xylem in the inner regions (Figure III.8.D–F). Tracheids within these bundles showed elongated forms with thick lignified cell walls, dark-pink staining, and lighter lumens (Figure III.7. E); these structures were more evident under fluorescence microscopy (Figure III. 8. F).

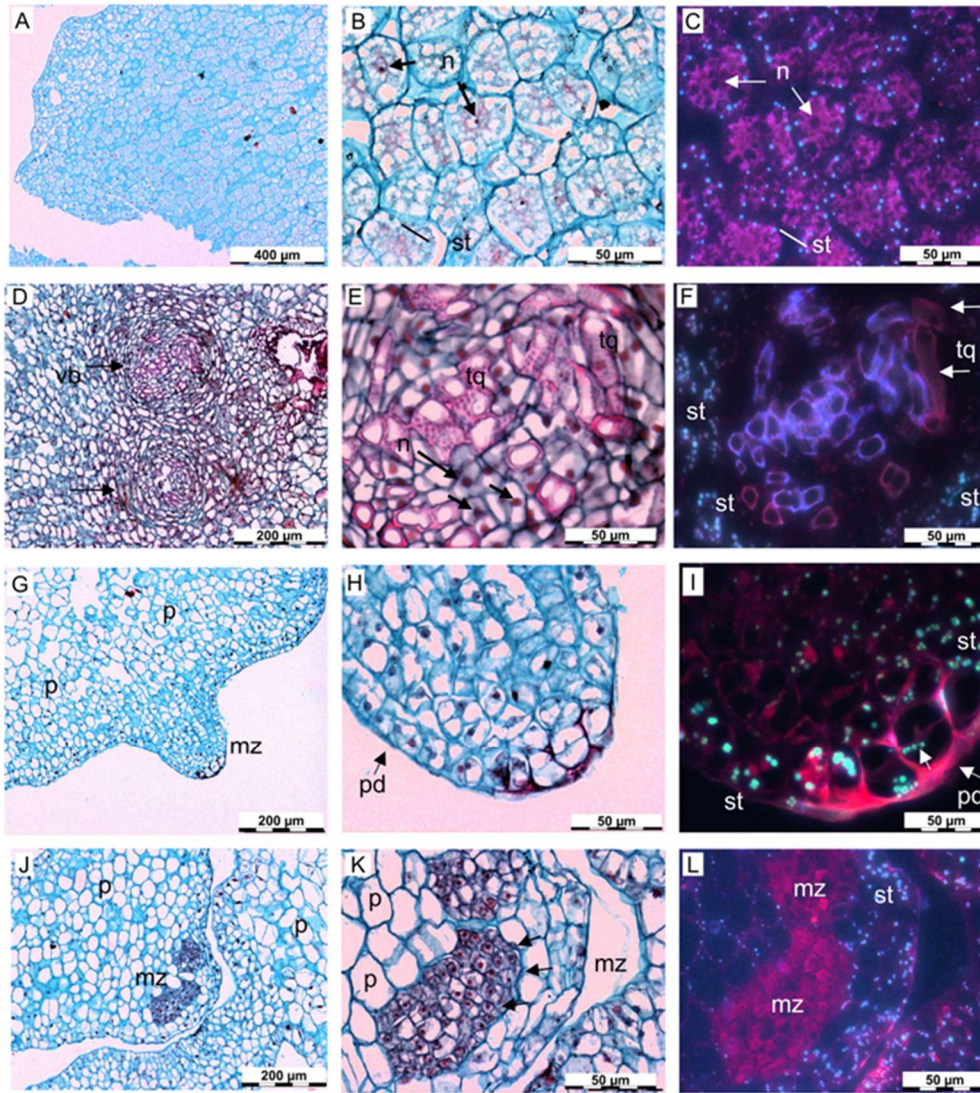


Figure III.8. Histological observations of high- (A–F) and low-efficiency (G–L) embryogenic calli. (A) Overview of a high-efficiency calli showing compact and well-organised cellular structure. (B, C) High-efficiency calli with prominent nuclei (n) and abundant starch deposits (st) under light and fluorescence microscopy, respectively. (D–F) Somatic embryo primordia in high-efficiency calli with clear vascular bundle formation (vb), including concentric xylem and phloem arrangement, and differentiated tracheids (tq) with lignified walls, highlighted by fluorescence (F). (G–I) Low-efficiency calli displaying loosely arranged parenchyma cells (p) and meristematic zones (mz) located peripherally. Cells exhibit reduced cytoplasmic content and lower starch accumulation, with protodermal organisation (pd). (J–L) Low-efficiency calli displaying meristematic zones (mz) with small actively dividing cells. Abbreviations: mz, meristematic zone; n, nucleus; p, parenchyma; pd, protodermis; st, starch; tq, tracheid; vb, vascular bundle.

In contrast, LE lines (Figure III. 8. G–L) exhibited less organised cellular architecture and tissue structure compared to HE lines, characterised by larger vacuolated cells, reduced cellular density, and meristematic zones confined to specific peripheral regions of the explants, indicating a loss of embryogenic competence (Figure III.8. G, J). These regions, characterised by higher cell density, contained clusters of embryonic cells arranged in layers or small groups. Cells in LE lines were generally larger, with less abundant cytoplasmic content. Although starch granules were visible in epidermis-like parenchyma cells, they were less abundant than in HE tissues.

In summary, histological analysis revealed clear structural differences between HE and LE embryogenic calli. HE lines displayed organised cell layers, high cytoplasmic content, abundant starch, and active somatic embryo primordia, often associated with vascular bundle formation. LE lines, on the other hand, displayed less distinct embryogenic zones, loosely arranged parenchyma cells, and fewer metabolic indicators. These results support the relevance of cellular architecture as a reliable marker of embryogenic potential and line efficiency.

III. 4.5. Flow Cytometry

Flow cytometry analysis of 45 samples across embryogenic lines revealed consistent ploidy levels among the different tissue types, including HE and LE embryogenic calli, abnormal somatic embryos, and leaf samples taken from normal and abnormal converted embryos. All samples displayed DNA content peaks corresponding to the diploid reference species *A. schoenoprasum*, indicating the maintenance of diploidy throughout SE and plant conversion. The results showed that 93.4% of the analysed samples exhibited the normal diploid level for olive (2×), as exemplified in Figure III. 9. A. Nevertheless, in 6.6% of the samples, variations in the relative DNA ratio were identified, as represented in Figure III. 9. B, where HE embryogenic calli presented 75% of the cells as diploid, and 25% exhibited aneuploidy, indicating chromosomal instability. The presence of a more pronounced intermediate peak suggests a higher proportion of cells in the S phase, indicating a higher rate of cell proliferation in this line. Furthermore, the CV% values were slightly higher, particularly in the intermediate peak (2.78%), reflecting greater heterogeneity in DNA replication.

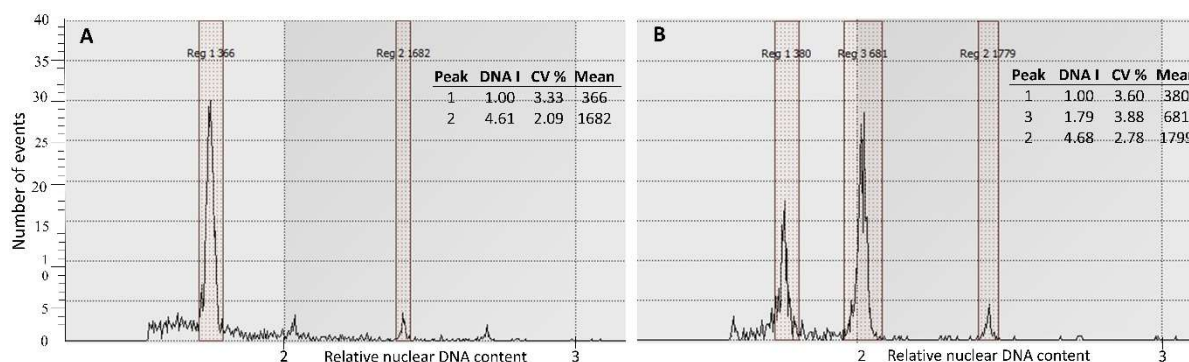


Figure III.9. Estimation of absolute nuclear DNA content in somatic embryogenic calli. The G1 peak of *Olea europaea* is indicated as Reg 1, while the G1 peak of the standard sample (*Allium schoenoprasum*) is indicated as Reg 2. The DNA histograms show both G1 and G2/M peaks. Panel (A) represents samples with stable diploid profiles (2×), while Panel (B) illustrates a sample with altered DNA content, suggesting chromosomal instability, as evidenced by the presence of additional or shifted peaks.

Flow cytometry analysis of abnormal somatic embryos identified in cv. ‘Galega Vulgar’ and cv. ‘Arbequina’ revealed that all samples were diploid (2×), indicating that the observed morphological abnormalities were not associated with changes in ploidy. This suggests that other factors, possibly related to environmental stress or genetic factors, may be contributing to the observed somatic embryo abnormalities. Leaves from regenerated plants exhibited stable diploid profiles, confirming that the SE process and plant regeneration maintained chromosomal integrity.

III. 5. DISCUSSION

Mature zygotic embryos from two olive cultivars (cv. Galega Vulgar’ and cv. Arbequina’) were successfully used as initial explants to establish and maintain HE and LE somatic embryogenic lines. Callogenesis was achieved in all explant types, with cv. ‘Galega Vulgar’ exhibiting higher calli formation in radicles and distal cotyledons. Similar results were reported by Pires et al. (2020) in the same cultivar, in which radicles and distal cotyledons showed the highest callogenesis rates under light conditions. In cv. ‘Arbequina’, no significant differences in callogenesis were observed between radicle and cotyledon explants. These findings are consistent with those previously described in the same cultivar (Mazri et al., 2012) but differ from the results of Trabelsi et al. (2003), who, despite working with cv. ‘Arbequina’ reported lower callogenesis rates than those obtained in the present study.

Rhizogenesis was observed in all explant types from both cultivars, with higher potential in calli derived from radicles. In this study, cv. ‘Arbequina’ showed significantly higher rhizogenesis rates than

cv. ‘Galega Vulgar’, regardless of explant type. These results align with Trabelsi et al. (2003). Overall, rhizogenic response appears to be genotype-dependent, with cv. ‘Arbequina’ showing greater morphogenic potential. As reported by Orinos & Mitrakos (1991), this response is typical of juvenile or immature tissues, particularly under auxin-based conditions. The formation of adventitious roots during SE induction from zygotic embryos is an expected phenomenon in plant tissue culture, as reported in several plant species, including *Malus domestica* (Paul et al., 1994) and *R. hybrida* (Du et al., 2024).

Regarding embryo formation, all explant types responded positively to the tested protocol, with radicles showing the highest response. The outstanding reaction of radicles for SE initiation, compared to cotyledons, has been previously reported in the cvs. ‘Koroneiki’ (Peyvandi et al., 2008), ‘Zard’ (Jafarzadeh-Bajestani et al., 2011), and ‘Picual’ (Cerezo et al., 2011). Peyvandi et al. (2008), in a study of the cvs. ‘Zard’ and ‘Mission’, observed that embryogenic potential decreases from the radicle toward the proximal regions of the embryo. This phenomenon is likely due to the higher density of young and competent cells in radicles, which exhibit greater potential for dedifferentiation and redifferentiation into somatic embryos (Rugini & Caricato, 1995). Additionally, the hormonal balance between auxin and cytokinin in radicles appears more suitable for SE initiation than in cotyledons (Su et al., 2011).

The results presented here confirm that embryogenic capacity can be sustained over successive cycles of secondary SE, enabling the identification of somatic embryogenic lines with different abilities to differentiate somatic embryos. Cyclic embryogenesis from zygotic embryos in olive has previously been achieved in several genotypes, as exemplified by studies conducted on the cv. ‘Picual’ (Pérez-Barranco et al., 2009; Cerezo et al., 2011, 2019), and cv. ‘Dahbia’ (Mazri et al., 2020) using ECO gelled medium. Liquid embryogenic cultures are widely employed to promote homogeneous cell proliferation (Malik et al., 2024) and to facilitate the investigation of secreted biomolecules (Pernis et al., 2023; Pires et al., 2025). In olive, our previous research aimed at establishing a methodological framework for the isolation and characterisation of proteins and metabolites associated with SE, underscoring the significance of this system by revealing a high diversity of secreted compounds in liquid embryogenic cultures (Pires et al., 2025). However, despite its advantages, the maintenance of embryogenic cultures in liquid medium presents considerable challenges, primarily due to the elevated risk of contamination. To address this limitation, cultures in the present study were initially propagated on ECO gelled medium to enhance proliferation, followed by transfer to liquid medium to mitigate the risk of culture loss.

The LE lines exhibited higher levels of phenolic oxidation and reduced proliferation compared to HE lines. These characteristics were evident from the first subculture on ECO gelled medium and persisted throughout the experiments. Moreover, the LE lines were more difficult to maintain in a proliferative state, and some embryogenic lines did not respond to the inoculation in liquid medium. Previous studies developed with the cv. ‘Chemlal’ (Titouh et al., 2022) reported similar results, highlighting the occurrence of phenolic oxidation from the first subculture, leading to tissue necrosis

and consequent loss of some lines. This oxidative response may be associated with stress-induced metabolic activity, as previously reported (Baharan & Mohammadi, 2018; Oulbi et al., 2021; Pernis et al., 2023). Stress response proteins, such as peroxidases, catalases, and superoxide dismutase, are known to regulate several physiological processes, including de novo organogenesis and lignification (Guo et al., 2017; Voxeur et al., 2025), and have been proposed as biochemical markers. A few of these tissue-level indicators, specifically peroxidases, were also found in the secretome by Pires et al. (2025). In addition, the authors reported the detection of alpha-amylase, an enzyme that plays a key role in the breakdown of starch into simple sugars. Its activity has been associated with providing an energy source for root and shoot development during the conversion of somatic embryos (Sun et al., 2024). Its accumulation in mature somatic embryos has been documented in *Musa* spp. (Kumaravel et al., 2020) and *Araucaria angustifolia* (Navarro et al., 2021), indicating active starch mobilisation and its role as an energy source during somatic embryo germination. In addition, the identification of alpha-amylase in the secretome of a highly competent embryogenic line may indicate its presence in the embryogenic calli, consistent with the detection of starch granules in HE lines revealed by histological analysis. This observation supports the hypothesis of active carbohydrate metabolism, in which alpha-amylase contributes to the hydrolysis of starch into simple sugars, enabling the mobilisation of energy reserves to sustain embryogenic development. Additionally, embryos from HE lines showed a greater capacity for plant regeneration than those from LE lines, as shown by somatic embryo conversion assays. This improved performance may be associated with more effective starch mobilisation, as evidenced by the presence of starch granules and the involvement of alpha-amylase in maintaining energy supply during embryo germination.

Because prolonged subculturing in SE systems has been linked to the induction of somaclonal variations (Sanchez-Romero, 2024), it is crucial to assess the genetic stability of in vitro protocols before their application. In this context, flow cytometry was employed to evaluate chromosomal stability in embryogenic calli, somatic embryos, and regenerated plants. Genetic and epigenetic alterations in somatic cells during multiple mitotic divisions of a single clonal explant can result in somaclonal variations (Leva et al., 2012). Such variations have been identified using cytogenetic techniques, such as flow cytometry, in several plant species, including *M. acuminata* (Escobedo-Gracia-Medrano et al., 2018), *V. vinifera* (Catalano et al., 2021), *Piper hispidinervum* (De Sousa et al., 2022), and *Camelina sativa* (Bahmankar & Rahnama, 2024). Data revealed a high proportion of samples with normal ploidy levels. However, instances of aneuploidy and abnormal embryo development were observed. Bradaï et al. (2016b) investigated the effects of genotype and culture age on somaclonal variation in olive plants regenerated via SE from seeds of the cv. 'Picual' using morphological and biometric analyses of vegetative and reproductive traits. Their findings demonstrated significant somaclonal variation, with biometric analyses revealing intraclonal differences in the development of quantitative traits. Genotype was identified as a critical factor, with older lines exhibiting higher variability (Bradaï et al., 2019).

Somaclonal variation in SE-derived plants was confirmed by RAPD and SSR markers analysis, with older lines showing greater genetic instability, primarily attributed to genotype dependency.

The present study highlights the influence of genotype on the morphogenic response, with cv. ‘Arbequina’ exhibited a higher SE potential across all explant types. Embryogenic cultures from both cultivars remained stable in liquid medium across successive subcultures. The availability of lines with different embryogenic potential enables further research on the role of biomolecules secreted into the culture medium. Although the genetic stability of the cultured lines was not a primary goal of this study, the findings underscore the importance of testing the genetic uniformity of plants derived from SE whenever this technique is used in olive for clonal multiplication.

III. 6. CONCLUSIONS

To the best of our knowledge, *in vitro* cultured lines with differentiated embryonic capacity were, for the first time, maintained with systematic behaviour in olive in a process of cyclic SE. This was achieved using a comprehensive protocol for SE, based on zygotic embryos from the cvs. ‘Galega Vulgar’ and ‘Arbequina’, as initial explants. Acquired data allowed us to confirm genotype dependency in the success of somatic embryo production within the same species. The use of the ECO culture medium enabled the maintenance of embryogenic calli without altering the efficiency of embryogenic lines across multiple subcultures. Regarding somatic embryo conversion, it was possible to conclude that the globular and torpedo stages are the earliest and most effective stages for converting somatic embryos into plants in all cultivars. However, the conversion efficiency is also influenced by the genotype and the embryogenic line, with HE lines showing better embryo plant conversion rates than LE lines. Chromosomal stability was evaluated by flow cytometry, revealing a high degree of genetic stability in embryogenic calli, somatic embryos, and regenerated plants across all studied somatic lines. Nevertheless, some situations of aneuploidy were detected, confirming the need for the genetic evaluation for all individuals obtained by SE whenever the technique is used for clonal propagation in olive. The availability of both high and low somatic embryogenic lines will enable further research focused on evaluating differences in biomolecules secreted into the culture medium. This will provide insights into the molecular mechanisms underlying SE recalcitrance and guide future studies aimed at elucidating the processes involved in SE signalling. Considering the importance of these biomolecules in cellular communication, we hypothesise that they may play a crucial role in understanding SE recalcitrance in certain genotypes.

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CHAPTER IV

Establishment of a Protocol for the Characterisation of Secreted Biomolecules in Somatic Embryogenic Cultures of *Olea europaea* L.

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IV. 1. ABSTRACT

Somatic embryogenesis (SE) involves the formation of embryo-like structures from somatic cells without fertilisation and is widely used for clonal propagation and genetic transformation. However, in olive (*Olea europaea* sp. *europaea*), SE remains challenging due to the recalcitrant behaviour of adult tissues when used as initial explants. Bioactive molecules released into the culture medium (conditioned medium, CM) by embryogenic cultures have been identified as modulators of the SE response. However, their potential role in enhancing SE efficiency in olive and overcoming tissue recalcitrance remains largely unexplored. To investigate the role of these biomolecules in olive SE, a protocol was established using SE cultures of cv. ‘Galega Vulgar’. Proteins and metabolites were separated by filtration, concentrated through lyophilisation, and precipitated using three methods: Acetone, TCA/Acetone, and Methanol/Chloroform. The efficiency of these methods was evaluated through total protein quantification and via SDS-PAGE electrophoresis. LC-MS/MS was employed to analyse secretome composition using the TCA/Acetone precipitation method. Additionally, metabolite profiles were analysed using ^1H NMR spectroscopy. The results led to the identification of 1096 (526 protein groups) *O. europaea* proteins, including well-known SE biomarkers such as kinases and peroxidases. NMR spectroscopy identified several metabolites secreted into the medium or resulting from the metabolic activity of secreted enzymes, confirming the applicability of the procedure. Although extracting secreted biomolecules from the culture medium presents significant challenges, the protocol established in this study successfully enabled the isolation and identification of both proteins and metabolites, revealing a valuable workflow for future in-depth analyses of secreted biomolecules in olive SE.

IV. 2. INTRODUCTION

Somatic embryogenesis (SE) is a nonsexual propagation process regulated by complex mechanisms (Juarez-Escobar et al., 2021) that leads to the formation of structures resembling zygotic embryos. These structures arise from the dedifferentiation of somatic cells, which can generate a complete plant without fertilisation. SE is widely used for clonal propagation in several plant species (Hazubska-Przybył et al., 2020), providing suitable material for genetic transformation or to assist breeding programs following the manipulation of plant genome approaches (e.g., mutagenesis and New Genomic Techniques) (Vidal et al., 2010; Heringer et al., 2018), synthetic seed production, and germplasm conservation (Guan et al., 2016). However, the mechanisms by which a somatic cell develops into a whole plant are complex and remain poorly understood. The ability to shift cell development from the vegetative to the embryogenic pathway is restricted to a limited number of cells (Pais, 2019) and is regulated by complex endogenous and exogenous factors. Various factors have been tested to optimise SE protocols across different plant species. These include exogenous factors (Trabelsi

et al., 2003; Brhadda et al., 2003; Capelo et al., 2010; Oulbi et al., 2018), such as the culture medium composition, type and concentration of plant growth regulators, and growth conditions, and endogenous factors, including genotype, explant type, and developmental stage (Mazri et al., 2012; Narváez et al., 2019; Cardoso et al., 2019; Pires et al., 2020). In olive tree (*Olea europaea* subsp. *europaea*), the explant type is highlighted as one of the most limiting factors on the establishment of SE protocols (see Sánchez-Romero, 2021). In fact, most SE protocols developed for olive trees are based on juvenile tissues as initial explants, usually radicles and cotyledons of mature embryos (Pritsa & Voyiatzis, 2004; Pires et al., 2020; Mazri et al., 2020; Titouh et al., 2022). However, this approach is less ideal for clonal propagation purposes due to the high genetic variability associated with zygotic tissues (Pires et al., 2020; Cerezo et al., 2011; Mazri et al., 2020). Obtaining SE from adult olive material remains challenging due to the recalcitrant behaviour exhibited by most tested genotypes (see review in Cardoso et al., 2019). Exceptions are known for the cvs. 'Canino' and 'Moraiolo' (Rugini & Caricato, 1995) with the development of a double regeneration system through organogenesis, and for the cvs. 'Chetoui' (Trabelsi et al., 2011), 'Dahbia' (Mazri et al., 2012), 'Picual' (Toufik et al., 2014), and 'Moroccan Picholine' (Oulbi et al., 2018).

Despite the availability of some SE protocols using olive adult plant tissues, those protocols are not routinely and widely used in olive propagation due to the high genotype dependency of SE response (see Sánchez-Romero, 2021 for details), a factor particularly critical, as even closely related genotypes can exhibit recalcitrance behaviour when using a highly efficient protocol established for a distinct genotype (Narváez et al., 2019; Mazri et al., 2020). In this context, discovering the molecular mechanism controlling SE efficiency is essential to overcome recalcitrance in olive. An important factor reported to affect SE in different plant species, but which has received little attention from the scientific community and has not been considered in any studies on olive trees, is the release of bioactive molecules by explants into liquid culture medium that could be used to modulate SE efficiency. This approach is based on the establishment of nurse-cell cultures, which means highly efficient liquid embryogenic cultures from which the culture medium is collected (commonly referred to as conditioned medium, CM) and further used to establish SE cultures using recalcitrant plant material (Hazubska-Przybył et al., 2020). The influence of CM composition derived from nurse-cell culture systems on inducing or modulating the SE response efficiency has long been observed (Eigel & Koop, 1989; Westcott, 1994; Komai, 2006; Hargreaves et al., 2017; Hazubska-Przybył et al., 2020). The use of this approach to modulate the SE response in recalcitrant genotypes has been particularly explored in gymnosperms. Dyachok et al. (2022) reported that extracts of media conditioned by embryogenic cultures of *Picea abies* stimulate the development of pro-embryogenic masses, suggesting that an endogenous lipophilic chitin oligosaccharide acts as a signal molecule in this process. Recently, Pernis et al. (2023) reported proteomic data on the secretome of *Pinus nigra* cell lines with contrasting embryogenic capacity, revealing that cell wall-related and carbohydrate-acting proteins were the most differentially

accumulated in the CM, with peroxidases (PODs), extensin, α -amylase, and basic secretory protease (BSP) being more abundant in highly embryogenic lines.

The effectiveness of the use of the CM has stimulated research aiming to identify the biomolecules that could play the crucial role in cell-to-cell communication and consequently induce/modulate SE response (Nic-Can et al., 2015). Within the secreted molecules that have been demonstrated to induce, or modulate, the SE response, there are extracellular proteins, growth regulators, vitamins, lipophilic molecules, and secondary metabolites (Awada et al., 2019, 2020; Ben-Amar, 2021). Some studies have analysed the CM from embryogenic cultures to identify proteins and metabolites that could serve as biomarkers of SE efficiency (Nic-Can et al., 2015; Ngcala et al., 2020; Pernis et al., 2023) or as modulators by supplementing the culture medium with these biomolecules (Egertsdotter, 1998; Couillerot et al., 2012; Guo et al., 2019; Gulzar et al., 2020). These investigations have demonstrated that specific extracellular proteins play a significant role in the differentiation and morphogenesis of somatic cells (Nic-Can et al., 2015), functioning as either promoters or inhibitors of SE (Duchow et al., 2016). For example, Arabinogalactan proteins (AGPs), a family of extracellular proteoglycans in plants, are implicated in many aspects of growth and development and have been directly linked to SE efficiency in *Areca catechu* (Li et al., 2023). Other proteins comprising the secretome and proposed as biomarkers of SE efficiency in different plant species were the Germin-like proteins identified in *P. balfouriana* (Li et al., 2015), the Heat shock proteins in *Sorghum bicolor* (Ngcala et al., 2020), and the Chitinases (Fráterová et al., 2013), PODs, and alpha-amylases in *P. nigra* (Pernis et al., 2023). Kreuger and van Holst (1993) showed that adding exogenous AGPs to the culture medium re-induced embryogenic competence in a non-embryogenic cell line in *Daucus carota*. Later studies confirmed AGPs as SE inducers, promoting somatic embryo formation. Similarly, Ben-Amar et al. (2010) found that exogenous chitinases boosted cell population and embryogenic cell multiplication in *Cucurbita pepo* cultures.

Studies focused on the identification and quantification of a wide range of biomolecules require accurate and high sensitive analytical platforms. As reviewed by Tchordadjieva (2006), gel-based techniques like sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional polyacrylamide electrophoresis (2DE), occasionally complemented with immunoblotting, were among the initial approaches employed for fractionating and analysing proteins secreted during SE. However, the development of advanced proteomic platforms, such as nano-liquid chromatography coupled to mass spectrometry (nLC-MS/MS), enables the detection and quantification of hundreds to thousands of proteins, including low-abundant proteins comprising the CM (Rodríguez-Celma et al., 2016), making it the most appropriate approach. Similarly, many other biomolecules have been identified as comprising the CM. From the metabolites that compose the extracellular metabolome of CM, it was reported that caffeine and chlorogenic acid are the most abundant, while hydroxybenzoic

acid and trans-cinnamic acid, found in very low concentrations, negatively influence SE in *Coffea canephora* (Nic-Can et al., 2015). In another study in *Solanum betaceum* (Caeiro et al., 2022), anthranilic acid was shown to inhibit the growth of established calli during SE. The results suggested a correlation between some secondary metabolites and dedifferentiation rates during induction, with a tendency to inhibit callus growth, likely due to metabolic effects.

Various techniques have been used for identifying and quantifying those metabolites that could be involved in modulating SE response. Nuclear Magnetic Resonance (NMR)- based metabolomics offers a non-targeted approach, providing a quantitative profile of metabolites within a sample. Unlike other techniques, such as chromatography (e.g., MS, UV, and electrochemical), NMR does not require the prior separation of metabolites before detection (Segers et al., 2019), making it an alternative for investigating the metabolome composition of the CM matrix. In *P. glauca*, Dowlatabadi et al. (2009) used 1D ^1H NMR spectroscopy to identify intracellular metabolites such as γ -aminobutyric acid, malate, 5-oxoproline, isoleucine, valine, branched-chain keto acids, short-chain keto acids, glutamine, and phenylalanine. Their findings highlighted that the embryonic development of white spruce cells can be effectively monitored, with key metabolites implicated in developmental processes, thus providing insights into the optimisation of SE developmental conditions.

Supported by prior knowledge of the importance of the CM as an inducer or modulator of the SE response, our objective was to establish a protocol for the simultaneous isolation of the secretome and extracellular metabolome to identify and quantify the proteins and metabolites comprising the CM matrix from olive somatic embryogenic cultures. This approach will support future research aimed at identifying the molecular mechanisms governing the SE response in this species, contributing to the optimisation of protocols for recalcitrant genotypes.

IV. 3. MATERIALS AND METHODS

IV. 3.1. Plant Material

Olea europaea subsp. *europaea* L. cv. 'Galega Vulgar' embryogenic lines were previously established using radicles and cotyledons of zygotic embryos cultivated in Agar-gelled Olive Medium culture (OMc) (Cañas & Benbadis, 1988) supplemented with 2.5 μM 6-dimethylallylamino-purine (2iP), 25 μM indole-3-butyric acid (IBA) (Orinos & Mitrakos, 1991) and 6 g L⁻¹ Agar-Powder (VWR, Lisbon, Portugal). Cultures at the induction phase were kept at 25 °C and 0 h photoperiod for 21 days. Developed calli were further transferred to an Agar-gelled expression OMc medium (6 g L⁻¹ Agar-Powder, VWR, Lisbon, Portugal) devoid of plant growth regulators to promote embryo differentiation. Cultures were maintained for 30 days under the same conditions of temperature and photoperiod as those used in the induction phase. Embryogenic calli and somatic embryos of a selected high

embryogenic line were transferred to an Agar-gelled Embryogenesis Cyclic Olive (ECO) culture medium (6 g L⁻¹ Agar-Powder, VWR, Lisbon, Portugal) following the protocol described by Pires et al. (2020).

To promote cyclic embryogenesis, the previously selected embryogenic line grown on ECO gelled medium was transferred to Erlenmeyer flasks containing 50 mL of liquid ECO medium with the same composition as the previous medium. To obtain sufficient material to further establish the experiment, the embryogenic cultures were maintained through three consecutive subcultures in fresh medium. Subcultures were performed every four weeks, with the cultures kept at 25 °C, in complete darkness, under 100 rpm orbital agitation.

To set up the experiment, approximately 200 mg of embryogenic calli was transferred into 100 mL Erlenmeyer flasks containing 50 mL of liquid ECO medium. Six Erlenmeyer flasks were prepared to further generate a pooled sample of CM (ECO culture medium collected four weeks after inoculation of embryogenic plant material). Simultaneously, six additional 100 mL Erlenmeyer flasks containing 50 mL of liquid ECO medium were prepared under the same conditions to serve as the control sample (ECO culture medium without inoculated embryogenic plant material).

IV. 3.2. Isolation of Secretome and Extracellular Metabolome from Conditioned Medium

The previously established Erlenmeyer flasks were left undisturbed for 5 min to allow the plant material to decant (procedure is represented in Figure IV.1). The CM was further collected from each Erlenmeyer flask in 50 mL tubes and centrifuged at 10,000× g for 10 min at 25 °C (Regente et al., 2008). The collected supernatant was then filtered through a 0.45 µm filter and thereafter through a 0.22 µm filter using a vacuum system (Millipore, Burlington, MA, USA). The plant material settled at the bottom of the Erlenmeyer flasks was collected, flash-frozen with liquid nitrogen, and stored at -80 °C until further processing (see Section IV.3.3).

A pooled sample was prepared by combining the filtered samples collected from the six Erlenmeyer flasks. Proteins and metabolites were then separated using a MacroSep™ Spin Filter 1K (PALL, New York, UK), which was pre-washed with 10 mL of RNase- and DNase-free sterile water, followed by centrifugation at 10,000× g for 10 min. The first step, the filtration step, was crucial for separating proteins from metabolites, retaining proteins (and some peptides larger than 1 kDa) above the filter while allowing metabolites to pass through. This process is particularly important for NMR analysis, as proteins, due to their large and complex structures, generate broad, overlapping signals in the NMR spectrum. These signals can obscure metabolite peaks, making accurate identification and quantification more challenging. Removing proteins not only enhances spectral clarity but also helps

concentrate metabolites, improving detection sensitivity in NMR. Meanwhile, the fraction containing the whole secretome was collected from the top of the 1K filter and divided into aliquots to test three different extraction methods, with and without sample lyophilisation. However, it is important to note that the use of centrifugal filtration devices may introduce a potential sample loss step. Proteins may adsorb onto the filter membrane, reducing their recovery, or they may precipitate and aggregate due to increased local concentration during filtration. These factors should be carefully considered when interpreting results and optimising protocols to minimise protein loss.

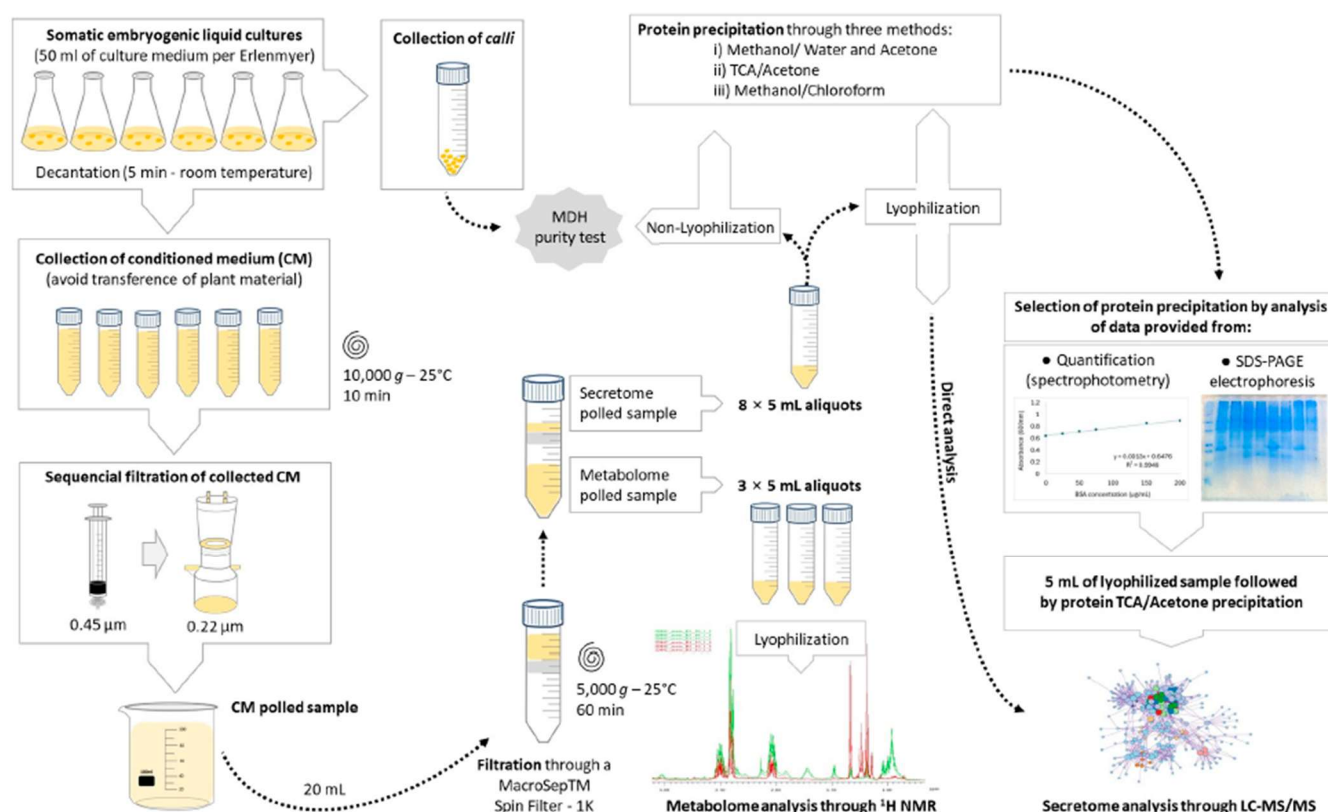


Figure IV.1. Workflow followed to collect and analyse the secretome and extracellular metabolome collected from liquid embryogenic cultures of *Olea europaea* L. Embryogenesis Cyclic Olive (ECO) culture medium was collected from replicates of an olive somatic embryogenic line that was initially selected and multiplied (see Section IV.a.3.1). This medium corresponds to the conditioned medium (CM). The collected CM was then filtered to separate the protein fraction from the metabolite fraction (for details, see Section IV.a.3.2). The purity of the protein extract was assessed by detecting malate dehydrogenase (MDH) activity, using plant material collected from the same cultures as a positive control (see Section 2.3). For secretome precipitation, two approaches were tested, lyophilisation and non-lyophilisation, in combination with three different protein precipitation protocols. The efficiency of each method was evaluated through total protein quantification and SDS-PAGE electrophoresis (details in Section IV.a.3.4). For secretome analysis via LC-MS/MS, two approaches were tested (see Section IV.a.3.5): (i) sample obtained using the most efficient protein precipitation method (lyophilisation of CM followed by total protein precipitation using the TCA/Acetone method); (ii) direct analysis of the lyophilised sample. For extracellular metabolome analysis, the

fraction obtained from CM filtration was divided into 5 mL aliquots, lyophilised, and further analysed through ^1H NMR (for details, see Section IV.a.3.6). For both secretome and extracellular metabolome analyses, a control sample was used, consisting of ECO medium with the same basal composition as CM but without the inoculation of plant material

Due to the system's limitation (maximum volume of 20 mL), multiple centrifugation steps were performed at $5000\times g$ for 60 min. The sample containing the whole secretome was collected from the top of the 1K filter, to which 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to protect the sample from proteases. The total volume of the polled sample (~40 mL) was divided into 8 samples of 5 mL each to test the three different precipitation methods, with or without sample lyophilisation. A 5 mL sample was lyophilised and directly used for LC-MS/MS analysis (see Section IV.3.5), and the remaining 5 mL sample was stored for further precipitation following the selected procedure and further analysed through LC-MS/MS.

The sample containing the whole extracellular metabolome was collected from the bottom of the 1K filter. After sample collection (CM polled sample after all filtration procedures), three 5 mL aliquots were lyophilised and stored at $-80\text{ }^{\circ}\text{C}$ until further processing (see Section IV.3.6).

IV. 3.3. Analysis of the Secretome Purity Using a Cytosolic Biomarker

To confirm that the secretome did not contain contaminants from the cell interior, an assay using malate dehydrogenase (MDH), a cytosolic biomarker (Genova & Lenaz, 2014), was conducted comparing the collected secretome (see procedure in Section IV.3.2) with the total soluble proteins extracted from plant material (used as a positive control). Embryogenic calli previously separated from the CM (Section IV.3.2) were ground with liquid nitrogen. A volume of 1.2 mL of extraction buffer [0.2 M Tris-HCL buffer (pH 8.2) solution containing 0.14 M NaCl, 0.05% (v/v) Tween-20, 0.2% (w/v) bovine serum albumin (BSA), 2% (w/v) Na_2SO_3 , 2% (w/v) polyvinylpyrrolidone (PVP) K25, insoluble PVP (1:1, w/w)] was added to 150 mg of grounded embryogenic calli. Samples were vortexed and incubated on ice for 30 min with agitation. The homogenate was then centrifuged at $14,000\times g$, for 15 min at $4\text{ }^{\circ}\text{C}$ (Diniz et al., 2019), and the supernatant was collected. The protein content of the extracts was quantified based on the Bradford method using the PierceTM 660 nm Protein Assay Reagent Kit (Thermo Scientific, Waltham, MA, USA).

An MDH analysis was performed using the sample extracted from plant material (positive control sample) and the collected secretome sample (see above IV.3.2). For that, 122.5 μL of each sample were mixed with 2% of IPG buffer (GE healthcare, Chicago, IL, USA) and applied by passive rehydration into immobilized nonlinear pH gradient 3-10 Immobiline Dry strips (7 cm) for 16 h at room

temperature in the Multiphor II system (GE Healthcare). Each strip was covered with 2 mL of mineral oil (dry strip cover fluid) to prevent evaporation. Isoelectric focusing (IEF) was performed in Pharmacia LKB Multiphor II, at 12 °C, for 50 min at a constant voltage of 200 V, followed by another 50 min at a constant voltage of 400 V (Loureiro et al., 2011), followed by vertical separation NATIVE-PAGE gel in 14% acrylamide gels. In the NATIVE-PAGE separation, the strip containing the proteins previously separated by isoelectric focusing was placed on top of a resolving gel [14% polyacrylamide with 0.370 M Tris-HCl (pH 8.3), 14% acrylamide/bis-acrylamide, 0.07% ammonium persulfate (APS), and 0.05% tetramethyl ethylenediamine (TEMED)] and electrophoresis was carried out in a Mini-PROTEAN System (Bio-Rad, Hercules, CA, USA), filled with running buffer (25 mM Tris, 190 mM glycine) at a constant voltage of 130 V until the running front reaches the end of the gel. Each sample was run in duplicate. NATIVE-PAGE gel was incubated, during 30 min, in 0.1 M Tris-HCL buffer pH 8.5 containing 0.025 (w/v) nicotinamide adenine dinucleotide (NAD), 10% (v/v) 1 M sodium hydrogen malate, 0.02% (w/v) nitroblue tetrazolium (NBT), 0.001% (w/v) phenazine methosulfate (PMS), in the dark at 40 °C (Shaw & Prasad, 1970). The appearance/absence of protein spots was indicative of the presence/absence of the MDH enzyme.

IV. 3.4. Establishment of a Procedure for Secretome Precipitation and Characterisation

IV. 3.4.1. Selection of the most efficient method for secretome precipitation

To precipitate the secretome from the collected CM pooled sample, three methods were tested: (i) Acetone precipitation (Damerval et al., 1986), (ii) Trichloroacetic Acid (TCA)/Acetone precipitation (Damerval et al., 1986), and (iii) Methanol/Chloroform precipitation (Wessel & Flügge, 1984). The most efficient method for total protein precipitation was selected based on the quantification procedure through spectrophotometry, and on the protein band pattern evaluated through SDS-PAGE gel electrophoresis (described below in Section IV.3.4.3).

Acetone precipitation method: Amounts of 5 mL lyophilised and 5 mL nonlyophilised sample were mixed with a methanol/H₂O solution (80:20) (previously stored at -20 °C) in the 1:0.5 (v/v) and 1:4 (v/v) ratio, respectively. After vigorously mixing for 15 s, the samples were centrifuged (15,000× g, 4 °C for 10 min). The resulting pellets, with and without lyophilisation, were mixed with 1 and 5 mL of ice-cold acetone, respectively, vigorously mixed for 15 s, and incubated overnight at -20 °C. The pellet (protein fraction) was collected by centrifugation under the conditions described above and washed with 1 and 5 mL of ice-cold acetone 90%, respectively, followed by centrifugation at 15,000× g, 4 °C, for 10 min. The purified pellets were air-dried for 1 h and then reconstituted in 1 mL of solubilization buffer (see Section IV.3.4.2).

TCA/Acetone precipitation method: Amounts of 5 mL lyophilised and 5 mL non lyophilized sample were mixed with a methanol/H₂O solution (80:20) and centrifuged as described above. Subsequently, the resultant pellets from the culture medium, with and without lyophilisation, were mixed with 1 and 5 mL of ice-cold acetone containing 20% TCA and 20 mM DTT, respectively, vigorously mixed for 15 s and incubated overnight at −20 °C. The pellet was collected by centrifugation under the conditions described above and washed with 1 and 5 mL of ice-cold acetone 90%, respectively, followed by centrifugation at 15,000× g, 4 °C, for 10 min. The purified pellets were air-dried for 1 h and then reconstituted in 1 mL of solubilization buffer (see Section IV.3.4.2).

Methanol/Chloroform precipitation: Amounts of 5 mL lyophilised and 5 mL nonlyophilised sample were mixed with ice-cold methanol in a 1:0.5 (v/v) and 1:4 (v/v) ratio, respectively, followed by vortexing for 15 s. Then, 5 mL of chloroform and 15 mL of Milli-Q sterile water were added to both samples, with vigorous mixing for 15 s between each reagent addition. Subsequently, the samples were centrifuged at 15000 ×g, room temperature, for 5 min. The pellet was collected and mixed with 20 mL of methanol. After gentle mixing, the samples were centrifuged at 15,000× g, 4 °C, for 10 min. The pellet was washed with 40 mL of ice-cold acetone 90%, followed by centrifugation at 15,000× g, 4 °C, for 10 min. The purified pellets were air-dried for 1 h and then reconstituted in 1 mL of solubilization buffer (see Section IV.3.4.2).

IV. 3.4.2. Protein solubilization and quantification

Following protein precipitation, the pellets were resuspended in 1 mL of solubilisation buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM Tris-HCl, pH 7.5) and vortexed until fully solubilised. Total protein quantification was carried out using the Pierce™ 660 nm Protein Assay Reagent Kit (Thermo Scientific). To generate the calibration curve, bovine serum albumin (BSA) concentrations of 125, 250, 500, 750, 1000, 1500, and 2000 µg/mL were used. In a 96-well microplate, 10 µL of each BSA solution or the same volume of each sample, in triplicate, was added to the wells. Subsequently, 150 µL of the Pierce™ 660 nm Protein Assay reagent was added to each well. Absorbance readings at 660 nm were taken using a Fluorescence multi-mode microplate reader GloMaxR (Promega, Charbonnières-les-Bains, France). For each plate, a calibration curve was plotted based on the average absorbance values of each BSA standard against protein quantity. By interpolation and accounting for the dilution factor, the total protein concentration for each triplicate sample was calculated.

IV. 3.4.3. Analysis of the secretome band patterns by SDS-PAGE

To evaluate the one-dimensional protein profile and determine the most effective protein precipitation method for the CM, the solubilised samples were subjected to SDSPAGE following the

method of Laemmli (1970). Each sample was tested in triplicate. The resolving gel (14% acrylamide) was prepared with 1.5M Tris-HCl pH 8.8, 0.1% of SDS, 14% acrylamide/bis-acrylamide, 5.45 mL of double-distilled water, 0.07% APS, and 0.05% TEMED. The stacking gel (4% acrylamide) was composed of 0.5M Tris-HCl pH 6.8, 0.1% SDS, 4% acrylamide/bis-acrylamide, 0.06% APS, and 0.11% TEMED. After polymerisation, the gels were placed in the Protean Mini system (Bio-Rad) with running buffer (0.025 M Tris, 0.192M glycine, 1% (w/v) SDS). The samples were mixed with sample buffer (0.125 M Tris-HCl, pH 6.8, 1% (w/v) SDS, 5% 2-mercaptoethanol, 20% glycerol, trace amounts of bromophenol blue), heated to 37 °C for 10 min, and then cooled on ice before being loaded into the gel wells. A total of 35 µL of each sample was loaded per lane, and 4 µL of molecular weight marker (NZYA Colour Protein Marker II, Nzytech, Lisbon, Portugal) was added to each run. Electrophoresis was performed at a constant voltage of 130 V. At the end of the electrophoretic run, the gels were fixed for one hour in a solution of 40% methanol/10% acetic acid and stained for two hours with Coomassie Brilliant Blue (CBB) G-250 solution (Candiano et al., 2004). Gels were scanned in a scanning Molecular Dynamics densitometer with internal calibration using the LabScan 6.0 software (GE Healthcare). Images were analysed using the GelAnalyzer software package (GelAnalyzer 2010a by Istvan Lazar, www.gelalyzer.com).

IV. 3.5. Analysis of the Secretome by LC-MS/MS

After sample lyophilisation and precipitation using the TCA/Acetone method, protein identification was carried out by LC-MS/MS analysis at the Proteomics Unit, National Centre for Biotechnology (Madrid, Spain). Protein cysteine residues were reduced and alkylated at 60 °C for 30 min with a buffer containing 5% SDS, 25 mM triethylammonium bicarbonate (TEAB), 5 mM tris(2-carboxyethyl) phosphine (TCEP), and 10 mM chloroacetamide (CAA). Protein digestion on S-Trap columns (Protifi, Huntington, NY, USA) was performed according to the previously established protocol (Ciordia et al., 2021). Briefly, the precipitated sample was digested at 37 °C overnight using 1 µg of trypsin. Alternatively, the lyophilised sample (without precipitation) was directly loaded in the S-Trap columns. Tryptic peptides were cleaned up using home-made Stage-Tips prepared from Octadecyl C18 solid-phase extraction disks (Empore™, 66883-U, Stellarton, NS, Canada), as previously described (Ciordia et al., 2022). Eluted peptides were dried in a speed vacuum and quantified by fluorimetry (QuBit) according to the manufacturer's instructions. Tryptic peptides were dried in a speed vacuum and quantified by fluorimetry (QuBit) according to the manufacturer's instructions.

For the analysis via nano-liquid chromatography coupled to electrospray ionisation tandem mass spectrometry (nanoLC-ESI-MS/MS), 1 µg of each sample was individually analysed using an Ultimate 3000 nano HPLC system (Thermo Fisher Scientific, Dreieich, Germany) coupled online to an Orbitrap Exploris™ 240 mass spectrometer (Thermo Fisher Scientific, Germany). Each sample [1 µg in 5 µL of

sample resuspended in mobile phase A: 0.1% formic acid (FA)] was loaded on a 50 cm×75 µmEasy-spray PepMap C18 analytical column at 45 °C. Tryptic peptides were separated at a flow rate of 250 nL/min using a 90 min gradient ranging from 6% to 95% mobile phase B (80% acetonitrile (ACN) in 0.1% FA). To avoid carry-over, two 40 min blank samples (mobile phase A) were systematically run between samples. The instrument sensitivity was monitored through frequent injection of Pierce™HeLa Protein Digest Standard (Thermo Scientific). Data acquisition was performed using a data-dependent top method, in full scan positive mode, scanning 375 to 1200 m/z. MS1 scans were acquired at an orbitrap resolution of 60,000 at m/z 200, with a normalised automatic gain control target of 300%, a radio frequency lens of 80%, and an automatic maximum injection time (IT). The top 20 most intense ions from each MS1 scan were isolated and fragmented with a higher-energy collisional dissociation (HCD) value of 30%. The resolution for HCD spectra was set to 15,000 at m/z 200, with a normalised AGC target of 50% and an automatic maximum IT. The precursor isolation window was 1.0 m/z, and dynamic exclusion (45 s) was applied. Precursor ions with single, unassigned, or six and higher charge states from fragmentation selection were excluded.

Raw data files were processed using the Proteome Discoverer 2.5.0.400 software (Thermo Scientific, Bremen, Germany), and database search was carried out using four search engines (Mascot (v2.8.0), MsAmanda (v2.4.0), MsFragger (v3.1.1), and Sequest HT against an *O. europaea* UniProtKB database (19 February 2021, 20,378 sequences) containing the most common laboratory contaminants (cRAP database with 69 sequences). Search parameters were set as follows: cysteine carbamidomethylation (+57.021464 Da); methionine oxidation (M) (+15.994915 Da), N-term acetylation (+42.010565 Da), and Gln→pyro-Glu (−17.026549 Da) as variable modifications. Precursor mass tolerances were set at 10 ppm and the fragment mass tolerance at 0.02 Da and trypsin/P was selected as a protease with a maximum of 2 missed cleavage sites. The false discovery rate (FDR) was calculated using the processing node Percolator (maximum delta Cn 0.05; decoy database search target) and an FDR ≤ 1% was considered for the validation of proteins and peptides. For protein identification, at least one unique peptide and a minimum of two peptide-spectrum matches (PSMs) were required. Protein abundance was also performed in Proteome Discoverer using the “Reporter Ions Quantifier” feature in the quantification workflow using the following parameters: unique+razor peptides; co-isolation threshold was set at 50%; signal to noise of reporter ions was 10; the normalisation was performed considering the total peptide amount. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE Archive—proteomics identification database (Perez-Riveral et al., 2022) (<https://www.ebi.ac.uk/pride/archive>, accessed on 29 November 2024) with the dataset identifier PXD058411 and 10.6019/PXD058411.

A functional enrichment analysis of the identified proteins was visualised as a network of biological terms using Metascape (<https://metascape.org>) (Zhou et al., 2019). The enrichment analysis

of proteins identified following the selected strategy was conducted using databases such as Gene Ontology (GO) (Gotz et al., 2008) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2016) to identify statistically enriched terms. The resulting functional network integrated biological terms associated with the proteins set, where the size of the nodes are proportional to the number of proteins linked to each term was carried out using STRING (<https://string-db.org/>). Cytoscape 3.10.3 (<https://cytoscape.org/>) was used to map SE-related proteins across different biochemical pathways.

IV. 3.6. Analysis of the Extracellular Metabolome by ^1H NMR Spectroscopy

The three lyophilised CM samples were further reconstituted in 1 mL of deuterated phosphate buffer (100 mM, pH 7.4) containing 0.1 mM trimethylsilylpropanoic acid (TSPd4), and then centrifuged at $10,000\times g$ for 10 min. The TSP-d4 signal at 0 ppm was used for both chemical shift referencing and shimming, ensuring consistent spectral resolution and line width across all samples. A 550 μL portion of each supernatant was then transferred to a 5 mm NMR tube for analysis. The same procedure was followed, using basal culture medium (ECO), not used for plant material subculture, but maintained under the same conditions (control sample).

NMR spectra were recorded at the University of Aveiro on a Bruker Avance III HD 500 spectrometer, operating at 500.13 MHz for ^1H observation. One-dimensional (1D) ^1H spectra were collected at 298 K, with a total of 32 K data points, a spectral window of 7002.80 Hz, a relaxation delay of 2 s, and 512 scans, using the ‘noesypr1d’ pulse sequence. Data processing was conducted using TopSpin 4.0.3 (Bruker BioSpin, Rheinstetten, Germany), applying cosine multiplication (ssb 2), zero-filling to 64 k points, manual phasing, baseline correction, and calibration to the TSP-d4 reference signal at 0 ppm. Two-dimensional (2D) ^1H - ^1H total correlation (TOCSY) spectra and J-resolved spectra were acquired for one sample to aid spectral assignment. For the semi-quantitative analysis of medium spectra, signal areas were measured using Amix-Viewer v. 3.9.15 (Bruker BioSpin, Billerica, MA, USA), and the variations were represented in a heatmap using Metaboanalyst 6.0. Since both ECO and cell-CM were incubated under identical conditions, and all sample processing steps were carefully standardised, this approach allowed us to effectively determine metabolite consumption and secretion patterns.

IV. 4. RESULTS AND DISCUSSION

IV. 4.1. Selection of the Most Efficient Method for Protein Precipitation

At the end of 21 days on induction OMc medium, radicles and cotyledons exhibited the development of calli (Figure A.1a). At this point, all explants showing callus formation were transferred to OMc expression medium devoid of growth regulators to induce embryo differentiation. After 60 days, various calli exhibited somatic embryo development (Figure A.1b), being further transferred to ECO culture medium to promote cyclic embryogenesis. Embryogenic lines that demonstrated a high number of somatic embryos were classified as highly competent embryogenic lines (Figure A.1c). In contrast, low-competent embryogenic lines (Figure A.1d) were characterised by a low number of developed embryos. The most competent embryogenic line, exhibiting a high number of somatic embryos, was selected and established in ECO liquid culture medium for subsequent secretome and extracellular metabolome analysis (conditioned medium—CM).

The CM, collected four weeks after the third subculture of the embryogenic line in ECO liquid medium, was first used to determine the most suitable protein precipitation method. Total protein quantification results across the three tested methods (Acetone, TCA/Acetone, and Methanol/Chloroform), with or without a prior sample lyophilisation step, revealed a dependency on the precipitation method, with lyophilisation being a crucial step (Table A.1). The selection of the appropriate extraction method plays a crucial role in protein recovery, even when using highly sensitive techniques such as mass spectrometry. The correct approach to extraction and solubilization helps maximise protein recovery while minimising interferences in subsequent steps, such as trypsin digestion. Although protein precipitation could introduce some protein loss, our results showed that the precipitation before LC-MS/MS analysis improved the protein recovery (see Section IV.3.2). Lyophilisation, commonly referred to as freeze-drying, is extensively employed across several sectors, including pharmaceuticals, cosmeceuticals, nutraceuticals, chemicals, and the food industry (ElNaker et al., 2021). This technique offers several advantages, such as concentrating the secretome (Rogulska et al., 2024), while not significantly affecting the stability of its proteins, lipids, and nucleic acids (Peng et al., 2021). Abila et al. (2022) also highlight the lyophilisation procedure to preserve not only nucleic acids and proteins but also other sensitive materials, including microorganisms and nanoparticulate systems.

Table IV.1. Total protein concentration ($\mu\text{g/mL}$) across three precipitation methods tested, lyophilised and non-lyophilised treatments.

Method	Lyophilised ($\mu\text{g/ml}$)	Non-Lyophilised ($\mu\text{g/ml}$)
Acetone	8.93	nt
TCA/Acetone	54.00	nt
Methanol/Chloroform	nt	nt

nt – not detected.

The TCA/Acetone method combined with sample lyophilisation was the procedure that yielded the higher protein concentrations when compared with the other two methods (Table IV.1). SDS-PAGE gel electrophoresis confirmed those results, allowing the clear identification of six distinct bands (numbered 1 to 6, with apparent molecular weights ranging from 63 to 35 kDa) (Figure IV.2).

Of the six analysed bands, the TCA/Acetone method was the only one present across all bands, showing higher protein concentration values compared to the other methods. However, significant differences were only observed in the lyophilised samples, specifically in band 5, corresponding to an apparent molecular mass of 30 kDa approximately, where the TCA/Acetone method outperformed the Acetone method. Notably, the band was not detected in samples processed with the Methanol/Chloroform method, which was expected considering the results achieved in the quantification procedure (results of statistical analysis presented in Table A.1).

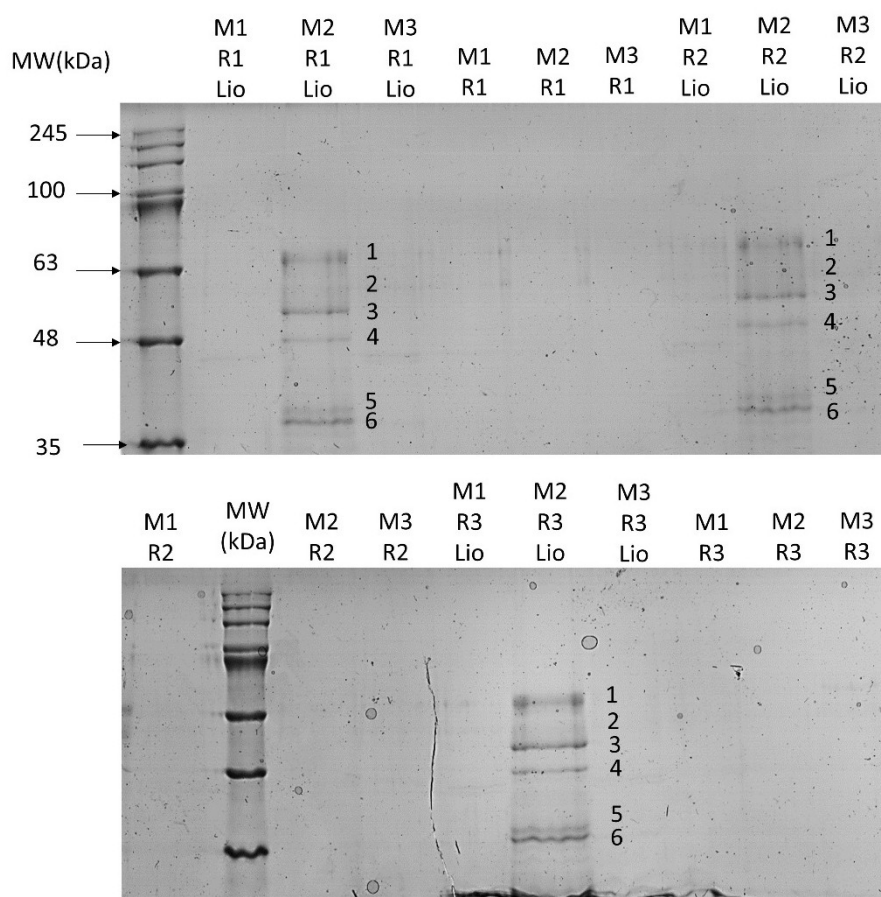


Figure IV.2. SDS-PAGE electrophoresis gel showing the band pattern achieved from analysis of samples precipitated with the three methods: Acetone precipitation method (M1), TCA/Acetone precipitation method (M2), and Methanol/Chloroform precipitation method (M3). The lyophilisation procedure is identified by Lio. R1, R2, and R3 correspond to each technical replicate performed for each precipitation

procedure. The six bands considered for the analysis of protein profiles are identified in the gel (numbered 1 to 6). MW—molecular weight marker (NZYA Colour Protein Marker II, Nztech) with the apparent size of each band indicated in kDa.

The TCA/Acetone precipitation method relies on protein denaturation under acidic or hydrophobic conditions, which improve protein concentration and contaminant removal (Wu et al., 2014). Compared to the two other tested methods, TCA/Acetone is well recognised for its ability to enhance protein yield, minimise degradation by protease activity, and reduce contamination from salts and polyphenols, factors that are critical for downstream applications (Shevchenko et al., 2006; Niu et al., 2018). By contrast, the Acetone precipitation method is limited by the requirement of a minimum 4:1 acetone-to-aqueous protein solution ratio, making it less practical for processing large sample volumes (Zhang et al., 2015). The Methanol/Chloroform precipitation method is specifically applied for recovering proteins from small, dilute samples, particularly those containing membrane proteins (Wessel & Flügge, 1984).

Based on the results, the TCA/Acetone precipitation method, combined with prior sample lyophilisation, was the only protein precipitation method that successfully enabled protein precipitation, thus allowing further analyses through high-throughput platforms. The same method has proven highly effective for precipitating proteins, including low-molecular-weight proteins and extracellular proteins, which often have low expression levels, in systems very similar to the liquid embryogenic cultures. There are examples of its application for concentrating extracellular enzymes released into the culture medium by *Pseudomonas* (Sharma et al., 2017) and to isolate extracellular vesicles secreted by *P. aeruginosa* during biofilm development (Saad et al., 2024).

To assess the purity of the protein extract obtained through the selected procedure (TCA/Acetone precipitation after prior sample lyophilisation), a protocol for the detection of cytosolic proteins was employed. The cytosolic biomarker MDH, an enzyme in the TCA cycle that catalyses the reversible reduction of oxaloacetate to malate in the presence of NADH (Genova & Lenaz, 2014), was employed. This approach has been widely used to evaluate the purity of plant cellular fluids, such as extracted apoplastic fluids, in various plant species (Guerra-Guimarães et al., 2015; Jaswanthi et al., 2019; Jiang et al., 2023).

In the present research, the presence of the expected MDH spots in samples obtained from plant material (embryogenic calli collected from the CM) and their absence in the CM extract samples (Figure A.2) confirmed the absence of cytosolic proteins in the CM sample. These results validate the effectiveness of the established workflow for isolating the secretome of liquid embryogenic cultures, enabling subsequent characterisation studies.

IV. 4.2. LC-MS/MS-Based Analysis of Secretome from Embryogenic Calli

The strategy consisting of direct solubilization of 5 mL lyophilised sample without precipitation, followed by digestion with an S-trap column resulted in the identification and quantification of only 261 proteins/protein groups. This suggests that although this simpler strategy could help reduce protein losses, the presence of interfering substances may have affected trypsin digestion, leading to lower peptide recovery and a reduction in the number of identified proteins. In comparison, a total of 1111 proteins and 538 protein groups (FDR < 1%, PSM $\geq$ 2, at least a unique peptide), of which 1096 proteins (526 protein groups) correspond to *O. europaea* proteins (Table A.2—Metascape results), were identified as part of the secretome of embryogenic calli using a strategy combining TCA/Acetone precipitation with prior sample lyophilization, protein digestion by S-trap and a proteomic analysis by LC-MS/MS. Of these proteins, it was possible to measure the abundance of 994 *O. europaea* proteins (493 protein groups) in the sample, confirming that this strategy is the most appropriate for the analysis of the secretome of *O. europaea* cv. 'Galega Vulgar' embryogenic lines. Nevertheless, it should be taken into account that only a single replicate was analysed using this final strategy. As a result, the outcomes might vary slightly with a higher number of replicates, particularly for the proteins identified with a single peptide.

The results obtained align with findings from similar proteomic analyses, although the number of identified proteins varies depending on the methodology used and the material analysed. For instance, in tissue cultures, an LC-MS/MS-based proteomic analysis of *Elaeis guineensis* (Ribeiro et al., 2019) identified approximately 4600 proteins and over 10,000 peptide sequences, significantly higher than the number identified in our study. In contrast, a recent study on the secretome of *P. nigra* (Pernis et al., 2023) identified and quantified 187 proteins. Many of these proteins were products of complex gene families with multiple functions. While our study identified a substantially higher number of proteins, this broader identification likely reflects the complex nature of the secretome and the interactions occurring during somatic embryogenesis in *O. europaea*. Nevertheless, further analyses incorporating biological replicates will be necessary to ensure the reliability of the results and to accurately interpret protein expression patterns and their role in SE. An enrichment analysis allowed the identification of diverse canonical pathways, with the identification of proteins with different biological functions, processes, and locations. Figure IV.3 represents different categories of protein ontology analysis. Each chart illustrates the percentage distribution of proteins associated with various biological categories, based on three primary aspects: biological component, biological function, and biological process. The results indicate a strong presence of proteins related to metabolism, catalytic activity, and intracellular processes, suggesting that the studied system is metabolically active and engaged in various cellular

processes and responses to stimuli, including stress response, as highlighted in previous reports (Domzalska et al., 2017; Peng et al., 2022; Pernis et al., 2023).

In analysing the biological component, the presence of proteins originating from intracellular organelles and the cytoplasm in the extracellular space can be attributed to exocytosis processes. Exocytosis is an energy-consuming process that expels secretory vesicles containing nanoparticles out of the cell membranes and into the extracellular space (Singh, 2016). These membrane-bound vesicles contain soluble proteins, membrane proteins, and lipids, and subsequently fuse with the cell membrane for secretion into the extracellular environment (Kalluri & LeBleu, 2020). Lysosomes, which contain a range of hydrolytic enzymes for degrading cellular materials, also play a crucial role in exocytosis (Tancini et al., 2020). Specifically, these organelles can extracellularly release their contents via lysosomal exocytosis, involving the fusion of lysosomes with the plasma membrane to discharge their enzymes and other substrates into the extracellular space (Ibata & Yuzaki, 2021). Furthermore, exocytosis can be induced by changes in environmental conditions or by cellular stress signals, reflecting the cell's ability to adapt and modulate its interactions with the microenvironment (Su et al., 2024). Thus, the detection of intracellular organelles and the cytosolic proteins in the CM may indicate an active cellular state, where communication and interaction with the external environment are essential to its functionality. The role of cytosolic proteins in regulating cellular processes related to organelle function and environmental interactions has been reported in various studies. For example, heat shock proteins (HSP) type 2 was found to be upregulated during SE induction in *Cyathea delgadii* (Domzalska et al., 2017), while cytosolic HSP18.2 and class II HSP17.6 were upregulated during somatic embryo development in *P. pinaster* (Morel et al., 2014).

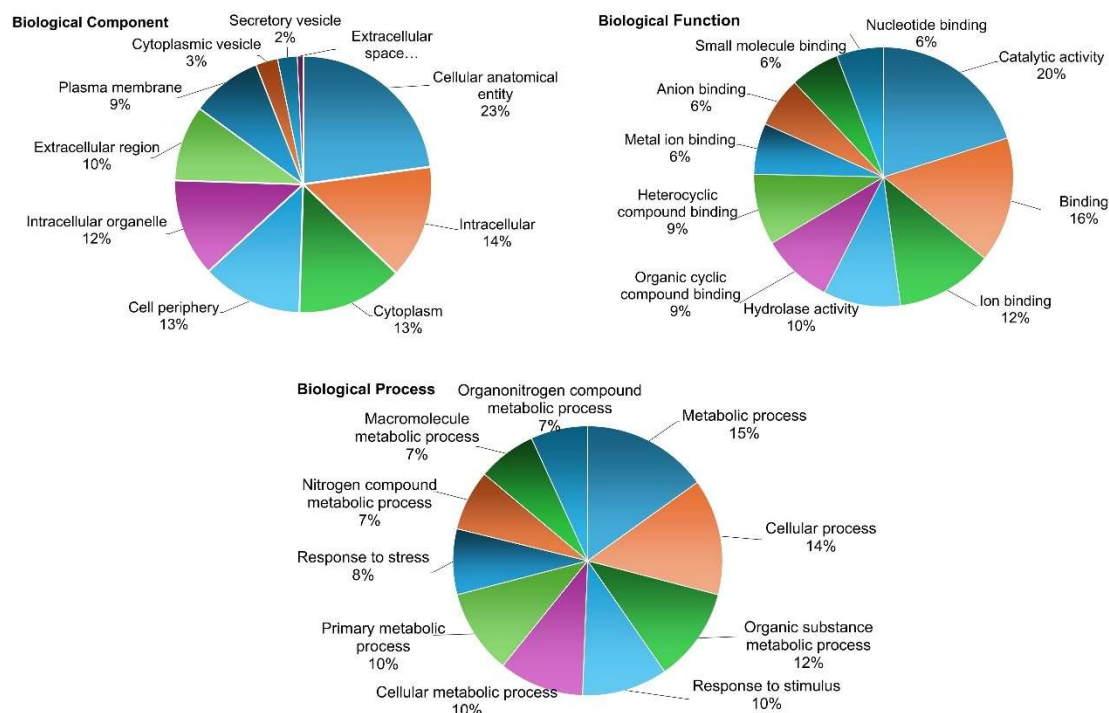


Figure IV.3. Distribution of proteins identified in the secretome of olive (*Olea europaea* subsp. *europaea* L.) somatic embryogenic liquid cultures. The circular charts illustrate the relative proportions of putative biological components, key functions, and associated processes of the proteins, highlighting their roles within the precipitated secretome.

Regarding biological putative functions, a total of 78 were identified. The most prevalent processes identified are related to metabolic functions, specifically cellular metabolism and the metabolism of organic substances. These metabolic processes play a crucial role during SE, as cells transition from a somatic to an embryogenic state. During this transition, cells undergo dedifferentiation, activate their cell division cycle, and reorganise their metabolic and physiological states (Wang et al., 2020). Among these processes, metal ions are vital, serving to bridge distant residues or domains within proteins, mediating interactions between proteins and ligands, and functioning within the active site as nucleophilic catalysts and electron transfer agents (Chaturvedi & Shrivastava, 2005). In proteins, metal ions are crucial for maintaining structural integrity, regulatory functions, and enzymatic activity, all of which are essential for sustaining homeostasis and supporting complex cellular networks (Selvaraj et al., 2024). The binding of specific metal ions can stabilise proteins or protein domains (Permyakov, 2021). Among the identified proteins, ascorbate peroxidase 1 (APX1) has been reported to play a significant role in SE, being involved in tissue protection. A study examining embryogenic and non-embryogenic calli from *Medicago arborea* (Galleggo et al., 2014) investigated POD activity. The results showed a decrease in soluble POD activity in the embryogenic calli at the time when somatic embryos

began to emerge. According to several authors, SE is a plant cell response upon abiotic stresses (Arnholdt-Schmitt et al., 2016), and the initiation and differentiation of SE is regulated by the redox balance within embryogenic tissues, and an increase in oxidative stress levels is necessary to promote the formation of embryogenic cells (Belmonte et al., 2005; Pasternak et al., 2005).

The enrichment analysis identified statistically significant terms, including GO terms and KEGG pathways, based on cumulative hypergeometric *p*-values and enrichment factors (Figure A.3). These terms were hierarchically organised into clusters using Kappa statistical similarity, with a threshold of 0.3, grouping terms that share common proteins. The most significant cluster includes terms related to the recognition of pollen, other glycan degradation, phenylpropanoid biosynthesis, and external encapsulating structure organisation, suggesting that the identified proteins play a central role in SE. The results suggest that the most enriched processes involve pollen recognition and the metabolism of carbohydrates and phenolic compounds (Figure A.4). Pollen recognition is likely associated with mechanisms of self-incompatibility or selective fertilisation. Among the identified proteins, RK3 stands out as a member of the plant receptor-like kinase (RLK) family. These proteins are composed of an extracellular domain, a single transmembrane segment, and an intracellular kinase domain. The extracellular region of RLKs is highly diverse, featuring specialised domains that enable ligand recognition and signal perception. This structural variability allows RLKs to function as key cell surface receptors, mediating crucial biological processes such as development and stress responses in SE (Li et al., 2019).

The functional enrichment analysis of the identified proteins was visualised as a network of biological terms using Metascape (Figure IV.4). The most significant terms include glycan degradation and phenylpropanoid biosynthesis. This aligns with findings from a study on *P. koraiensis* (Peng et al., 2022), where different SE cell lines were analysed, revealing significant enrichment in phenylpropanoid biosynthesis, starch and sucrose metabolism, and glycan degradation pathways in responsive SE cells compared to blocked or non-SE cell lines. Additionally, the organisation of external encapsulating structures relates to the organisation of the cell wall or extracellular structures (Zhang et al., 2023). Starch and sucrose metabolism are indicated as key metabolic processes involved in SE. In fact, carbohydrates can act as signalling molecules and regulators of gene expression, as part of signalling networks connecting the environment with plant metabolism, development, and growth (Dobrenel et al., 2013). Among the identified proteins, α -amylase (AMY, including AMY1 and AMY2), a hydrolase that catalyses the breakdown of internal α -(1–4)-glycosidic bonds in starch, glycogen, and related oligosaccharides, play a key role in energy and carbon metabolism by processing environmental polysaccharides (Ju et al., 2019). AMYs are typically secretory proteins that are biosynthesised in the secretory tissues, such as the scutella epithelium and the aleurone layer, before being secreted into the starchy endosperm (Damaris et al., 2019). In a recent study using SE suspension cell cultures of *P. nigra*,

Pernis et al. (2023) identified the AMY protein secreted into the culture medium. The authors found that this protein was more abundant in the medium of lines with high embryogenic capacity. The detection of this enzyme in the secretome in this study may indicate an important role in SE and its potential as a molecular marker.

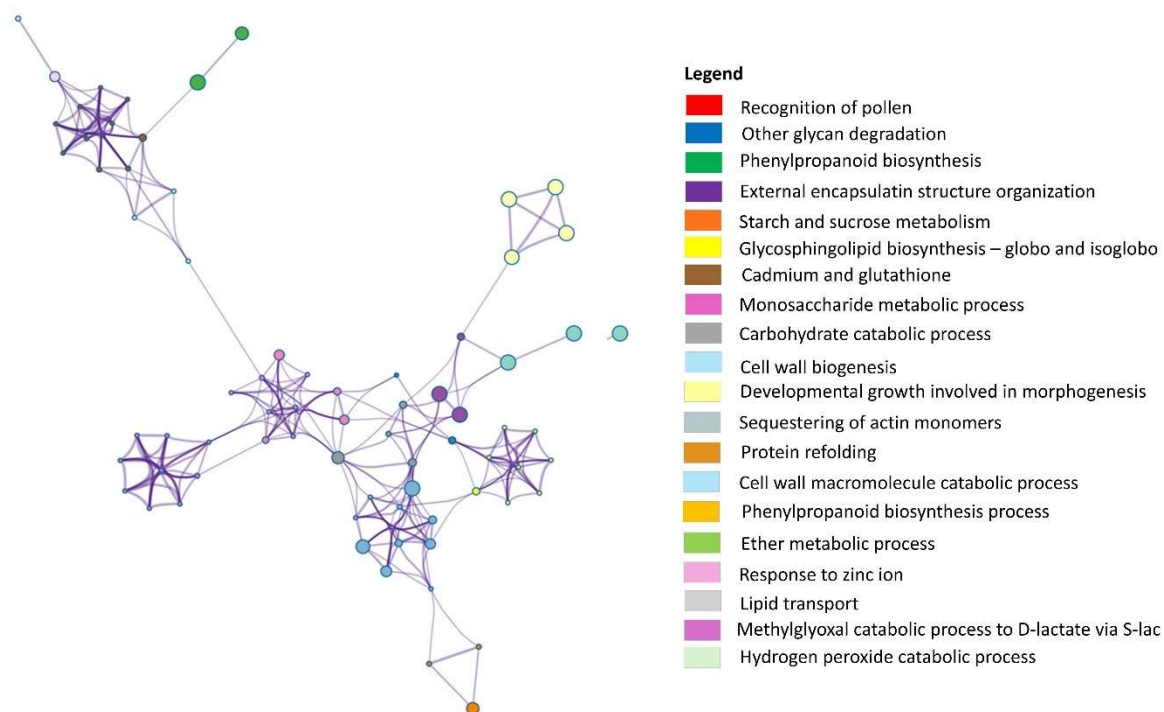


Figure IV.4. Network of identified biological terms constructed using Metascape (<https://metascape.org>), where each circle represents a term, and the size of the circle indicates the number of associated proteins. The colours represent clusters of related terms. The connections (edges) between nodes reflect functional similarity, with the thickness of the connections indicating the degree of similarity (score > 0.3). Larger nodes represent terms that are widely represented by the identified proteins, suggesting significant relevance of these processes in the proteomic analysis. Thicker connections between terms indicate high similarity, highlighting interrelated biological processes.

The generated network reveals various interrelated clusters of biological terms, reflecting the biological processes associated with the set of proteins identified in the secretome of the embryogenic line. Terms with a higher number of associated proteins indicate groups that share proteins or have related biological functions, suggesting that these processes are highly relevant within the context of SE. The interactions among these terms highlight potentially interconnected biological pathways. Smaller clusters can also be observed in Figure IV.4, such as the group of terms associated with the hydrogen peroxide (H_2O_2) catabolic process, represented by proteins like mitochondrial Peroxiredoxin-IIF (PRXIIF) and thioredoxin peroxidase (TPX1). These proteins are linked to pathways related to antioxidant activity, catalytic activity, and POD activity. H_2O_2 is a significant REDOX molecule that,

at high concentrations, induces oxidative damage to biomolecules. In a study on SE in olive, Oulbi and co-workers (2021) observed higher POD activity in embryogenic cultures compared to non-embryogenic cultures for the zygotic explants of cv. ‘Picholine Marocaine’ and cv. ‘Dahbia’. Their results suggest that POD enzymes may be involved in secondary or adaptive mechanisms during SE and could potentially serve as biochemical markers. Ultimately, the results highlight the important role of secreted proteins in SE, particularly the involvement of plant α -amylases in carbohydrate metabolism and H_2O_2 in catabolic processes and signalling pathways. A better understanding of these secreted proteins improves our knowledge of the metabolic adaptations that support SE and the viability of plant cells in culture.

IV. 4.3. Detection of Changes in the Exametabolome Composition Through 1H NMR Spectroscopy

NMR spectroscopy enabled the identification of various components in both the ECO basal medium (negative control) and the CM collected for growing embryogenic calli. In total, 20 metabolites were unambiguously identified, including amino acids, sugars, organic acids, and alcohols. Figure IV.5A shows representative spectra retrieved from both samples. Several amino acids were identified in the ECO medium, likely originating from the casein hydrolysate used in its preparation. The hydrolysed form of casein plays a crucial role in SE by providing essential nutrients that support cellular growth and development. Casein is a phosphoprotein that comprises four distinct subunits (α s1-, α s2-, β -, and κ -casein) varying in their primary structure and the type and extent of post-translational modifications (Swaisgood, 2007). Casein hydrolysates are a rich source of calcium, phosphate, various microelements, vitamins, and, most importantly, a mixture of up to 18 amino acids (Wang et al., 2020).

The results indicate that embryogenic cells consumed significant amounts of several amino acids, including methionine ($-75.5 \pm 13.4\%$ vs. ECO medium), tryptophan ($-74.4 \pm 12.2\%$), lysine ($-76.2 \pm 12.1\%$), phenylalanine ($-88.7 \pm 13.1\%$), tyrosine ($-92.8 \pm 12.7\%$), branched chain amino acids—valine, leucine, and isoleucine ($-88.4 \pm 14.0\%$)—and alanine ($-84.9 \pm 11.6\%$) (Figure IV.5B). The aromatic amino acids tyrosine and phenylalanine are known to serve as crucial substrates for producing secondary metabolites that plants use to counteract stress (Simpson et al., 2021). Additionally, embryogenic cells consumed $58.6 \pm$ the pyroglutamate (formed through the non-enzymatic cyclisation of glutamine) present in the ECO medium. Amino acids in the glutamine/glutamate family play key roles in initiating and accelerating the incorporation of ammonia and nitrite into organic nitrogen metabolism (Greenwell & Ruter, 2018). These amino acids function as nitrogen storage compounds (Okumoto et al., 2016), act as intermediates for incorporating ammonia into amino acids, and serve as building blocks for proteins or nucleotides (Cruzat et al., 2018). Thus, these amino acids are an efficient nitrogen source for direct protein synthesis (Efzueni et al., 2014) and facilitate nitrogen assimilation

during long-distance metabolic transport (Lee et al., 2023). A recent study identified amino acids as key regulators of morphogenesis during SE in *Litchi chinensis* Sonn. cv. 'Feizixiao' (Wang et al., 2023), demonstrating their positive influence on callus proliferation and embryo induction. When supplied in specific mixtures, amino acids significantly enhanced somatic embryo regeneration. These findings highlight their role not only as an efficient nitrogen source for protein synthesis and metabolic transport but also as crucial factors in optimising in vitro regeneration protocols, supporting our observations of their contribution to nitrogen assimilation during SE.

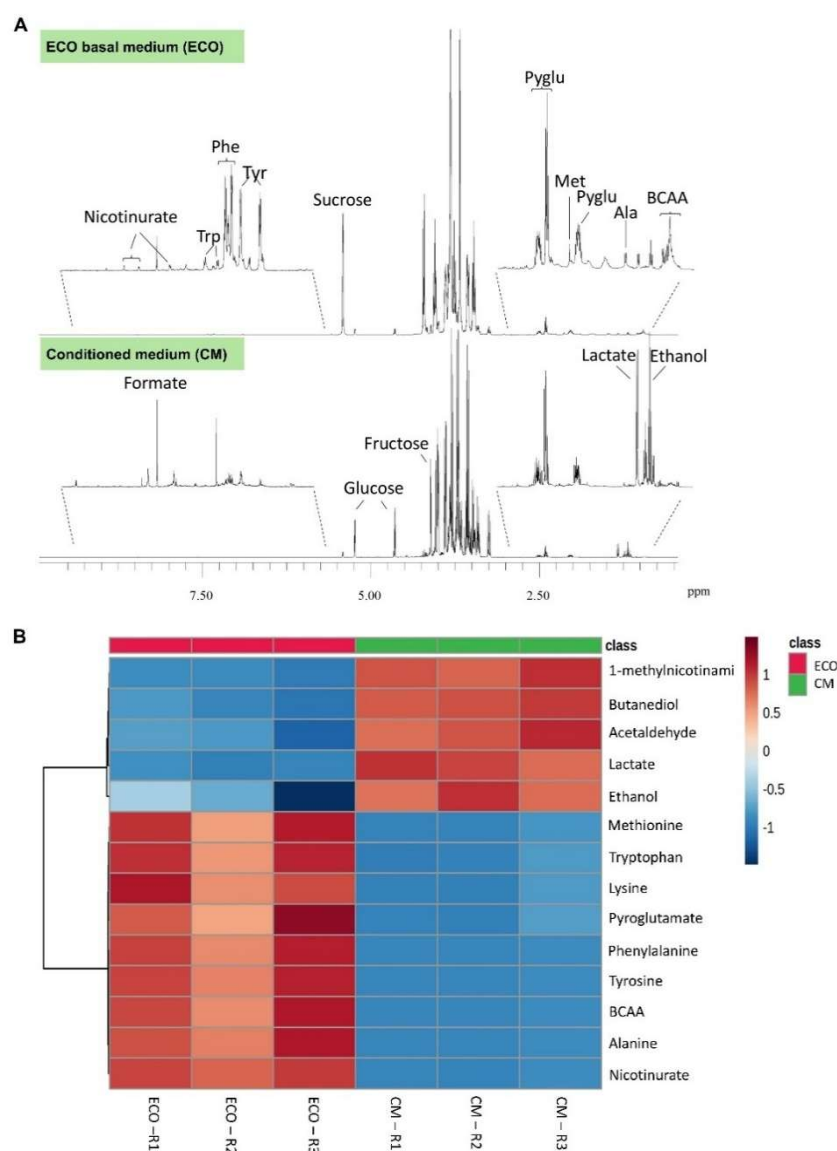


Figure IV.5. NMR analysis of conditioned medium (CM) samples (ECO medium collected four weeks after inoculation of embryogenic plant material), and ECO samples (ECO medium not inoculated with embryogenic plant material but maintained under the same photoperiod and temperature conditions as those of the CM samples). (A) Representative ^1H NMR spectra with key signals assigned to major metabolites. (B) Heatmap displaying the relative abundance of metabolites

with statistically significant differences between the two sample types. R1, R2, and R3 correspond to the three replicates considered for each type of sample.

Regarding the metabolites secreted into the medium by embryogenic cells, they included 1-methylnicotinamide (MNA), 2,3-butanediol, acetaldehyde, lactate, and ethanol (Figure IV.5B). The secretion of MNA suggests that the cells may be methylating nicotinamide as part of a detoxification or regulatory process to maintain NAD⁺ homeostasis. The consumption of nicotinurate (conjugate of nicotinic acid and glycine, both used in the preparation of the ECO medium) is consistent with the active regulation of NAD⁺ metabolism.

The secretion of 2,3-butanediol, acetaldehyde, lactate, and ethanol suggests a metabolic shift that could involve anaerobic or microaerobic processes. These metabolites are typically associated with fermentation pathways, which may serve to regenerate NAD⁺ and sustain glycolytic flux under specific metabolic conditions. For instance, the production of 2,3-butanediol involves the conversion of pyruvate to acetoin, followed by its reduction to 2,3-butanediol. This pathway helps regenerate NAD⁺ from NADH, allowing glycolysis to continue. Similarly, acetaldehyde is a key intermediate in ethanol fermentation, where pyruvate is decarboxylated to form acetaldehyde, which is then reduced to ethanol by alcohol dehydrogenase. Lactate production, typically arising from lactic acid fermentation, results from the reduction of pyruvate in the absence of sufficient oxygen, again facilitating NAD⁺ regeneration. While these metabolic shifts are often linked to low oxygen availability, we acknowledge that our study does not directly measure dissolved oxygen levels in the culture medium. Thus, although our results are consistent with metabolic adaptations observed under hypoxic conditions, we cannot conclusively attribute them to oxygen limitation. Additionally, alternative biochemical pathways could contribute to the detected metabolites, and the presence of fermentation intermediates does not necessarily confirm a predominant reliance on anaerobic metabolism.

Moreover, some of these metabolites could result from extracellular enzymatic activity rather than direct cellular secretion. If cells release enzymes capable of metabolising medium components, these transformations could influence the observed metabolite profile. Passive diffusion of small molecules between cells and the extracellular environment may also contribute. However, the coordinated presence of these metabolites, particularly intermediates of known fermentation pathways, suggests that they primarily originate from active cellular metabolism rather than random extracellular reactions. Future studies incorporating direct oxygen measurements, as well as gene expression or enzymatic analyses, will be necessary to further elucidate the metabolic state of these cells.

In the context of SE, sucrose in the basal medium is essential for the maintenance and maturation of somatic embryos, serving as a primary source of metabolic energy and carbon during embryo

development (Juarez-Escobar et al., 2021; Lee et al., 2023). These compounds act as osmotic agents, helping to preserve the integrity of the plasma membrane (Bartos et al., 2018), and may also function as reserve compounds or contribute to desiccation stress tolerance during SE process (Pescador et al., 2008). An analysis of ^1H NMR spectra showed that sucrose was hydrolysed into glucose and fructose, which were clearly detected in the CM spectra (Figure A.4). In fact, plant α -amylases are enzymes that contribute to the hydrolysis of starches, while sucrose itself is hydrolysed into glucose and fructose by specific enzymes, such as invertase (Xie et al., 2016). Consequently, the production of glucose and fructose from sucrose may be influenced by the activity of amylases, which can enhance the efficiency of this hydrolysis, particularly under stress conditions or during cellular development. Interestingly, the total amount of glucose and fructose detected was similar to the initial amount of sucrose, suggesting that there was minimal consumption of these sugars by the cells (Figure A.4). This observation likely raises a question about the key biological roles played by and the production of metabolites such as 2,3-butanediol, acetaldehyde, lactate, and ethanol, which are typically derived from the breakdown of glucose through glycolysis and subsequent fermentation. One possible explanation for this apparent discrepancy is that the cells may be utilising an alternative metabolic pathway or an external source of carbon to produce these metabolites. For instance, the cells might be relying on internal reserves, such as starch or other polysaccharides that are not directly reflected by the sugars measured in the medium. Another possibility is that the observed production of fermentation by-products could result from alternative pathways involving other carbon sources or the regeneration of NAD^+ through anaerobic or microaerobic conditions, independently of the sugar consumption.

Overall, our results highlight the complex metabolic processes occurring in embryogenic plant cells, including the active regulation of NAD^+ homeostasis, the utilisation of amino acids and alternative carbon sources, and the engagement of fermentation pathways under oxygen-limited conditions. These findings contribute to a better understanding of the metabolic adaptations that support SE and the survival of plant cells in culture.

IV. 5. CONCLUSIONS

The present study outlines an efficient protocol for separating proteins and metabolites secreted by olive (*O. europaea* subsp. *europaea* L.) embryogenic cultures during cyclic embryogenesis in ECO liquid culture medium. The application of LC-MS/MS and NMR techniques proved highly effective for identifying and relatively quantifying these secreted biomolecules, underscoring their potential for a detailed analysis of cellular secretions. The results reveal that numerous proteins and metabolites are secreted into the extracellular environment, accumulating as they are released by explants during the SE process. The findings of this study lay the groundwork for a deeper understanding of the molecular mechanisms governing SE. By establishing an efficient workflow, this research enables future

comparisons between high- and low-efficiency embryogenic lines to identify key molecules that regulate and enhance SE in olive.

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CHAPTER V

Integrated LC-MS/MS and ¹H NMR approach revealed differences in secreted biomolecules associated with somatic embryogenic efficiency in *Olea europaea* L.

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V. 1. ABSTRACT

Somatic embryogenesis (SE) is an *in vitro* propagation system in which somatic cells are reprogrammed to form somatic embryos. Despite its potential, SE remains inefficient in recalcitrant species such as *Olea europaea*. Improving SE requires a better understanding of the mechanisms underlying embryogenic competence, which is strongly influenced by exogenous factors. Proteins and metabolites secreted into the culture medium (conditioned medium, CM) may modulate embryogenic competence. Here, we performed integrated proteomic (LC-MS/MS) and metabolomic (^1H NMR) analyses of CM from embryogenic lines of *O. europaea* with contrasting embryogenic efficiencies: high-efficiency (HE) and low-efficiency (LE). Secretome analysis revealed significant differences in protein abundance, particularly in pathways related to redox regulation, amino acid metabolism, and cell wall dynamics. Metabolomic profiling identified amino acids and metabolites associated with central metabolism and stress responses. Differences in carbon metabolism were also detected, with HE lines showing markers of efficient carbon utilisation, including elevated acetate levels, whereas LE lines accumulated lactate, suggesting a shift toward fermentative metabolism. Overall, embryogenic competence appears closely associated with coordinated metabolic regulation, highlighting CM as a promising source of biomarkers for improving SE efficiency.

V. 2. INTRODUCTION

Somatic embryogenesis (SE) is an *in vitro* culture system that enables the regeneration of entire plants without the requirement for sexual fertilisation (Wang et al., 2023). Although SE offers several advantages, making it the preferred method for plant regeneration in genetic transformation, large-scale propagation of elite genotypes, and germplasm conservation (Guan et al., 2016; Sánchez-Romero, 2021), most protocols developed for woody species rely on the use of zygotic embryos as initial explants (Pais, 2019; Ballesteros et al., 2024). Nevertheless, the use of zygotic embryos constitutes a drawback, as regenerated plants may not necessarily retain the same genetic characteristics as the donor plants (Egertsdotter et al., 2019; Pires et al., 2020). Protocols based on adult tissues are generally considered the most appropriate, but in woody plant species, it is usually difficult and strongly genotype-dependent, with several agronomically relevant species displaying recalcitrance, thus limiting their applicability.

Proteomic and metabolic studies have extensively examined SE; however, most work has been conducted at the tissue level, typically by comparing explants or cell lines with differing abilities to develop somatic embryos (Heringer et al., 2015; Caeiro et al., 2022). Over the past decade, several groups have shifted attention to the role of extracellular biomolecules in the initiation and maintenance of embryogenic cell cultures in important major crop species (Ngcala et al., 2020; Pernis et al., 2023; Pires et al., 2025a), focusing on how secreted factors shape SE systems. Proteins and metabolites

released at specific developmental stages and under defined physiological conditions, via distinct secretory mechanisms, can modulate the cellular microenvironment and influence SE outcomes (Agrawal et al., 2010; Nic-Can et al., 2015). Through effects on cell wall matrix dynamics, these molecules support intercellular signalling and structural organisation, thereby promoting the establishment and maintenance of SE cultures. Comparative studies between high-efficiency (HE) and low-efficiency (LE) embryogenic lines further suggest that secreted proteins, known as the secretome, may serve as both mediators and markers of embryogenic competence (Pernis et al., 2023; Pires et al., 2025a). Consistent with this, the secretome released into the extracellular space appears to contribute to the acquisition and maintenance of SE potential (Ben-Amar, 2021). In planta, the secretome comprises proteins exported by cells or tissues to the extracellular space, known as the apoplast (Alexandersson et al., 2013). In *in vitro* liquid culture systems, the culture medium may provide an extracellular-like context that resembles the apoplastic environment. Nevertheless, few studies have been published, and those that have are limited to a small number of species. Proteomic analyses of CM have revealed the presence of several proteins recurrently associated with embryogenic cultures across species. Among these, germin-like proteins (GLPs), first identified in *Pinus caribaea* var. *hondurensis* (Domon et al., 1995), are proposed markers of SE, with enriched expression during early embryogenic stages in *Saccharum* spp. (Heringer et al., 2015). More recently, GLPs were detected in the extracellular protein fraction of *Olea europaea* embryogenic cultures (Pires et al., 2025b). Also, enzymes involved in carbohydrate metabolism, particularly α -amylases, have also been identified in CM, including *Pinus nigra* (Pernis et al., 2023) and *O. europaea* (Pires et al., 2025b). In addition, peroxidases (PODS) have been reported in the secretome of high embryogenic cell lines in *P. nigra* (Pernis et al., 2023), *Sorghum bicolor* (Ngwenya et al., 2024), and *O. europaea* (Pires et al., 2025b), supporting their association with embryogenic efficiency. Moreover, stress-related proteins, including heat shock proteins (HSP) and the chaperone-binding immunoglobulin protein, are recurrent components of plant extracellular proteomes and have been identified in the CM of embryogenic suspension cultures of *S. bicolor* (Ngcala et al., 2020; Ngwenya et al., 2024) and *O. europaea* (Pires et al., 2025b). Collectively, these studies highlight that, despite progress in SE secretome characterisation, knowledge remains fragmented across species and protein classes, underscoring a persistent mechanistic gap.

In addition to proteins, a high diversity of metabolites is secreted by cells to their extracellular environment, composing the exometabolome, which serves as a crucial interface between plant cells and their environment. To date, most metabolomic studies in SE have focused on specific metabolite classes or individual metabolic pathways, rather than adopting a holistic view of the process. Advanced analytical techniques have been employed to identify discriminatory metabolites and infer metabolic networks associated with SE (Brito De Moura et al., 2025). For example, liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based analyses have revealed stage-specific accumulation patterns of nucleotides, lipids, flavonoids, and hydroxycinnamoyl derivatives in SE of *Gossypium*

hirsutum (Guo et al., 2019), while targeted metabolite profiling in embryogenic cultures of *Camellia sinensis* identified developmental changes in phytohormones and phenolic compounds, including indole-3-acetic acid, gibberellin A4, and chlorogenic acid (Awon et al., 2024). Additionally, nuclear magnetic resonance (NMR) spectroscopy has also been successfully applied for metabolic fingerprinting of embryogenic systems, enabling the detection of key metabolites such as sugars and amino acids associated with embryo formation and maturation (Caeiro et al., 2022).

This study aimed to generate comprehensive secretome and metabolome profiles to identify potential biomarkers associated with SE in *O. europaea* cv. 'Arbequina'. To this end, somatic embryogenic lines exhibiting different abilities to differentiate somatic embryos during cyclic embryogenesis (HE and LE) were selected for proteome analysis through LC-MS/MS and metabolome analysis through ^1H NMR. The results provide new insights into the complex regulation of SE and highlight potential molecular markers for SE in olive.

V. 3. MATERIALS AND METHODS

V. 3.1. Establishment of Embryogenic Lines Exhibiting Different Capacities to Differentiate Embryogenic Embryos

Embryogenic lines obtained from zygotic embryos of *O. europaea* cv. 'Arbequina', with varying competence to differentiate somatic embryos, were successfully established following the protocol previously described by Pires et al. (2020; 2025a). Explants were dissected and inoculated onto gelled induction olive medium (OMc) (Cañas & Benbadis, 1988), supplemented with 2.5 μM 6-dimethylallyl amino-purine (2iP), 25 μM indole-3-butyric acid (IBA) (Orinos & Mitrakos, 1991), and gelled with 6 g L^{-1} Agar-Powder (VWR, Portugal). The cultures were maintained at $25 \pm 1^\circ\text{C}$ in the dark for 21 days. Following the induction phase, explants were transferred to the expression phase, using a hormone-free OMC medium, and cultures were kept under the same growth conditions as previously described.

After the observation of somatic embryos differentiation, after two subcultures of 30 days each, embryogenic calli were subcultured onto liquid olive cyclic embryogenesis medium (ECO), supplemented with 50 mg L^{-1} Myo-inositol, 20 g L^{-1} sucrose, 550 mg L^{-1} L-glutamine, 0.5 μM 2iP, 0.44 μM 6-benzyl adenine (BA), 0.23 μM IBA, 1 g L^{-1} casein hydrolysate (Pérez-Barranco et al., 2007), and gelled with 2.5 g L^{-1} Sigma-Phytigel. To select embryogenic cell lines with distinct capacities for somatic embryo differentiation, embryogenic calli were subcultured on ECO gelled medium for two consecutive 30-day subcultures and subsequently transferred to ECO liquid medium for a period of three months. After three subcultures, three HE and three LE (working as biological replicates) embryogenic lines were selected according to the method described by Pires et al. (2025a).

V. 3.2. Establishment of the Experiment for Secretome and Exometabolome Analysis

To establish the experiment for secretome and metabolome analysis, approximately 200 mg of embryogenic calli from each selected embryogenic line was transferred to 100 mL Erlenmeyer flasks containing 50 mL of liquid ECO medium. Six flasks (corresponding to technical replicates) were prepared from each somatic embryogenic line. Cultures were maintained for three consecutive subcultures of 30 days each at 25 ± 1 °C, in the dark, under continuous agitation at 105 rpm.

In parallel, six 100 mL Erlenmeyer flasks containing 50 mL of ECO liquid medium and no embryogenic plant material were prepared and submitted to same conditions, working as control replicates.

V. 3.3. Secretome and Exometabolome Isolation from Conditioned Medium

After subcultures the Erlenmeyer flasks were left undisturbed for 5 min to allow the plant material to settle and further processed according with procedure previously established by Pires et al. (2025b). Briefly, the conditioned medium (CM) was collected from each flask into 50 mL centrifuge tubes and centrifuged at $10,000 \times g$ for 10 min at 25 °C to remove cellular debris. The resulting supernatant was sequentially filtered through 0.45 μm and 0.22 μm membrane filters using a vacuum filtration system (Millipore, Burlington, MA, USA). Subsequently, the CM from each embryogenic line was separated using a MacroSep™ Spin Filter 1K (PALL), previously equilibrated by washing with 10 mL of RNase- and DNase-free sterile water and centrifuged at $10,000 \times g$ for 10 min. The filtrate collected from the upper chamber of the 1 kDa filter, corresponding to the total secretome, was supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity. For LC-MS/MS analysis, the secretome component collected from the six technical replicates of each embryogenic line was pooled, yielding a final volume of approximately 45 mL per biological replicate. Following recovery, pooled CM samples were lyophilised, and total proteins were extracted following the procedure described below

The filtrate collected below the 1 kDa filter, corresponding to the extracellular metabolome, was processed separately. From each technical replicate of each embryogenic line (biological replicate), 5 mL of CM was collected and further lyophilised and directly sent for analysis through ^1H NMR spectroscopy.

V. 3.4. Protein Analysis

V. 3.4.1. Analysis of the secretome by LC-MS/MS

The whole proteome of each biological sample was analysed by nano-liquid Chromatography coupled to Electrospray Ionization Tandem Mass Spectrometry (nanoLC-ESI-MS/MS) at the Proteomics Unit, National Centre for Biotechnology (Madrid, Spain).

Protein cysteine residues were reduced and alkylated at 60°C for 30 min with a buffer containing 5% sodium dodecyl sulfate (SDS), 25 mM triethylammonium bicarbonate (TEAB), 5 mM tris(2-carboxyethyl) phosphine (TCEP), and 10 mM chloroacetamide (CAA) (Ciordia et al., 2024). Protein digestion on S-Trap columns (Protifi, Huntington, NY, USA) was performed according to the previously established protocol (Ciordia et al., 2021). Briefly, the precipitated samples were digested overnight at 37°C with 1 µg of trypsin. Tryptic peptides were cleaned up using in-home-made Stage-Tips prepared from Octadecyl C18 solid-phase extraction disks (Empore™, 66883-U), as previously described (Ciordia et al., 2021). Eluted peptides were dried in a speed vacuum and quantified by fluorimetry (QuBit) according to the manufacturer's instructions. Tryptic peptides were dried in a speed vacuum and quantified by fluorimetry (QuBit) according to the manufacturer's instructions.

For the analysis, 1 µg of each sample was individually analysed using an Ultimate 3000 nano HPLC system (Thermo Fisher Scientific) coupled online to an Orbitrap Exploris™ 240 mass spectrometer (Thermo Fisher Scientific). Each sample [1 µg in 5 µl of sample resuspended in mobile phase A: 0.1% formic acid (FA)] was loaded on a 50 cm × 75 µm Easy-spray PepMap C18 analytical column at 45°C. Tryptic peptides were separated at a flow rate of 250 nL/min using a 90-minute gradient ranging from 6% to 95 % mobile phase B (80 % acetonitrile (ACN) in 0.1% FA). To avoid carry-over, two 40-minute blank samples (mobile phase A) were systematically run between samples. Data acquisition was performed using a data-dependent top method, in full scan positive mode, scanning 375 to 1200 m/z. MS1 scans were acquired at an orbitrap resolution of 60,000 at m/z 200, with a normalised automatic gain control target of 300%, a radio frequency lens of 80%, and an automatic maximum injection time (IT). The top 20 most intense ions from each MS1 scan were isolated and fragmented with a Higher-energy collisional dissociation (HCD) value of 30%. Resolution for HCD spectra was set to 15,000 at m/z 200, with a normalised AGC target of 50% and an automatic maximum IT. Precursor isolation window was 1.0 m/z and dynamic exclusion (45 s) was applied. Precursor ions with single, unassigned, or six and higher charge states from fragmentation selection were excluded.

V. 3.4.2. Data processing

Raw data files were processed using the Proteome Discoverer 2.5.0.400 software (Thermo Scientific, Bremen, Germany), and database search was carried out using four search engines (Mascot

(v2.8.0), MsAmanda (v2.4.0), MsFragger (v3.1.1), and Sequest HT) against an *O. europaea* UniProtKB database containing the most common laboratory contaminants (cRAP database with 69 sequences). Search parameters were set as follows: cysteine carbamidomethylation (+57.021464 Da); methionine oxidation (M) (+15.994915 Da), N-term acetylation (+42.010565 Da), and Gln→pyro-Glu (-17.026549 Da) as variable modifications. Precursor mass tolerances were set at 10 ppm, and the fragment mass tolerance at 0.02 Da, and trypsin/P was selected as a protease with a maximum of 2 missed cleavage sites. False discovery rate (FDR) was calculated using the processing node Percolator (maximum delta Cn 0.05; decoy database search target) and the validation of proteins, peptides, and peptide spectral matches (PSMs) peptides with a FDR ≤ 1 %. Protein abundance was also performed in Proteome Discoverer using the “Reporter Ions Quantifier” feature in the quantification workflow using the following parameters: unique+razor peptides, co-isolation threshold was set at 50%, signal to noise of reporter ions was 10, and the normalisation was performed considering the total peptide amount. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2025) partner repository with the dataset identifier PXD079057 and 10.6019/PXD079057

Annotation was carried out in KOBAS against the *Arabidopsis* database.

V. 3.4.3. Secretome data analysis

Label-free quantification (LFQ) intensity data from secretome analysis were processed in R using the limma package. Zero values were treated as missing, and data were log2-transformed. Quality control included assessment of intensity distributions, Pearson correlation, and principal component analysis (PCA) using complete cases. Proteins were retained if detected in at least two of three replicates in at least one group. Missing values were imputed using a left-shifted normal distribution (mean = minimum – 1.8, SD = 0.3). Differential abundance between HE and LE lines was evaluated using linear models with empirical Bayes moderation, and p-values were adjusted using the Benjamini–Hochberg method. Due to limited sample size ($n = 3$ per group), proteins with $p < 0.05$ and $|\log_2\text{FC}| \geq 1$ were considered differentially abundant proteins (DAPs). Heatmaps were generated using heatmap, and pathway analysis was performed using Kyoto encyclopedia of genes and genomes (KEGG) and Plant Reactome annotations, with downstream visualisation conducted using ggplot2.

V. 3.5. Analysis of the Exometabolome by ^1H NMR Spectroscopy

Lyophilised samples were dissolved in 1 mL of deuterated phosphate buffer (PBS 100 mM, pH 7.4), containing 0.1 mM 3-(trimethylsilyl)propionic acid (TSP-d4). The samples were then vortexed for 1 min to ensure homogeneous mixing and transferred to Eppendorf tubes. Next, the samples were

centrifuged at $13,000 \times g$ and $4\text{ }^{\circ}\text{C}$ for 5 min. For spectral acquisition, 550 μL of the supernatant was transferred to a 5 mm NMR tube.

Spectra were acquired on a Bruker Avance III HD spectrometer operating at 500.18 MHz for ^1H observation (University of Aveiro, Portuguese NMR Network), at 298 K. Standard one-dimensional (1D) ^1H spectra were recorded with 32k points, a spectral width of 7002.80 Hz, a relaxation delay of 2 s, and 512 scans, using the “noesypr1d” pulse sequence. Spectra were processed using TopSpin 4.0.3 (Bruker BioSpin, Rheinstetten, Germany), applying exponential multiplication (LB 0.3 Hz), manual phasing and baseline correction, and calibration to TSP-d4 (0.0 ppm). Metabolite assignment was based on matching 1D ^1H spectral data to reference spectra available in Chenomx (Edmonton, Canada) and BBIOREFCODE. Quantification of identified metabolites was performed using signal deconvolution and fitting to reference spectra from the Chenomx library through the Profiler module of Chenomx NMR Suite v10.1 Professional. For signals that could not be unambiguously assigned, relative quantification was performed through spectral integration using AMIX 4.0.2 (Bruker BioSpin).

V. 3.5.1. Exometabolome data analysis

Metabolite data were processed and analysed in R. Raw metabolite intensities were log-transformed using a $\log(1 + x)$ transformation to reduce skewness and stabilise variance, and missing values were imputed with half of the minimum detected value. PCA was performed using the `prcomp` function with unit variance scaling to assess global metabolic variation. Partial Least Squares Discriminant Analysis (PLS-DA) was conducted using the `mixOmics` package to evaluate group separation, and model performance was assessed by repeated M-fold cross-validation (5 folds, 10 repeats), with classification accuracy reported as Balanced Error Rate (BER). Variable Importance in Projection (VIP) scores were calculated, and to specifically identify metabolites discriminating between HE and LE lines, a separate PLS-DA model was constructed using only these groups, with metabolites showing $\text{VIP} > 1$ considered important contributors. Two-way ANOVA was performed to assess the effects of embryogenic group and line, as well as their interaction. FDR correction was applied, and metabolites with adjusted p -values < 0.05 were considered significant. Data visualisation was carried out using `ggplot2` in R. Pathway enrichment analysis was conducted using KEGG via MetaboAnalyst (Pang et al., 2024), and pathways with FDR-adjusted p -values < 0.05 were considered significantly enriched.

V. 4. RESULTS

V. 4.1. Identification of Secretome Signature Associated with Embryonic Efficiency

The characterisation of secretome profile from both HE and LE embryonic lines revealed the identification of 1.681 proteins, corresponding to 1002 protein groups (protein FDR confidence High; FDR<1%) (Table B.1). The distribution of log2-transformed protein intensities across samples was assessed using boxplots (Figure V.1A), showing comparable median values and overall distributions, indicating effective normalisation and high data consistency. Although slightly higher variability was observed within LE samples, overall intensity distributions were similar across conditions. Furthermore, correlation analysis, as shown in the heatmap (Figure V.1B), showed that biological replicates are clustered according to their respective conditions, with strong intra-group correlations, particularly among HE samples. LE samples were also grouped, although with slightly lower correlation values, suggesting increased variability within this group.

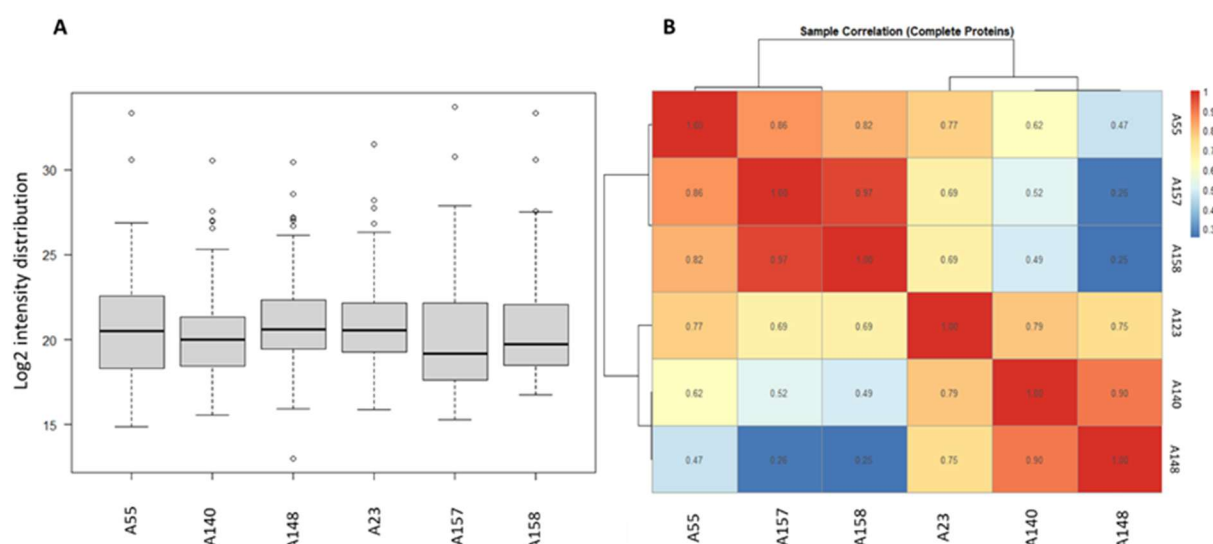


Figure V.1. Evaluation of data distribution and sample similarity across high-efficiency (HE) and low-efficiency (LE) lines. (A) Boxplot of log2-transformed protein intensity values across all samples. Each box represents the distribution of protein abundances for individual biological replicates, showing median, interquartile range, and overall variability; (B) Heatmap of sample-to-sample Pearson correlation coefficients based on log2-transformed protein intensities. Rows and columns represent individual biological replicates. Colour scale indicates the strength of correlation, with higher values reflecting greater similarity between samples. Hierarchical clustering was performed to evaluate relationships among samples.

Of the 865 quantified protein groups (FDR < 1%, PSM $\geq$ 2, at least a unique peptide), 173 were identified as differentially abundant between HE and LE embryogenic lines (Table B.2). As depicted in the volcano plot, DAPs show a clear separation from non-significant proteins (Figure B.1). Indeed, most DAPs were detected exclusively in one of the conditions, including 81 proteins detected exclusively in HE lines and 92 in LE. This pattern highlights the presence of distinct proteomic signatures associated with HE and LE embryogenic lines. Overall, these results suggest that the two conditions differ substantially at the proteomic level, reflecting underlying biological differences between groups.

Protein abundance was further visualised as a heatmap to establish a differential proteome signature associated with embryogenic efficiency (Figure V.2). The heatmap shows the top 20 most regulated proteins identified by the linear model, emphasising the differences between HE and LE samples. Several proteins showed consistent up-regulation in HE samples and down-regulation in LE samples, as well as the opposite pattern, indicating a strong association between protein abundance and embryogenic competence. These contrasting expression profiles enabled a clear discrimination between HE and LE embryogenic lines.

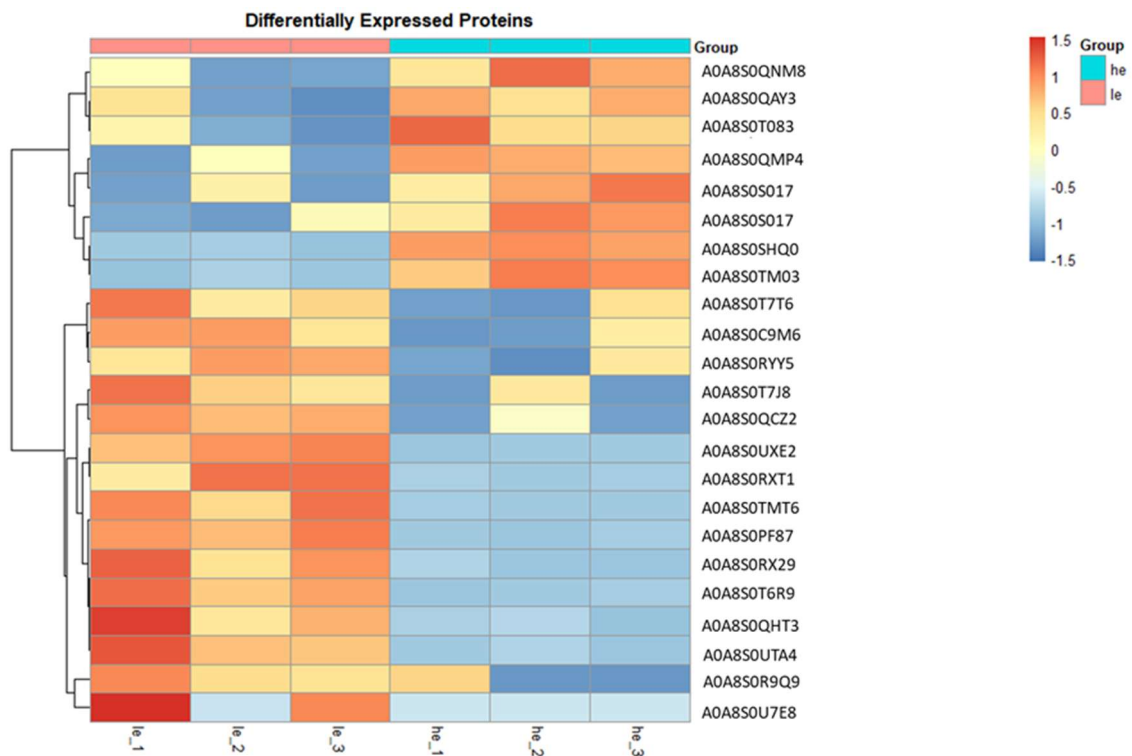


Figure V.2. Heatmap illustrating the top regulated proteins (UniProt accessions) between high-efficiency (he: samples he_1, he_2, and he_3) and low-efficiency (le: samples le_1, le_2, and le_3) somatic embryogenic lines. Proteins were selected according to the linear model results using a threshold of $p < 0.05$ and $|\log_2 \text{ fold change}| \geq 1$. Rows represent protein groups and columns correspond to biological replicates from each condition. Colour intensity reflects relative protein abundance, with red

indicating higher expression and blue indicating lower expression levels. Hierarchical clustering was applied to both proteins and samples based on similarity in expression profiles.

Functional annotation of the DAPs was performed using pathway classification from KEGG and Plant Reactome databases (Figure V.3). KEGG pathway-based functional analysis revealed that most proteins were associated with amino acid metabolism (45%), followed by central metabolism (20%) and secondary metabolism (20%). In comparison, a smaller proportion was related to cellular regulation (15%). This distribution indicates a strong representation of metabolic processes, particularly those involved in primary metabolism (Figure V.3A). Similarly, the Plant Reactome-based analysis (Figure V.3B) showed that amino acid metabolism was also the most significantly enriched category (31.2%), reinforcing its central role in the embryogenic efficiency. In addition, pathways related to hormone signalling (25%) and stress and immunity (18.8%) were prominently represented, along with development (18.8%), suggesting the involvement of regulatory and adaptive biological processes. Cellular regulation accounted for the lowest proportion of pathways (6.2%), consistent with the KEGG results.

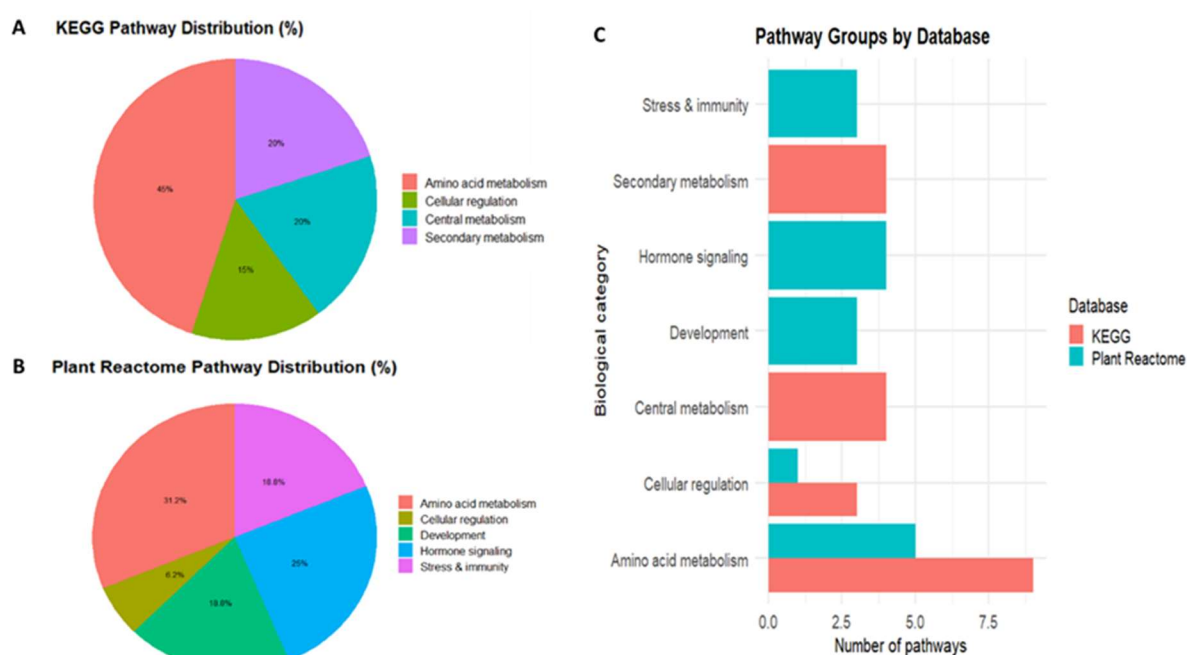


Figure V.3. Functional classification and pathway distribution of differentially expressed proteins (DAPs) based on pathway enrichment analyses. (A) Distribution of proteins across biological pathways according to Kyoto encyclopedia of genes and genomes (KEGG); and (B) Plant Reactome, expressed as percentages of total annotated proteins; (C) Number of pathways identified per biological category in KEGG and Plant Reactome databases.

To further compare pathway representation between databases, the number of pathways per biological category was evaluated (Figure V.3C). Amino acid metabolism exhibited the highest number of pathways in both databases, with a more pronounced representation in KEGG. In contrast, cellular regulation showed the fewest pathways in both analyses. Overall, these results highlight a predominant involvement of metabolic pathways, alongside contributions from regulatory, developmental, and stress-related processes.

V. 4.2. Metabolite Profiling

The exometabolome of different embryogenic lines was assessed using ^1H NMR spectroscopy. A total of 34 spectral features were detected, of which 16 metabolites were unambiguously identified and quantified. These included amino acids (valine, leucine, isoleucine, alanine, phenylalanine, tyrosine, tryptophan, glutamate and pyroglutamate), sugars (sucrose, glucose and fructose) and organic acids (acetate, formate, lactate and fumarate), while the remaining signals corresponded to unidentified compounds.

As shown in Table V.1, statistical analysis revealed a strong effect of group across the dataset, with all metabolites showing significant differences ($\text{FDR} < 0.05$). Notably, most metabolites exhibited highly significant differences between groups ($\text{FDR} < 0.001$), indicating a consistent group-driven metabolic variation. This effect was observed across multiple metabolite classes, including amino acids, sugars and organic acids. A significant effect of line was also detected for most metabolites, although with more variable significance levels compared to the group effect. In contrast, no interaction effects between group and line were detected. Overall, these results indicate that group-related differences represent the dominant source of variation in the dataset, while line-specific effects contribute to a lesser extent.

Table V.1. Statistical analysis of exometabolites detected by ^1H NMR spectroscopy. FDR-adjusted p -values are presented for the effects of group and line on each metabolite. Significance levels are indicated as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Unidentified compounds are denoted as “Un” followed by their corresponding chemical shift (ppm).

Metabolite	Group (FDR)	Line (FDR)	Group Sig	Line Sig
Methionine	1.02E-21	0.00121	***	**
Asparagine	3.54E-16	0.000875	***	***
Sucrose	3.54E-16	0.00189	***	**
Phenylalanine	4.04E-15	0.000471	***	***
Tryptophan	2.17E-14	0.000187	***	***
Alanine	6.98E-14	0.000452	***	***
Tyrosine	1.40E-13	8.40E-05	***	***

Leucine	9.08E-11	8.56E-05	***	***
Valine	2.42E-09	0.000106	***	***
Un1.24	4.98E-09	0.0195	***	*
Isoleucine	1.12E-08	4.82E-05	***	***
Lactate	1.12E-08	6.39E-05	***	***
Glutamate	1.80E-08	4.86E-06	***	***
l_MeNicotinamide	5.49E-08	1.06E-05	***	***
Un1.15	5.62E-08	0.000471	***	***
Un4.52	9.70E-08	0.000187	***	***
Lysine	3.56E-07	0.00104	***	**
Un4.89	4.52E-07	0.00011	***	***
Un4.45	1.88E-06	0.00113	***	**
beta_Fru	4.32E-06	3.85E-06	***	***
Un5.07	4.43E-06	2.03E-06	***	***
alpha_Fru	5.56E-06	6.49E-05	***	***
Un3.10	9.11E-06	2.03E-06	***	***
Un8.29	9.76E-06	1.15E-06	***	***
beta_Glc	1.49E-05	5.84E-06	***	***
Un5.025	1.84E-05	1.64E-06	***	***
Pyroglutamate	4.35E-05	0.0108	***	*
Un8.42	4.36E-05	5.14E-05	***	***
alpha_Glc	1.00E-04	1.75E-05	***	***
Un7.56	0.00012	1.15E-06	***	***
Un1.83	0.000297	0.000927	***	***
Acetate	0.000538	0.0106	***	*
Formate	0.00124	1.64E-06	**	***
Fumarate	0.0156	0.000831	*	***

To visualise the global metabolic differences between samples, PCA was conducted (Figure V.4A). The analysis showed that the first two components explained 49.5% (PC1) and 27.1% (PC2) of the total variance, revealing the overall distribution of samples. A partial separation between groups was observed, with control samples forming a distinct cluster, while HE and LE samples showed some overlap. Notably, a higher dispersion of samples was observed within the LE group, indicating greater intra-group variability compared to HE and control samples, which exhibited more compact clustering patterns.

PLS-DA was applied to evaluate differences between experimental groups (Figure V.4B). The model showed a clear separation between groups, consistent with the patterns observed in PCA. The first two components explained 69.9% and 30.1% of the variance, respectively, and the model demonstrated good predictive performance (BER = 0.11), indicating reliable classification of samples despite the observed intra-group variability.

Significant differences between HE and LE groups were further illustrated by the relative abundance of selected metabolites (Figure V.4C). Several metabolites were found at higher levels in HE

lines, including sucrose, acetate, α - and β -fructose, and β -glucose. In contrast, MeNicotinamide accumulated at higher levels in LE lines, together with unidentified compounds such as Un1.24, Un4.45 and Un4.49. Overall, these results highlight distinct metabolite accumulation patterns between HE and LE groups.

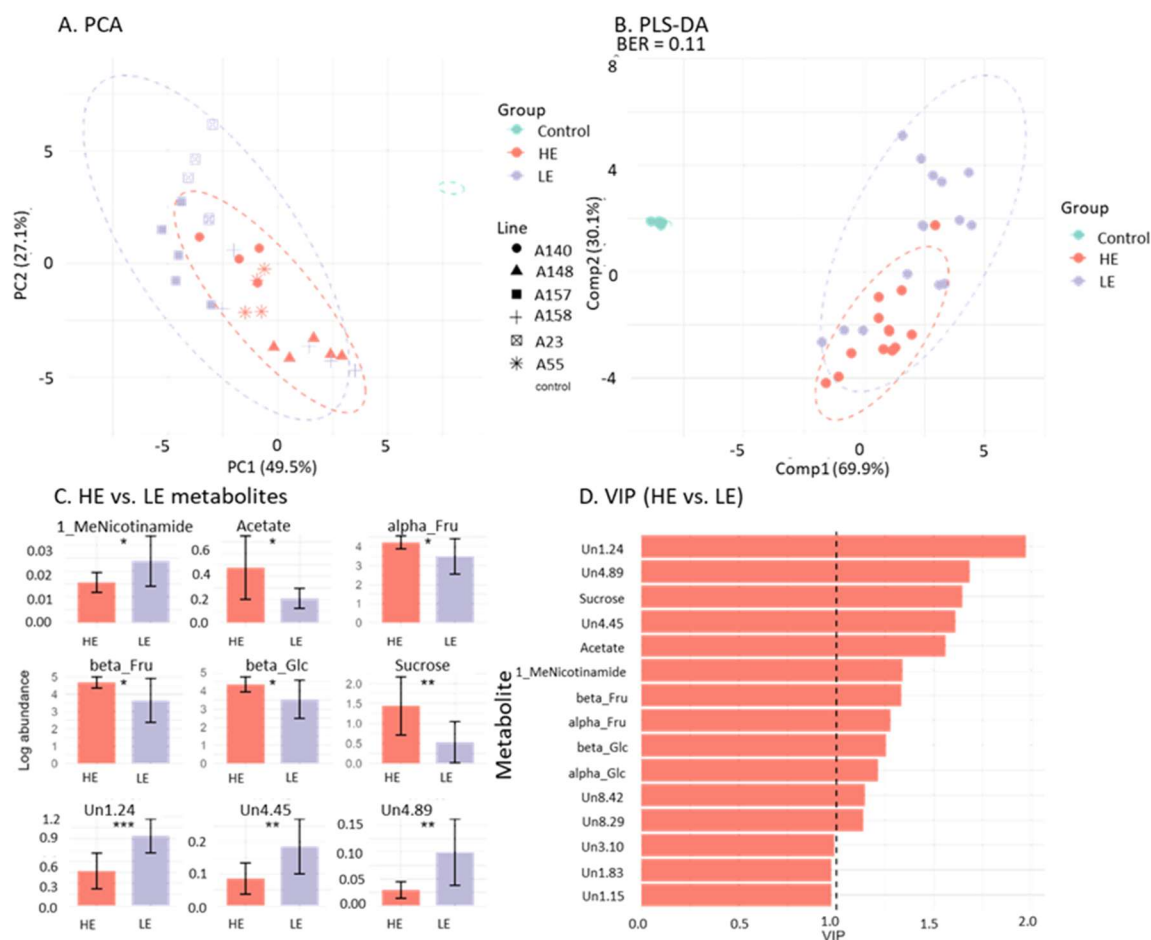


Figure V.4. Multivariate and univariate analysis of metabolomic profiles. (A) Principal Component Analysis (PCA) of metabolomic data showing overall sample distribution; (B) Partial Least Squares Discriminant Analysis (PLS-DA) illustrating supervised separation of groups; (C) Bar plots showing mean metabolite levels ($\pm$ SD) for the top nine metabolites significantly different between high efficiency (HE) and low efficiency (LE) lines (FDR-adjusted $p < 0.05$). Statistical significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) and (D) Variable Importance in Projection (VIP) scores from PLS-DA identifying metabolites contributing most to discrimination between HE and LE. The dashed line indicates the threshold for importance (VIP = 1).

Variable Importance in Projection (VIP) scores from the PLS-DA model were used to identify the metabolites contributing most to the discrimination between HE and LE groups (Figure V.4D). The highest VIP scores were observed for Un1.24 and Un4.89, followed by sucrose, Un4.45 and acetate.

Additional important contributors included 1-methylnicotinamide and sugars such as α - and β -fructose and glucose (α - and β -forms), all exceeding the threshold for significance (VIP > 1).

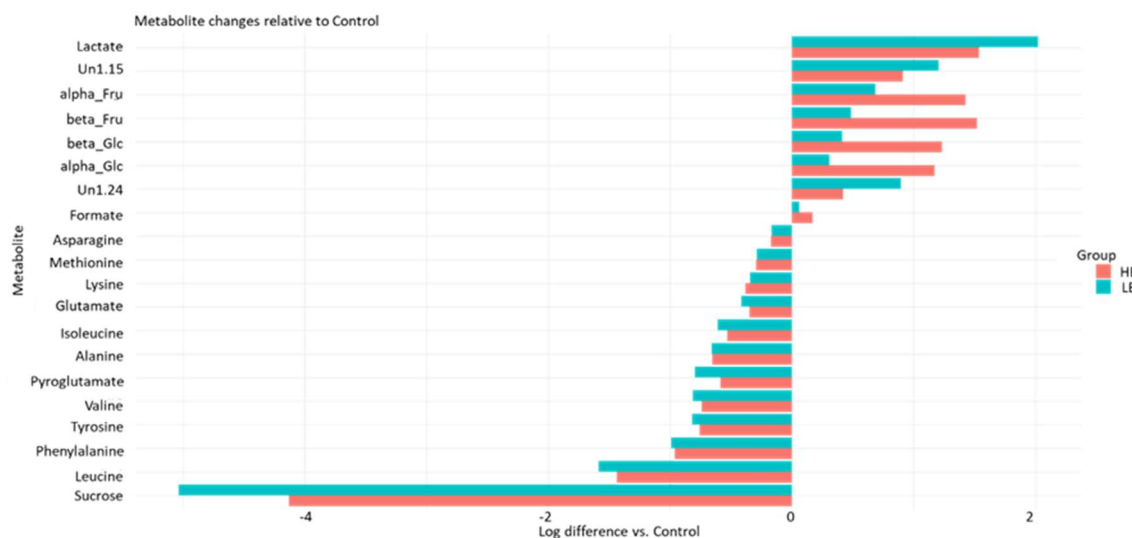


Figure V.5. Metabolite changes relative to the acellular culture medium (control), expressed as log differences. Negative values indicate depletion of metabolites from the culture medium, while positive values indicate accumulation in the conditioned medium (CM). Data are shown for high-efficiency (HE) and low-efficiency (LE) embryogenic lines.

Metabolite changes relative to the control condition (acellular culture medium) revealed distinct consumption and accumulation patterns across embryogenic lines (Figure V.5). Most metabolites showed negative log differences, indicating depletion from the medium. Sucrose exhibited the strongest decrease, particularly in LE lines, while leucine also showed pronounced depletion, with slightly lower levels in LE compared to HE. Other amino acids, including phenylalanine, tyrosine, valine and pyroglutamate, showed moderate decreases, generally with slightly lower values in LE lines. Glutamate and several amino acids such as lysine, methionine and asparagine remained close to zero, indicating minimal variation relative to control. Notably, alanine levels were also affected, which, together with the detected abundance of alanine–glyoxylate transaminase in the secretome (Table B.1), suggests active interconversion between alanine and glyoxylate, linking amino acid metabolism with carbon flux. In contrast, several metabolites showed positive log differences, indicating accumulation in the CM. Formate displayed a slight increase, more pronounced in HE lines. Sugars, including glucose and fructose (α and β), showed marked accumulation, particularly in HE lines. Lactate exhibited the highest positive values overall, with a stronger accumulation in LE lines.

Overall, these patterns indicate differential carbon utilisation between HE and LE lines, with LE lines showing stronger substrate depletion and higher lactate accumulation, while HE lines exhibit greater accumulation of soluble sugars in the CM. The involvement of alanine metabolism further

supports a connection between nitrogen and carbon metabolic pathways during subculture.

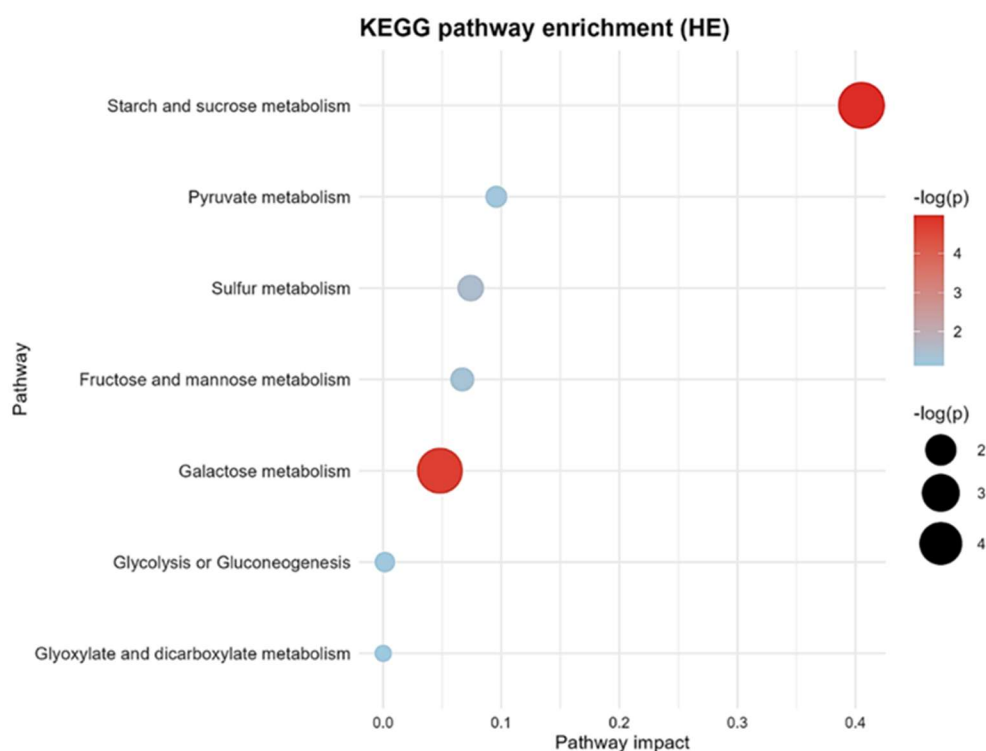


Figure V.6. Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis for high-efficiency (HE) embryogenic lines. Circle size and colour indicate statistical significance ($-\log(p)$).

KEGG pathway enrichment analysis for HE lines identified several metabolic pathways associated with the observed metabolite changes (Figure V.6). Among these, starch and sucrose metabolism showed the highest pathway impact and the strongest statistical significance, followed by galactose metabolism, which also exhibited high significance despite a lower impact value. Fructose and mannose metabolism and sulfur metabolism displayed moderate pathway impact and significance, while pyruvate metabolism showed a similar impact but lower statistical relevance. In contrast, pathway topology analysis indicated a relatively low impact and statistical significance for glycolysis/gluconeogenesis and glyoxylate and dicarboxylate metabolism. Although glycolysis displayed a low pathway impact score, differential accumulation of some glycolysis-related proteins was observed in HE lines, including triosephosphate isomerase and phosphoglycerate kinase (Table B.2). Overall, the enrichment profile highlights carbohydrate metabolism pathways as the most prominent features associated with HE lines. These findings are consistent with patterns identified in both univariate and multivariate analyses, including PCA and PLS-DA, as well as with the differential accumulation of key metabolites and enzymes between HE and LE lines.

Taken together, the combined univariate, multivariate, and pathway analyses indicate that metabolic variation between embryogenic lines is primarily driven by differences in carbon metabolism, particularly in pathways related to sugar utilisation and associated metabolic processes, including glycolysis.

V. 5. DISCUSSION

The integration of proteomic and metabolomic data revealed a coordinated reprogramming of metabolic and regulatory pathways associated with embryogenic competence. A strong enrichment of amino acid metabolism was observed through both KEGG and Plant Reactome analyses of the secretome, indicating an active involvement of these pathways at the protein level. Although several amino acids were detected by NMR, their presence in the control medium (acellular culture medium) indicates that they mainly reflect the basal composition of the medium rather than differential accumulation between HE and LE lines. These include branched-chain (valine, leucine, and isoleucine), aromatic (tryptophan, tyrosine, and phenylalanine), and metabolically interconnected amino acids such as glutamate and alanine. In this context, the enrichment of amino acid metabolism pathways in the proteomic dataset likely reflects changes in enzymatic activity and metabolic flux rather than variations in extracellular metabolite levels. This is consistent with previous observations that metabolite abundance does not necessarily reflect pathway activity. Relatively stable compounds may remain at steady-state levels despite dynamic metabolic regulation (Ishihara et al., 2015). Furthermore, the detection of aromatic amino acids such as tryptophan, phenylalanine, and tyrosine may indicate functional metabolic differences, as these compounds are key precursors for hormone biosynthesis and secondary metabolism.

These findings are broadly consistent with the enrichment of amino acid metabolism pathways observed in both KEGG and Plant Reactome analyses. Among these, glutamate represents a central node in nitrogen metabolism and is closely associated with glutathione (GSH) biosynthesis, a key component of cellular redox regulation (Nehela & Killiny, 2023; Riseh et al., 2026). In the present study, this functional context is supported by the detection of glutathione S-transferase L3-like isoform X1, although changes in its abundance were not statistically strong. The presence of pyroglutamate may be associated with amino acid and glutathione-related metabolism, potentially reflecting alterations in redox balance. GSH plays a central role in cellular redox homeostasis by scavenging reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), through both enzymatic and non-enzymatic mechanisms (Sood, 2025). In plants, GSH is an essential component of the ascorbate–glutathione cycle, acting as an electron donor for the reduction of dehydroascorbate (DHA) to ascorbate (Foyer & Kunert, 2024). This cycle constitutes one of the major antioxidant systems controlling intracellular redox balance. In the context of SE, GSH has been reported to influence cell proliferation and embryogenic

development in several plant species, including *Picea glauca* (Stasolla, 2004), *Arabidopsis thaliana* (Kudelko & Gaj, 2019), and *P. koraiensis* (Gao et al., 2022). Interestingly, glutamate levels did not differ significantly between HE and LE lines, suggesting that differences in embryogenic competence may be associated with downstream regulatory processes rather than global changes in nitrogen metabolism. In addition, the increased abundance of alanine–glyoxylate transaminase suggests a potential link between amino acid and central carbon metabolism, indicating a coordinated adjustment of nitrogen-related metabolic fluxes.

Consistent with this, the upregulation of PODs and germin-like proteins (GLPs) observed in the secretome further supports the activation of antioxidant defence mechanisms. Class III PODs are directly involved in ROS detoxification, particularly in the apoplast, where they modulate H₂O₂ levels and participate in cell wall remodelling processes (Li et al., 2024). These enzymes function in the removal of excess ROS while also contributing to cell wall strengthening through oxidative reactions involved in lignification and cross-linking processes (Rao et al., 2025). Their activity is coordinated with cellular redox regulatory networks (Liu et al., 2015). Their presence in the secretome reflects extracellular redox-related processes and supports their role in redox homeostasis. Similarly, GLPs are associated with ROS metabolism and stress signalling, often exhibiting superoxide dismutase activity and contributing to the regulation of oxidative balance in the extracellular space (Dunwell et al., 2008). GLPs have also been implicated in developmental processes and defence responses, reinforcing their role in mediating stress-related signalling pathways (Govindan et al., 2024). PODs and GLPs have been widely reported in extracellular fractions and CM, where they are recognised as indicators of SE competence (Domon et al., 1995; Heringer et al., 2018; Ngwenya et al., 2024; Pires et al., 2025b). In the present study, these redox-related proteins are also evident in the differential expression patterns displayed in the secretome heatmap (Figure V.2), supporting their coordinated regulation in embryogenic lines. PODs were upregulated in HE lines, highlighting enhanced extracellular ROS modulation and cell wall remodelling in embryogenic tissues, consistent with the role of controlled H₂O₂ dynamics in promoting cell differentiation during SE (Dong & Lin, 2021; Maslennikova et al., 2023). In contrast, GLPs were more abundant in LE lines, indicating a predominance of stress-related ROS responses that may compromise the regulated oxidative environment required for optimal embryogenic progression.

Further support for the involvement of redox regulation and cell wall-related pathways is provided by some DAPs identified in the heatmap. Receptor-like kinases such as THESEUS1 (THE1) sense cell wall integrity. They link extracellular cues to intracellular responses and integrate developmental and hormonal signals to regulate growth and stress adaptation (Zwartkruis et al., 2026). THE1 modulates cell wall stiffness and coordinates turgor pressure via abscisic acid (ABA) biosynthesis and signalling (Bacete et al., 2022) and acts as a receptor for Rapid Alkalinization Factor (RALF)

peptides (Gonneau et al., 2018) in *A. thaliana*. THE1 modulates cell wall stiffness and coordinates turgor pressure via abscisic acid (ABA) biosynthesis and signalling (Fehér, 2015). The observed differential expression may reflect its contribution to coordinating structural and signalling changes associated with embryogenic competence. Additionally, the upregulation of ubiquitin domain-containing DSK2A-like proteins in LE lines highlights the role of selective protein turnover. Acting as shuttle factors in ubiquitin-mediated pathways, these proteins promote targeted degradation of regulatory proteins, ensuring precise control of developmental transitions in *Jatropha curcas* (Medina-Hernández et al., 2025). Their increased abundance in LE suggests a stronger involvement of protein turnover processes, possibly reflecting differences in the regulation of developmental progression.

In addition, in this study, HE lines exhibited elevated levels of both α - and β -glucose and fructose, reflecting changes in sugar availability and central carbon metabolism. This pool of sugars may result from active cellular uptake via sucrose/ H^+ symporters and intracellular hydrolysis of sucrose by invertase (EC 3.2.1.26) into glucose and fructose (Ma et al., 2019). In contrast, lower sugar levels in LE lines could indicate reduced metabolic activity. The elevated levels of glucose, fructose and sucrose observed in the CM of HE lines align with previous reports in *Coffea arabica* (Bartos et al., 2018) and *Solanum betaceum* (Caeiro et al., 2023), highlighting that both the presence and utilisation of these soluble sugars are associated with embryogenic competence. Supporting this, sucrose synthase (SUSY) was present in HE lines (Table B.1), together with some glycolysis-related enzymes such as phosphoglycerate kinase and triosephosphate isomerase, indicating altered sucrose cleavage and downstream carbon metabolism.

Consistent with the observed enrichment of pyruvate metabolism and the upregulation of SUSY in HE lines, other small intermediate metabolites, including acetate and lactate, were detected, with acetate particularly enriched in HE lines. As a central metabolite linking carbohydrate and lipid metabolism, acetate can be generated from acetaldehyde via pyruvate decarboxylation or ethanol dehydrogenation (Lin & Oliver, 2008). It also serves as a precursor for acetyl-CoA, connecting central metabolism to biosynthetic and regulatory processes (Hann et al., 2023). This increase suggests enhanced acetyl-CoA availability, potentially supporting cellular reprogramming and proliferation during SE through histone acetylation (Cai et al., 2011). Lactate was also identified in the CM. Produced via pyruvate reduction by lactate dehydrogenase (Jain et al., 2020), it enables nicotinamide adenine dinucleotide (NAD^+) regeneration under conditions of limited mitochondrial respiration or redox imbalance in *Oryza sativa* and *A. thaliana* (Chatterjee et al., 2023). In plant systems, lactate accumulation is associated with metabolic constraints that restrict efficient oxidative metabolism (Igamberdiev & Eprintsev, 2016). The higher levels of lactate in LE lines, therefore, suggest a shift in pyruvate metabolism toward fermentative pathways, reflecting altered metabolic regulation. This pattern is consistent with observations in the non-embryogenic callus of *S. betaceum* (Cordeiro et al.,

2023). In contrast, the observed differences in glycolysis-related proteins in HE lines suggest a differential routing of pyruvate towards biosynthetic and respiratory pathways. Taken together, these findings indicate that the metabolic fate of pyruvate differs between HE and LE lines, with HE lines favouring pathways associated with growth and development, while LE lines exhibit features consistent with altered metabolic balance.

These observations underscore the role of the CM as a dynamic extracellular environment shaped by the secretory activity of nurse cultures. Differences in CM composition and activity between HE and LE lines reflect the capacity of these cells to modulate metabolic and signalling networks, directly influencing the reprogramming, proliferation, and developmental progression of SE.

V. 6. CONCLUSION

The present study revealed clear differences in the composition of the secretome and exometabolome of CM between embryogenic lines with contrasting embryogenic capacities in *O. europaea*. The integration of proteomic and metabolomic data highlighted a coordinated regulation of metabolic and signalling pathways associated with embryogenic competence, particularly those related to amino acid metabolism, redox homeostasis, and central carbon metabolism.

The HE embryogenic lines were characterised by a metabolic profile indicative of efficient carbon utilisation and controlled oxidative balance, supported by the differential abundance of extracellular proteins involved in redox regulation and cell wall dynamics, including class III PODs and receptor-like kinases such as THE1, as well as metabolites linked to acetyl-CoA metabolism. In contrast, LE embryogenic lines exhibited features associated with metabolic constraints, including increased lactate accumulation and a predominance of stress-related responses, suggesting a less favourable environment for embryogenic reprogramming.

These results highlight the culture medium as a dynamic and informative component of the SE system, reflecting the physiological state of the cultures and their capacity to modulate developmental processes. In particular, the composition of CM from HE embryogenic lines may contribute to the establishment of a favourable extracellular environment capable of promoting cellular reprogramming, improving the embryogenic response of recalcitrant explants. The identification of metabolites and proteins associated with embryogenic competence further supports the use of extracellular profiling as a strategy to develop biomarkers and optimise culture conditions. Future studies should explore the functional role of these extracellular components and their potential application in enhancing SE efficiency in olive.

V. 7. REFERENCES

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CHAPTER VI

Mimicking nurse cultures as drivers of embryogenic competence in *Olea europaea* L

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VI. 1. ABSTRACT

Somatic embryogenesis (SE) is a key technique for plant regeneration, widely used for large-scale clonal propagation and diverse biotechnological applications. However, in *Olea europaea*, SE efficiency remains limited, particularly when adult explants are used, due to their recalcitrance and reduced embryogenic competence. Nurse cultures have been proposed as a strategy to improve *in vitro* morphogenic responses by modifying the culture environment through the release of growth-promoting factors, including extracellular proteins, metabolites, and signalling molecules. In this context, conditioned medium (CM) obtained from high-efficiency (HE) embryogenic lines established from zygotic embryos was collected, filtered, and applied to low-efficiency (LE) embryogenic lines to reprogram the embryogenic response. In parallel, calli obtained from petiole explants taken from *in vitro* plants of the recalcitrant cultivar ‘Galega Vulgar’ were cultured on induction medium under liquid or gelled conditions supplemented with different CM concentrations. Subsequently, the resulting calli were transferred to expression medium devoid of plant growth regulators, while maintaining CM application. CM supplementation enhanced callus proliferation and somatic embryo formation in LE lines, with embryo numbers increasing with higher CM concentrations. In calli developed from adult explants, CM promoted the formation of globular-like and morphogenic structures, particularly under liquid conditions. These findings highlight the role of extracellular factors in modulating morphogenic responses and indicate that their effectiveness is influenced by culture conditions, especially liquid systems, representing a promising strategy to improve regeneration efficiency in olive.

VI. 2. INTRODUCTION

Somatic embryogenesis (SE) is a process in which somatic cells, when exposed to specific induction stimuli and suitable *in vitro* culture conditions, acquire embryogenic competence and develop into bipolar structures with distinct root and shoot poles (Elhiti & Stasolla, 2022). These structures resemble zygotic embryos and are capable of regenerating complete plants, independently of vascular connections to the maternal tissue (Martínez & Corredoira, 2024). This technique represents a powerful tool in plant biotechnology, with applications in large-scale clonal propagation, germplasm conservation, and plant breeding, including genetic engineering approaches (Guan et al., 2016; Cardoso et al., 2019). In addition, SE, defined as a morphogenic response triggered by exogenous stress factors, provides a valuable model system for studying plant cell totipotency and developmental reprogramming (Arnholdt-Schmitt et al., 2016; Loyola-Vargas & Ochoa-Alejo, 2016).

Advancing understanding of the molecular mechanisms controlling SE is essential to improve induction efficiency and enable somatic embryo formation in recalcitrant genotypes and adult-derived explants (Martínez & Corredoira, 2024). SE induction relies on the activation of complex signalling

networks that coordinate extensive gene expression reprogramming (Méndez-Hernández et al., 2019) in response to multiple factors. Type and concentration of plant growth regulators, culture conditions, and explant characteristics, including type and developmental stage, have been highlighted as the main factors influencing SE efficiency across plant species (Cardoso et al., 2010; Nic-Can et al., 2015), including olive (Cardoso et al., 2019; Pires et al., 2025a, b). Although SE is efficiently achieved across a wide range of plant species, including several horticultural crops such as *Vitis vinifera* (Cardoso et al., 2010), *Solanum lycopersicum* (Saeed et al., 2019), *Brassica juncea* (Faisal et al., 2021), and *Daucus carota* (Lee & Choi, 2024), as well as conifer species such as *Abies* spp. (Salaj et al., 2019), *Pinus taeda* (Maruyama & Hosoi, 2019), and *Picea abies* (Varis et al., 2018, 2021), many woody plants of agronomic importance, including *Olea europaea* L., remain highly recalcitrant to somatic embryo induction, even when zygotic embryos are used as the initial explant (Pires et al., 2025a). The recalcitrance to SE in olive has been mainly associated with the ontogenetic maturity of the donor tissue, as embryogenic competence is markedly higher in juvenile material than in adult-derived explants (Rugini et al., 1995; Mazri et al., 2013; Pires et al., 2025a). Adult olive tissues exhibit a reduced capacity for developmental reprogramming, which has been linked to stable epigenetic constraints, including DNA methylation patterns, that limit the activation of embryogenesis-related genes (Narváez et al., 2019). In addition, deficiencies in the perception and transduction of key embryogenic signals, particularly those associated with auxin and stress responses, have been reported to compromise SE induction in olive, contributing to the strong genotype dependence and low induction efficiency observed in most protocols (Rugini & Silvestri, 2016).

To address the limitations associated with SE in recalcitrant species, nurse culture techniques were introduced in the 1980s as a strategy to stimulate somatic embryo formation. This approach was based on the proposition that nurse cultures modify the *in vitro* environment through the release of growth-promoting and signalling factors essential for SE induction (Eigel & Koop, 1989). Research focused on identifying molecules secreted into the culture medium, and their subsequent application to enhance SE efficiency has been successfully reported in *Lilium japonicum* (Komai et al., 2006), *Coffea* spp. (Nic-Can et al., 2015), *Pinus* spp. (Hargreaves et al., 2017), and, more recently, in *P. nigra* (Pernis et al., 2023). In this context, the culture medium, referred to as conditioned medium (CM), acts as a reservoir of secreted biomolecules – including phytohormones, proteins, metabolites, and other signalling compounds – that play key regulatory roles in modulating both proliferative and differentiation processes underlying SE (Ben-Amar, 2021; Quintana-Escobar et al., 2025). In this regard, liquid culture systems provide a particularly suitable experimental platform; they create a dynamic, non-adherent microenvironment that enhances cell–cell communication and promotes the secretion and accumulation of extracellular matrix components and bioactive factors (Franko & Ferraz, 2024).

In this study, the ability of culture-derived extracellular signals from high-efficiency (HE) embryogenic lines of *O. europaea* L. to enhance embryogenic competence in recalcitrant tissues was investigated. The HE embryogenic lines previously selected from cv. ‘Arbequina’ (Pires et al., 2025a) were established in liquid culture medium to allow the collection of CM. This CM was then applied, at different concentrations, to previously selected low-efficiency (LE) embryogenic lines of the same cultivar (Pires et al., 2025a), as well as to adult explants (petioles) of cv. ‘Galega Vulgar’ was maintained *in vitro* through microcuttings subculture at the Laboratory of Plant Breeding and Biotechnology, University of Évora. This experimental approach enabled the functional validation of secreted factors released by HE embryogenic lines and provided insight into their capacity to modulate embryogenesis SE and mitigate recalcitrance in olive.

VI. 3. MATERIAL AND METHODS

VI. 3.1. Plant Material

Mature olive fruits of *O. europaea* cv. ‘Arbequina’ were harvested at full ripeness from open-pollinated orchards at the Coleção Nacional de Referência de Cultivares de Oliveira, Instituto de Investigação Agrária e Veterinária (Elvas, Portugal). Fruit pulp was removed, and seeds were collected, washed, and stored at $4 \pm 1^\circ\text{C}$ for 30 days. Subsequently, the endocarp was manually broken, and seeds were extracted for surface disinfection following the procedure described by Pires et al. (2020).

VI. 3.2. Establishment of Somatic Embryogenesis from Juvenile and Adult Plant Tissues

Zygotic embryos, collected from previously disinfected seeds, were used to collect juvenile plant tissues used as initial explants for indirect SE induction. Explants were established in induction olive medium (OM) modified with macroelements replaced with the ones proposed by Bourgin & Nitsch (1988) (OMc), supplemented with 2.5 μM 6-dimethylallylamino-purine (2iP), 25 μM indole-3-butyric acid (IBA), and gelled with 6 g L⁻¹ Agar-Powder (VWR, Portugal), following the protocol of Pires et al. (2020). Embryogenic lines were maintained through cyclic SE in liquid ECO culture medium to promote cyclic embryogenesis and evaluate their embryogenic competence (Pires et al. 2025a).

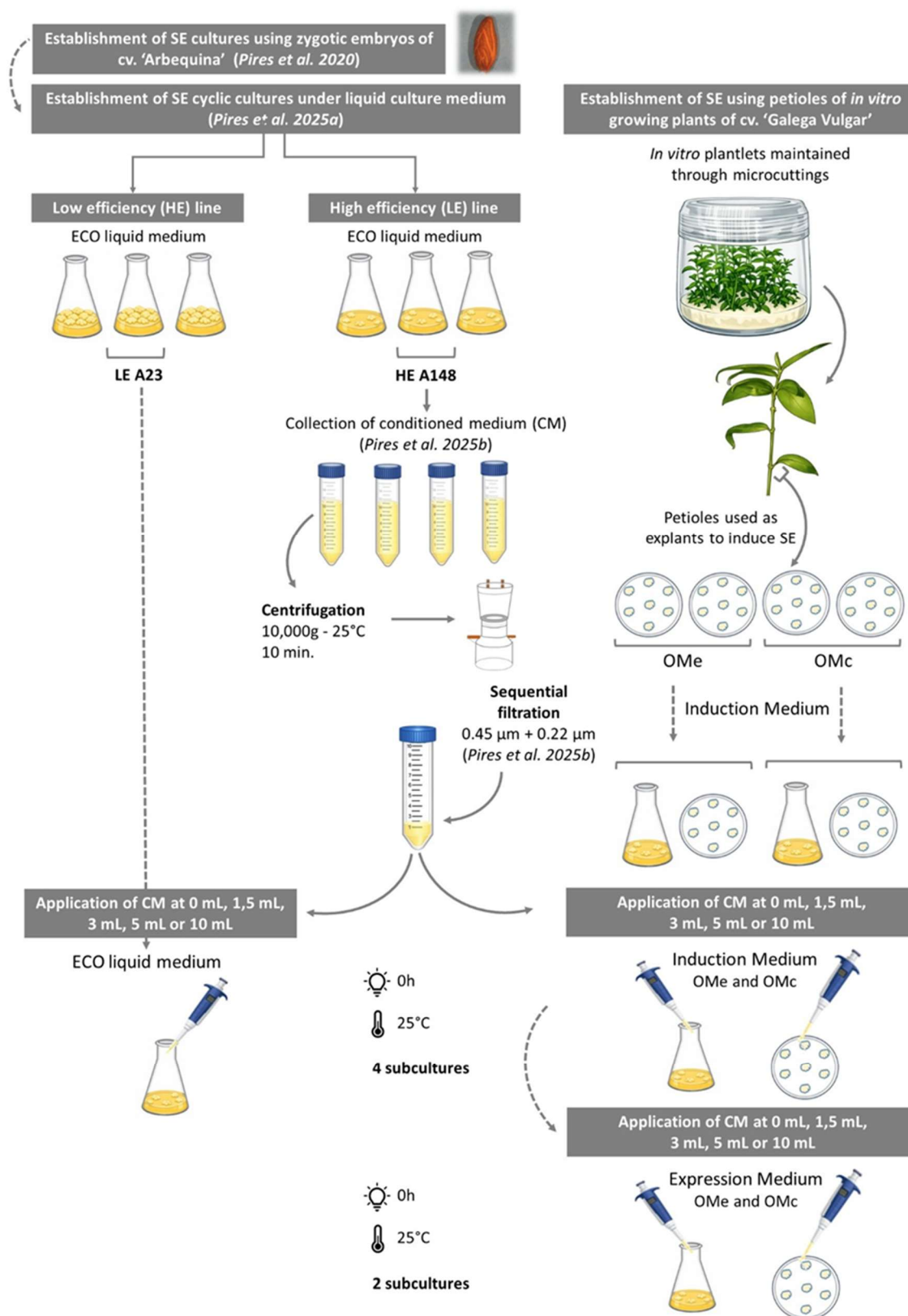


Figure VI.1. Workflow followed in this study. Embryogenic lines with high (HE) and low efficiency (LE) were maintained in liquid ECO medium. Conditioned medium (CM) was collected from HE line A148, properly processed, and applied at different concentrations to LE line A23 cultures. In parallel,

petiole explants derived from *in vitro* plants were induced in gelled medium to promote calli formation. The resulting calli were then transferred to induction medium under both liquid and gelled conditions, with or without CM supplementation. Finally, calli were transferred to expression medium (without plant growth regulators) under both culture systems, while maintaining CM application. The quality of the calli developed in each of the conditions were investigated through histological analysis.

In parallel, adult plant tissues were also used for SE induction. For this purpose, petiole explants from *O. europaea* L. cv. 'Galega Vulgar', clone 1441, was excised from leaves of plants maintained under *in vitro* through microcuttings subculture at the Laboratory of Plant Breeding and Biotechnology, University of Évora. Leaves were aseptically separated from the donor plants, and petioles were isolated and established in two distinct culture medium: the OMc (Cañas & Benbadis, 1988) medium supplemented with 25 μM IBA and 2.5 μM 2iP (Orinos & Mitrakos, 1991), as previously used for the induction of juvenile tissues, and the modified olive medium (OM) for SE (OMe) (Rugini & Caricato, 1995) supplemented with 2.5 μM naphthaleneacetic acid (NAA) and 2.25 μM zeatin, following the protocol described by (Trabelsi et al., 2011) for the induction of embryogenic cultures from mature olive tissues. The medium pH was adjusted to 5.8 and solidified with 6 g L⁻¹ Agar-Powder (VWR, Portugal) prior to autoclaving. The initial establishment was carried out on gelled medium to minimise contamination risks. For each culture medium, a total of 100 petioles were inoculated, distributed in 6 cm diameter Petri dishes, with five petioles placed per Petri dish. After 21 days of culture, the developed calli were divided into two groups: approximately 200 mg were transferred to 100 mL Erlenmeyer flasks containing 50 mL of liquid medium with the same composition and maintained under orbital agitation at 105 rpm, while the remaining calli were kept on the gelled medium to allow comparison between liquid and gelled culture systems. Subcultures were performed every four weeks using medium of the same composition.

VI. 3.3. Exploring the Applicability of CM as SE Modulator

For the evaluation of CM applicability, the HE line named A148, which consistently exhibited the highest number of somatic embryos across successive subcultures, was selected to collect the culture medium (corresponding to the CM) four weeks after subculturing. The CM was recovered from each of the four Erlenmeyer flasks into 50 mL tubes after four weeks of culture of the selected cell line and centrifuged at 10,000 $\times$ g for 10 min at 25 °C. The resulting supernatant was sequentially filtered through 0.45 μm and 0.22 μm pore-size filters using a vacuum filtration system (Millipore, Burlington, MA, USA), following the workflow outlined in Figure 1. After filtration, CM was applied to culture medium at different volumes (1.5 mL, 3 mL, 5 mL, and 10 mL) and to different explant types: i) to LE

embryogenic calli derived from zygotic explants subcultured to liquid culture medium with same composition (the line A23, which exhibited a reduced capacity for somatic embryo formation in 2.2.) and ii) to calli developed from adult plant tissue (petioles) subcultured in gelled and liquid culture induction medium. The application of CM in both LE embryogenic calli (liquid medium) and calli derived from petioles (gelled and liquid media) was performed by pipetting the corresponding volume across four subcultures, each lasting 30 days.

A control treatment condition (without CM application) was considered for each plant material and medium used (liquid/gelled). Four biological replicates (Erlenmeyer or Petri dishes) were considered for each plant material and CM volume combination. Cultures were kept at $25 \pm 1^\circ\text{C}$ in the dark, and control samples were maintained for the same number of subcultures as the CM-treated cultures, each lasting 30 days. Liquid cultures were maintained under orbital agitation at 105 rpm.

Calli of petioles achieved in gelled culture medium were further transferred to expression OMc and OMe medium devoid of plant growth regulators for two subcultures. In each subculture, both liquid and gelled cultures continued to be supplemented with the same volumes of CM as initially used. The cultures were maintained under the same growth conditions described above.

VI. 3.4. Histological Analysis

To study the morphological responses of olive petiole explants cultured under SE induction conditions, histological analyses were performed on explants grown in different culture medium and exposed to all CM treatments (0 mL, 1.5 mL, 3 mL, 5 mL, and 10 mL). Explants were collected at the end of the first subculture in the expression medium and fixed for 48 h in FAA solution (formaldehyde: acetic acid:70% ethanol, 1:1:8, v/v) (three explants per condition). Sample dehydration, paraffin infiltration, and embedding were performed according to the histological protocol previously described by Pires et al. (2025a). Sections of 5–7 μm thickness were obtained using a rotary microtome, and stained with Safranin O (0.01%, w/v) and Fast Green FCF (0.5%, w/v in 95% ethanol). After, the sections were subjected to two successive washes in xylene (2 min each), allowed to dry, and subsequently mounted using DPX mounting medium (Sigma-Aldrich, Madrid, Spain). Histological analyses were performed using an automated Leica DM6000B microscope (Wetzlar, Germany), and digital images were acquired with a Leica CC7000 camera (Wetzlar, Germany).

VI. 3.5. Statistical Analysis

Statistical analyses were performed using SPSS version 28.0. Normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene’s tests, respectively. Comparisons of mean

somatic embryo numbers per mg of calli in LE embryogenic lines subjected to different applications of CM derived from HE embryogenic lines were performed using one-way analysis of variance (ANOVA), or the nonparametric Kruskal–Wallis test when the assumptions of parametric analysis were not met. Data variability was expressed as the mean $\pm$ standard deviation, and statistical significance was set at $p < 0.05$.

VI. 4. RESULTS

VI. 4.1. Effects of Conditioned Medium on Low-efficiency Embryogenic Lines

To evaluate the effect of CM derived from HE lines as a modulator of embryogenic performance, a LE embryogenic line exhibiting the lowest somatic embryo formation was selected and cultured in the presence of different CM concentrations over four consecutive subcultures.

The results revealed a marked variation in the number of somatic embryos formed per mg of calli depending on the volume of CM applied (Table VI.1). Across all subcultures, control treatments without CM consistently showed the lowest values and did not differ significantly from cultures supplemented with 1.5 mL CM. In the first subculture, supplementation with 10 mL CM led to the highest number of somatic embryos, though statistically significant differences were observed only in comparison with the control and the 1.5 mL treatment. As subculturing progressed, somatic embryos formation generally increased, with the highest values being recorded in the fourth subculture. In the third subculture, no significant differences were detected between the 3 and 5 mL CM treatments. In the final subculture, CM volumes of 3, 5, and 10 mL resulted in a similarly high number of somatic embryos, with no significant differences among treatments. Notably, CM volumes equal to or higher than 3 mL were sufficient to induce the highest embryogenic response in the later subcultures.

Table VI.1. Number of somatic embryos per gram of calli in low-efficiency (LE) embryogenic lines cultured in ECO liquid medium supplemented with different volumes of conditioned medium (CM) over four subcultures. Different lowercase letters indicate statistically significant differences ($p \leq 0.05$). Data are presented as mean $\pm$ standard error.

Volume (ml)	Subcultures			
	First	Second	Thirth	Fourth
0	3.00 $\pm$ 1.00 a	4.33 $\pm$ 2.03a	3.33 $\pm$ 2.33 a	2.00 $\pm$ 0.58 a
1.5	6.67 $\pm$ 3.18a	7.67 $\pm$ 2.19 ac	9.00 $\pm$ 1.73 a	4.67 $\pm$ 1.86 a
3	11.67 $\pm$ 1.76 ab	14.67 $\pm$ 1.45 bc	13.67 $\pm$ 0.88 ab	24.33 $\pm$ 2.33 ab
5	14.0 $\pm$ 0.58 ab	18.33 $\pm$ 2.40 b	20.00 $\pm$ 2.52 b	32.00 $\pm$ 0.58 b
10	16.7 $\pm$ 1.67 b	20.33 $\pm$ 1.45 b	30.67 $\pm$ 2.96 c	37.33 $\pm$ 2.33 b

The morphological evaluation of LE embryogenic line subjected to different volumes of CM revealed clear differences in tissue organisation and embryogenic response among treatments (Figure VI.2). In the control and in cultures supplemented with 1.5 mL of CM, calli displayed a predominantly compact structure, with limited tissue fragmentation, frequent necrotic areas, and low embryogenic activity. In contrast, higher CM volumes induced marked morphological changes, with calli becoming increasingly fragmented and nodular, a structural pattern typically associated with active proliferation of embryogenic tissue. These treatments exhibited a substantially higher number of somatic embryos, frequently organised in embryogenic masses, which hindered the clear discrimination of individual developmental stages. Nevertheless, embryos at globular and torpedo stages could be identified, particularly in cultures supplemented with the highest (5 and 10 ml) CM volumes.

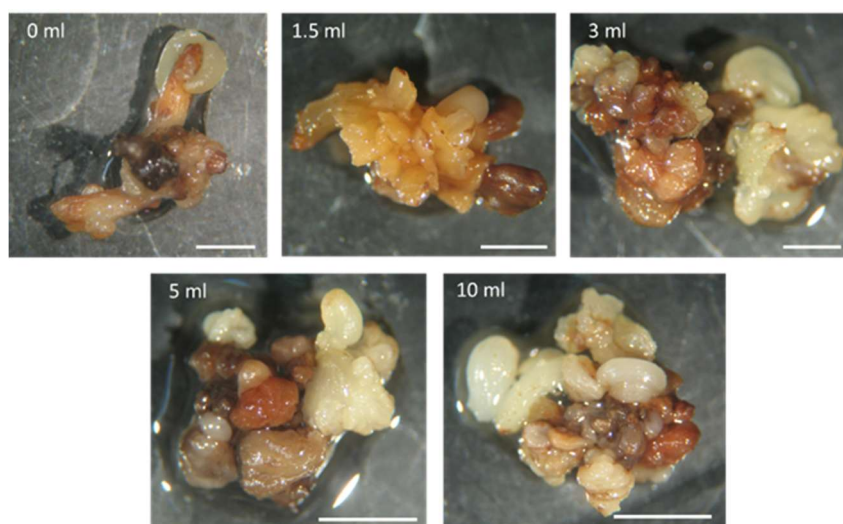


Figure VI.2. Embryogenic calli from A23 low-efficiency (LE) line cultured in ECO liquid medium under control conditions and supplemented with different volumes of high-embryogenic (HE) embryogenic line conditioned medium (CM) after 4 subcultures. Bars: 0.5cm.

Additionally, higher CM volumes induced a progressive shift in tissue appearance, with calli transitioning from compact milky masses to translucent structures, in line with the greater abundance of somatic embryos

VI. 4.2. Callus Induction from Adult Petiole Explants

After 30 days in gelled induction culture medium, callogenesis and necrosis rates were recorded for petioles cultured in OMc and OMe. Significant differences were observed between the two culture media for both evaluated parameters (Figure VI.3).

High callogenesis rates were obtained in both culture medium, however, petioles cultured in OMe exhibited a significantly higher callogenesis rate (95.5 ± 2.6) when compared with those on OMc (79.3 ± 5.4). Conversely, necrosis rates were significantly lower in OMe (6.1 ± 3.0) than in OMc (19.0 ± 5.2) (Figure VI.3).

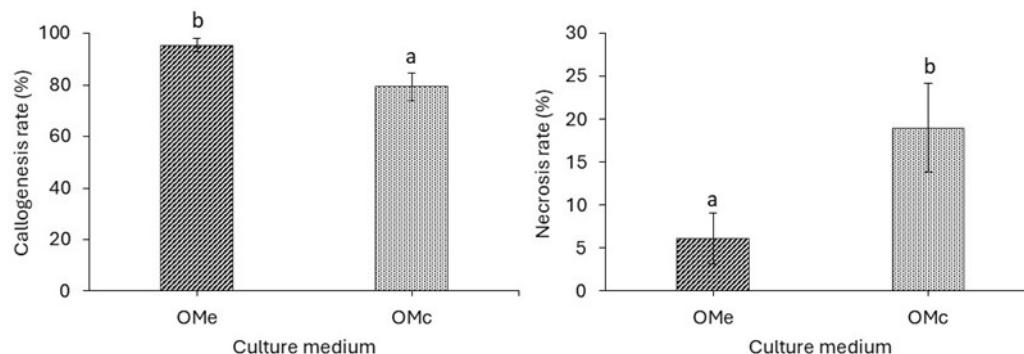


Figure VI.3. Callogenesis and necrosis rates (%) of petioles after 30 days in OMe and OMc medium. Different letters indicate statistically significant differences ($p \leq 0.05$) between lines. Data are presented as the mean $\pm$ standard error.

Regarding the morphology of the formed calli, petioles cultured in OMe medium produced calli with a whitish colour, exhibiting a more compact structure in certain regions and friable areas. Notably, nodular regions were observed, protruding from the surrounding tissue (Figure VI.4). In comparison, calli developed in OMc medium showed reduced growth, a more compact structure, and a yellowish colouration, with several explants exhibiting brownish tissue in oxidised areas. Remarkably, calli developed exclusively at the cut edges of the petioles, specifically at the regions connecting the petiole to the stem and leaf.

VI. 4.3. Effect of Conditioned Medium and Culture System on Callus Proliferation

To evaluate the effect of CM on calli developed from adult plant tissues, petiole-derived calli were subcultured to induction culture medium with the same mineral and hormonal composition, either in liquid or gelled form, supplemented with different CM concentrations, and compared with a control treatment devoid of CM application. CM supplementation in the gelled induction medium did not result in clear improvements in calli proliferation relative to the control treatment. Across the different CM concentrations tested, calli generally exhibited reduced proliferation and a lower degree of granular organisation, regardless of the CM volume applied. Morphological variation was nevertheless observed among treatments. Calli ranged in colour from light cream to darker brownish tones, with some explants displaying localised areas of oxidation or tissue necrosis. In fact, higher CM concentrations tended to

show darker calli tissues and more frequent oxidation. In addition, the formation of nodular or granular structures appeared limited and less clearly defined in the control and at 1.5 mL CM (Figure VI.4). Overall, the different CM concentrations did not promote a clear enhancement of calli differentiation under gelled induction conditions (Figure VI.4).

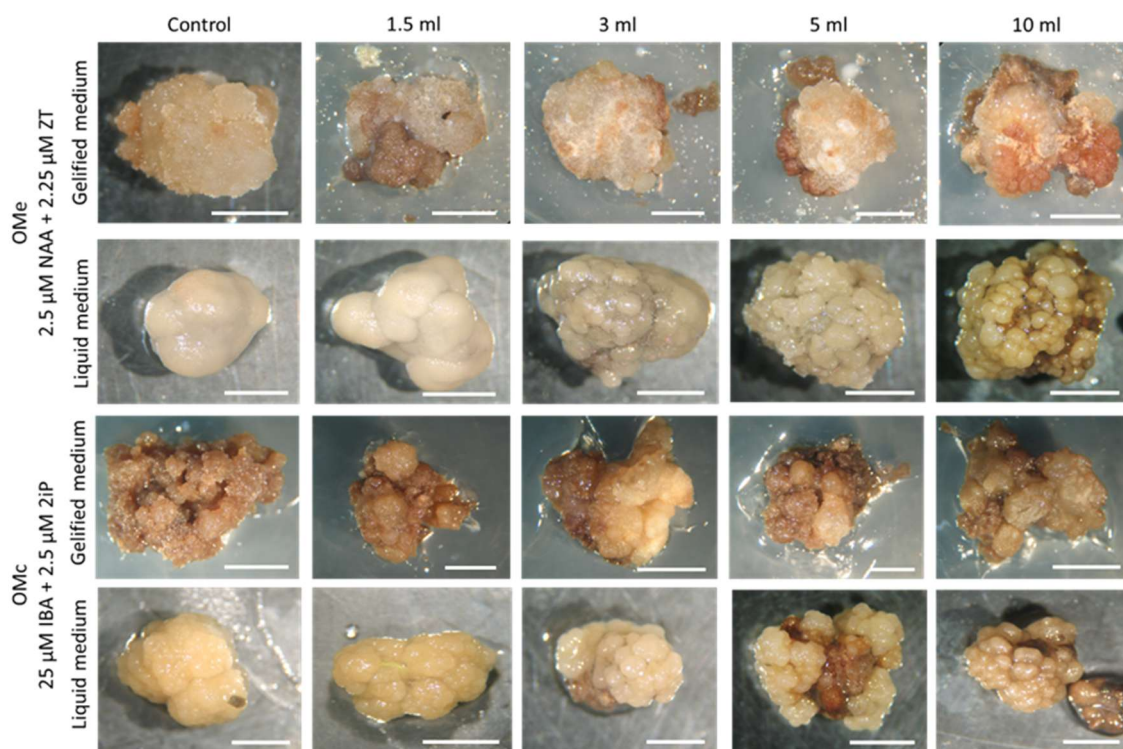


Figure VI.4. Morphology of petioles-derived calli after three subcultures of 30 days each in gelled and liquid induction medium supplemented with different concentrations of conditioned medium (CM). Abbreviations: OMe, modified olive medium OM for somatic embryogenesis SE; OMc, modified olive medium. Bars: 0.5 cm.

Calli cultured in liquid induction medium supplemented with CM displayed progressive morphological changes depending on the concentration applied (Figure VI.4). In the control treatment (0 mL CM), calli exhibited a compact and relatively smooth surface, with a homogeneous appearance and limited visible granulation. A comparable morphology was observed following the application of 1.5 mL CM; however, slight modifications in surface organisation became evident, including the emergence of discrete protuberances and a slightly more irregular texture. At intermediate concentrations (3 mL and 5 mL CM), these protrusions became more pronounced, forming nodular structures that gave the calli a markedly granular appearance. In some explants, the tissue appeared more heterogeneous, with localised compact regions interspersed with more friable areas. At the highest concentration tested (10 mL CM), calli exhibited an increasingly nodular surface organisation, characterised by the presence of numerous protruding structures that contributed to a more granular and

aggregated appearance. Regarding colour, calli cultured in OMe medium exhibited a light, cream-colored appearance with uniform texture. In contrast, calli maintained in OMc medium appeared more yellowish and frequently presented localised areas of tissue necrosis.

VI. 4.4. Calli Response during the Expression Phase

Following the induction phase, the calli were transferred to expression medium, devoid of plant growth regulators, to evaluate their developmental response under both liquid and gelled culture conditions, while maintaining CM supplementation at the same concentrations.

After two subcultures in expression medium, the calli exhibited an increased incidence of tissue necrosis, as shown in Figure 5. This response was particularly evident in explants maintained on gelled culture medium, where several calli displayed darker brown colouration and localised necrotic areas (Figure VI.5). In liquid culture conditions, although tissue necrosis was also observed, calli generally retained a lighter colouration and exhibited the formation of numerous granular structures, particularly in the 3, 5, and 10 mL CM treatments. The explants not supplemented with CM (control condition) and those treated with 1.5 mL of CM, did not show the formation of granular structures and exhibited highly necrotic tissue, which led to the loss of a considerable number of explants during this phase. Despite these differences in tissue viability, no clear morphological differences were observed between the two mineral compositions of the culture medium tested. Calli in both culture media (gelled and liquid) developed similar structural features, characterised by compact masses of proliferating cells and the presence of nodular or globular-like structures on their surface, when growing in higher volumes of CM (Figure VI.5).

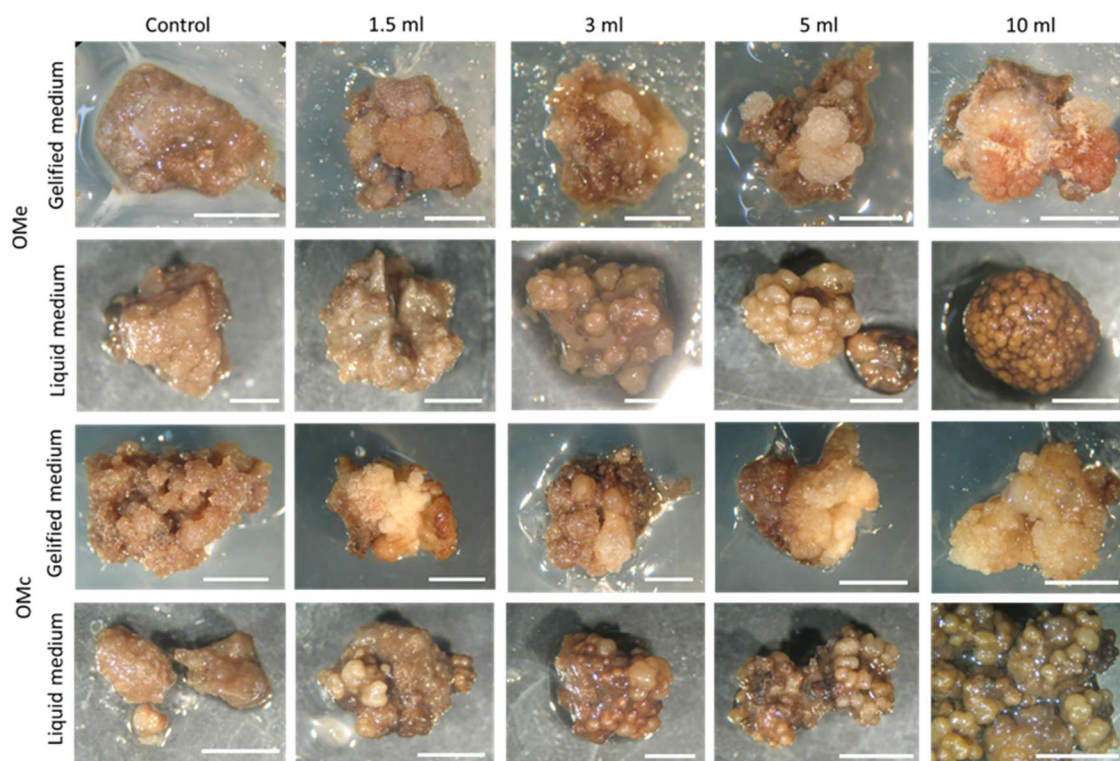


Figure VI.5. Representative morphology of calli obtained from adult petiole explants after two subcultures of 30 days each in expression medium devoid of plant growth regulators and supplemented with conditioned medium (CM). Cultures were maintained under liquid and gelled conditions using the same CM concentrations applied during the induction phase. Calli display compact structures with prominent nodular or globular-like formations on the surface, with colouration ranging from cream to brownish tones and occasional localised necrosis. Abbreviations: OMe, modified olive medium (OM) for somatic embryogenesis (SE); OMc, modified olive medium. Bars: 0.5 cm.

As illustrated in Figure VI.5, several calli displayed well-defined, rounded nodular structures distributed across the callus surface, forming compact aggregates with a granular appearance. These structures varied slightly in size and colouration, ranging from light cream to brownish tones, and in some cases were organised into clusters, resembling early globular formations. Although these structures were the most prominent morphogenic features observed during the expression phase, the development of clearly differentiated somatic embryos was not detected at this stage.

Based on these observations, the calli obtained under these conditions were subsequently collected and processed for histological analysis to characterise their cellular organisation further and to determine the presence of potential meristematic regions or embryogenic structures.

VI. 4.5. Histological Analysis of Calli Developed on Medium Supplemented with CM

Histological observations were undertaken to characterise the cellular organisation of explants cultured under control conditions in both induction culture medium. In agreement with the external morphological assessment, no organised embryogenic or meristematic structures were identified in the control treatments maintained on gelled OMc (Figure VI.6A) and OMe (Figure VI.6B) culture medium. Also, localised areas of tissue degeneration were detected, characterised by disruption of cellular integrity and irregular staining patterns. These degenerative features were more evident in explants cultured in gelled OMc medium (Figure VI.6A). The sections were predominantly composed of large, highly vacuolated parenchymatous cells exhibiting expanded central vacuoles, thin cytoplasmic layers, and inconspicuous nuclei. The reduced nucleus-to-cytoplasm ratio and the limited staining intensity were consistent with a differentiated, non-meristematic cellular condition. Cellular arrangement lacked clear spatial organisation, and neither a continuous protoderm-like layer nor defined structural domains were observed.

By contrast, sections of calli derived from the OMe (Figure VI.6C) and OMc (Figure VI.6D) liquid medium, still lacking the application of CM, displayed regions in which nuclei were more readily discernible, and cells appeared slightly more densely arranged, although without evidence of coordinated structural reorganisation.

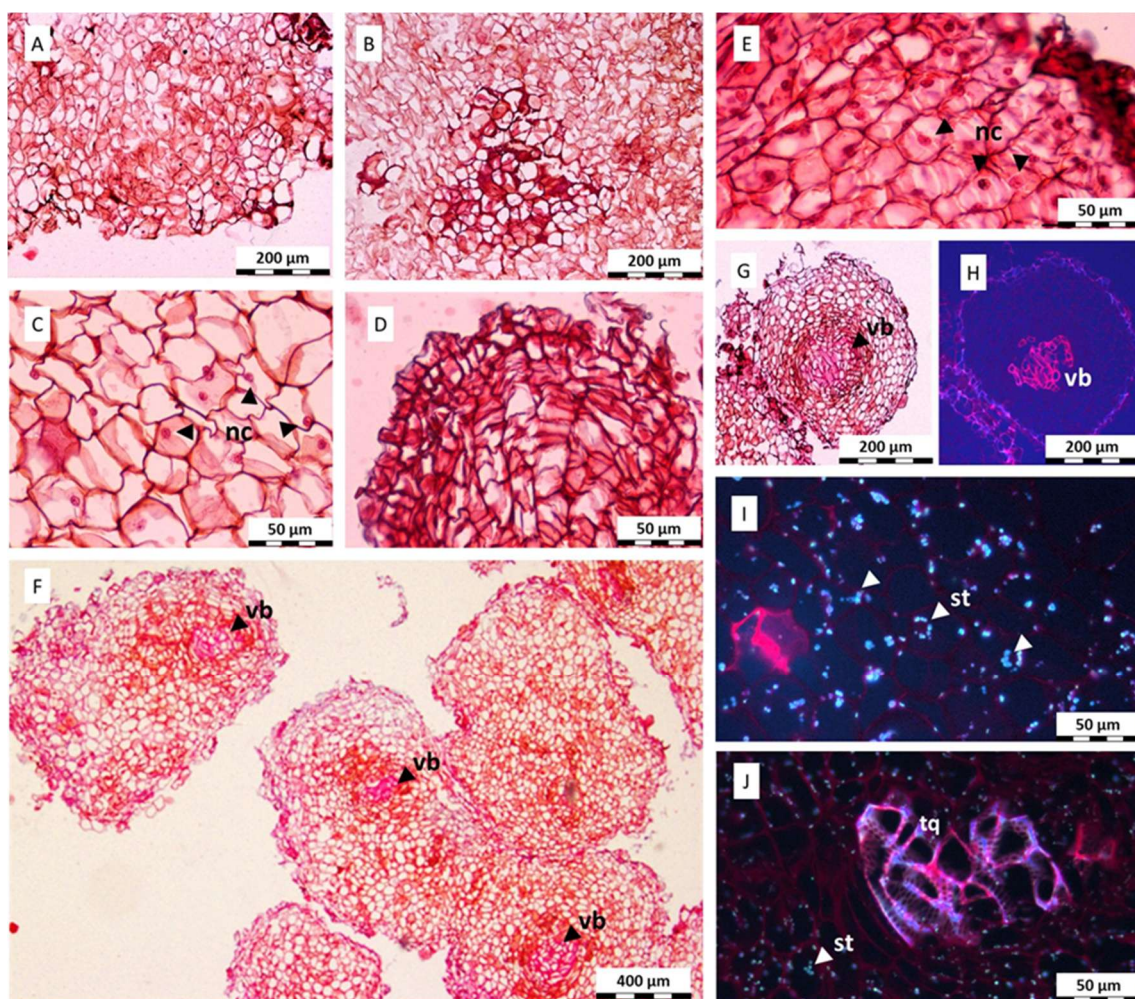


Figure VI.6. Histological observations of petioles after 4 weeks in expression culture medium. (A) Control in OMc gelled medium; (B) Control in gelled OMe medium; (C) Control OMe in liquid medium; (D) Control OMc in liquid medium; (E) OMe in liquid medium supplemented with 1.5 mL CM; (F) Nodules after 5 ml CM application in OMe medium; (G and H) OMe medium with 10 ml CM showing concentric xylem and phloem arrangement; (I) showing abundant starch deposits and (E–F) differentiated tracheids (tq) with lignified walls highlighted by fluorescence (J). Abbreviations: OMe: modified olive medium (OM) for somatic embryogenesis (SE); OMc: modified olive medium; nc, nucleus; st, Starch grains; tq, tracheid; vb, vascular bundle.

Within the calli masses, samples supplemented with 1.5 mL CM in OMe liquid medium (Figure VI.6E) exhibited a subtle gradient in cellular morphology. Cells located in the inner regions were somewhat smaller and more closely packed than those at the periphery, where cellular enlargement and extensive vacuolisation predominated. Nevertheless, even within these relatively compact regions, no distinct meristematic layer or organised developmental pattern could be identified.

Histological analysis of explants treated with CM in liquid culture medium revealed clear structural differences compared with untreated tissues. Sections from treatments containing 3 and 5 mL CM showed more densely arranged cells with prominent, clearly defined nuclei under light microscopy, as well as localised regions of increased cellular organisation (Figure VI.6F). Meanwhile, explants exposed to 10 mL CM exhibited a markedly more structured tissue organisation (Figure VI.6G–J). In these samples, nodular structures, likely corresponding to localised meristematic regions within the callus and resembling meristematic nodules, suggest the establishment of discrete and spatially organised developmental domains. Well-defined vascular bundles were detected, displaying a concentric organization in which the inner region exhibited xylematic tissue surrounded by outer layers of phloem tissue. Within the xylem tissues, differentiated tracheids were clearly identifiable, with cells showing elongated morphology, thickened lignified cell walls, intense pink staining under light microscopy, and lighter luminal regions – features that were particularly evident under fluorescence microscopy (Figure VI.6H and J). The outer zones were occupied by cortex-like cells exhibiting abundant starch deposits (Figure VI.6I), indicating enhanced metabolic activity. A thin layer of epidermis-like cells (Figure VI.6F–J) was observed surrounding the nodular structures (Figure VI.6G, H).

Although enhanced cellular organisation and vascular differentiation were observed, no evidence of organised shoot or root primordia was detected under any treatment.

VI. 5. DISCUSSION

The use of CM as a source of extracellular signalling molecules has been investigated for its ability to support cell growth and morphogenesis. It contains a complex mixture of biologically active compounds that together may constitute an embryogenic signature associated with stress control and the maintenance of highly proliferative embryogenic lines (Pires et al., 2025b). Several of these molecules released into the culture medium have been identified in *Curcubita pepo* (Ben Amar et al., 2010), *P. nigra* (Pernis et al., 2023), and *Sorghum bicolor* (Ngwenya et al., 2024) as embryo-promoting factors that influence the cellular environment and regulate embryogenic competence (Ben-Amar et al., 2021). Among these components, extracellular factors appear to play a particularly important role in cell signalling, cell nursing, and the maintenance of embryogenic potential (Quintana-Escobar et al., 2025). These molecules may contribute to the establishment of a favourable microenvironment that supports proembryonic masses transferred to liquid systems (Komai et al. 2006; Pires et al. 2025b). Evidence for such effects has been reported in several plant species, including *Saccharum* spp. (Oropeza et al., 2001), *Cichorium* spp. (Couillerot et al., 2012), and *P. nigra* (Pernis et al., 2023). Moreover, the application of extracellular proteins derived from highly embryogenic lines has previously been shown to enhance the embryogenic competence of low-embryogenic lines, as demonstrated in *C. pepo* (Ben-Amar et al., 2010). Consistent with these observations, supplementing LE lines with CM derived from HE lines in

the present study, across four subcultures, promoted increased proliferation and improved somatic embryo formation. These results support the hypothesis that extracellular factors secreted by embryogenic tissues into the culture medium play a key role in modulating SE response.

Considering the results obtained in the previous experiment and considering the nurse culture theory, CM derived from HE embryogenic lines – originally obtained through the induction of zygotic embryos in cyclic culture medium – were subsequently applied to adult explants to overcome the recalcitrance typically associated with these tissues. Marked differences in explant morphology were observed between cultures established in liquid medium and those maintained on gelled medium, suggesting the culture environment and the medium's hormonal composition may influence adult tissues' responses to the extracellular factors present in CM (Franco & Ferraz, 2024). In the control treatments, where CM was not applied, clear differences in calli morphology were observed between the two culture systems. According to Ascough & Fennell (2004), explants cultured on gelled medium tend to exhibit a degree of polarity in their responses, as only tissues in direct contact with the medium have immediate access to nutrients and regulatory signals. Cells located further from the medium must rely on diffusion through neighbouring tissues, which may lead to differential signal perception across the explant surface. Consequently, peripheral cells may receive different developmental cues compared with those directly exposed to the culture medium (Vis et al., 2020). In contrast, in liquid cultures, the explant is surrounded by the medium, allowing all cells to absorb nutrients directly and plant growth regulators (Nirmal et al., 2023). Regarding CM concentrations, morphological changes were observed, with higher concentrations promoting the differentiation of granular-like structures in the calli. However, this effect was not evident in the gelled medium, where CM was applied directly onto the surface of the explants. By the end of the subculture period, the gelled medium had absorbed the CM unequally, which may have affected its distribution and availability.

In the expression phase, the calli were transferred to culture medium with the same mineral composition devoid of plant growth regulators, while maintaining CM supplementation at the same concentrations. Although plant growth regulators are essential for inducing somatic embryos, woody species often require a subsequent phase with reduced or no growth regulators to allow the expression of cellular totipotency (Pais, 2019). In the present work, calli structure was preserved after transfer to the expression medium, and higher CM concentrations promoted the formation of numerous globular-like structures. Nevertheless, an increase in tissue necrosis was observed in the explants; despite this, the continued application of CM appeared to help maintain overall calli viability. Histological observations indicated that these globular-like structures likely correspond to meristematic nodules within the calli. These findings indicate that not only the application of CM, but also its use under liquid culture conditions, may contribute to the formation of meristematic regions within the explants. Liquid culture systems are known to enhance cellular proliferation and the establishment of organised

morphogenic centres (Etienne & Berthouly, 2002). This observation is consistent with the results obtained in the present study. Although a certain degree of tissue organisation was observed at higher CM concentrations in the gelled medium, histological analysis did not confirm the presence of well-defined meristematic nodules.

Meristematic nodules are specialised structures composed of organised cellular domains, typically including a central organisation centre surrounded by cortex-like parenchymatous cells and an epidermis-like outer layer (Fan et al., 2024). Although meristematic nodules may resemble globular embryos during SE in their external appearance, they can be clearly distinguished through histological analysis (Xu et al., 2022). In this work, histological analysis of adult tissues revealed the formation of nodular structures with defined cellular organisation, consistent with meristematic nodules or pro-embryogenic masses described in SE systems. Such structures have been associated with the acquisition of embryogenic competence, typically characterised by organised cellular domains (Bogdanović & Čuković, 2025) and the presence of starch granules (Steinmacher et al., 2011; Espinoza et al., 2017; Pires et al., 2025a), indicative of high metabolic activity. Additionally, the presence of an epidermis-like outer layer agrees with previous reports of early morphogenic structures delimited by peripheral cell layers, reflecting the onset of tissue organisation and polarity during embryo initiation (Espinoza et al., 2017). These structures exhibit high regenerative potential, genetic stability, and sustained cellular activity (Bansal et al., 2023). Due to these properties, they are considered highly suitable for plant regeneration through organogenesis and have also been explored for applications such as *in vitro* phytochemical production and plant genetic transformation (Pandey et al., 2025). The identification of similar structural features in calli tissues may therefore indicate regions with enhanced morphogenic potential. In fact, meristematic nodules have been reported to be successful for *in vitro* regeneration in several plant species, including herbaceous species like *Xanthosoma sagittifolium* (Bansal et al., 2023), and *Ocimum basilicum* (Montone et al., 2025) and woody plant species like *Populus euphratica* (Ferreira et al., 2009), and *Paeonia ostii* (Fan et al., 2024).

However, the regeneration patterns associated with meristematic nodule formation are strongly influenced by genotype and by the type and concentration of plant growth regulators (Trindade & Pais, 2003). In the present study, both growth regulator combinations tested in the culture medium were able to induce callogenesis from petiole explants; however, the OMe medium proved to be more favourable for the development of morphogenic tissues. The OMc medium, supplemented with IBA and 2iP, has shown good results when the initial explants are zygotic embryos (Orinos & Mitrakos, 1991), as reported in several recent protocols for olive SE (Mazri et al., 2020; Pires et al., 2020; Pires et al., 2025a). Nevertheless, when applied to petiole explants, this medium resulted in higher levels of tissue necrosis and less meristematic nodules formation. By contrast, the medium supplemented with NAA and zeatin promoted higher callogenesis rates and reduced levels of tissue necrosis. Under these conditions, the

calli displayed structural features that were more compatible with the establishment of morphogenic regions. The results obtained suggest that the formation of meristematic areas may have been favoured not only by the application of 10 mL of CM but also by the composition of the OMe culture medium. In the expression phase, the absence of plant growth regulators, particularly auxins, may have inhibited the morphogenic process, as these compounds play an essential role in apical meristem formation and in the downstream transcriptional regulation of genes involved in cell wall remodelling and morphogenetic events (Traas, 2018). In addition, cytokinins such as 6-benzylaminopurine (BAP) and zeatin have been reported to promote the direct regeneration of meristematic nodules in several plant species. For example, BAP has been shown to stimulate meristematic nodule formation in *Musa* spp. (Sipen & Davey, 2012) and *Portulaca oleracea* (Shekhawat et al., 2015), whereas zeatin and TDZ have been associated with this process in *Diospyros kaki* (Yokoyama et al., 2011) and *P. ostii* (Xu et al., 2022). In addition, the removal of these regulators may have contributed to the increased incidence of tissue necrosis, suggesting that the explants experienced physiological stress under regulator-free conditions.

VI. 6. CONCLUSIONS

The present study shows that CM derived from HE lines established from zygotic embryos can effectively modulate the developmental pathways of LE embryogenic lines, enhancing their morphogenic response across successive subcultures and CM concentrations. These results support a central role for extracellular factors released by embryogenic tissues in regulating cell proliferation and differentiation during SE. In adult explants of olive cv. ‘Galega Vulgar’, higher CM volumes promoted the formation of meristematic nodules, particularly under liquid culture conditions, highlighting the culture system as a critical determinant of morphogenic outcomes, likely through improved exposure of tissues to signalling molecules and nutrients. Although fully developed somatic embryos or organogenic structures were not obtained within the timeframe of this study, the induction of meristematic nodules represents a major step towards overcoming the recalcitrance of adult tissues.

To the authors’ knowledge, this is the first report demonstrating that CM application modulates morphogenic responses in *O. europaea*, including the induction of meristematic nodules, representing an important advance for SE and *in vitro* regeneration systems in this species. The generation of such structures, particularly in liquid systems, provides a valuable framework to investigate morphogenic processes regulated by the physical state of the culture medium and may offer an alternative pathway for *in vitro* plant regeneration, complementary to both classical SE and conventional micropropagation. Overall, the findings indicate that CM-based strategies have the potential to alleviate recalcitrance in otherwise non-responsive tissues and lay a solid foundation for optimising SE protocols and improving regeneration efficiency in recalcitrant olive cultivars, especially from adult explants, thereby contributing to advances in clonal propagation and biotechnological applications in olive.

VI. 7. REFERENCES

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CHAPTER VII

Comparative analysis of SP3 and S-Trap sample preparation protocols for proteomic profiling associated with somatic embryogenesis efficiency in *Olea europaea* L.

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VII. 1. ABSTRACT

Somatic embryogenesis (SE) is an asexual plant propagation technique in which embryo-like structures are generated from somatic cells. It is a powerful tool for clonal propagation, genetic transformation, and germplasm conservation, but not all species respond efficiently to SE, limiting its application. To overcome this recalcitrance, recent studies have investigated the physiological, molecular, and cytological processes associated with SE in different plant species. Proteomics has emerged as a high-throughput approach to investigate global protein dynamics, and it has been increasingly applied to characterise SE-related molecular mechanisms. However, even using these large-scale proteomic strategies, the impact of sample preparation on data quality has never been systematically demonstrated. Here, we compared, for the first time, two sample preparation strategies for the analysis of SE cultures by liquid chromatography–tandem orbitrap mass spectrometry (LC–MS/MS), including i) single-pot solid-phase-enhanced sample preparation (SP3), performed on an OT-2 automated platform, and ii) the Suspension Trapping (S-Trap). For this purpose, we analysed the proteome of *Olea europaea* embryogenic lines with high and low efficiencies, maintained through secondary SE. More than 9,000 protein groups were identified in each method, most of which (7,735 proteins) were shared between workflows, while only a small proportion were uniquely detected by either workflow. Protein quantification was also highly consistent, with 8,454 and 8,365 protein groups quantified for SP3 and S-Trap, respectively, and 7,075 in common. Both workflows achieved comprehensive proteome coverage, with SP3 capturing additional functional categories, such as mitochondrial components, membrane-associated proteins, and vesicle trafficking. S-Trap provided complementary but comparatively more restricted coverage, while both approaches consistently captured key biological processes related to metabolism and intracellular organisation. Overall, the results demonstrate that SP3 and S-Trap workflows provide highly consistent and complementary proteomic coverage, supporting their combined or alternative use for comprehensive proteomic studies of SE.

VII. 2. INTRODUCTION

Somatic embryogenesis (SE) is a developmental process in which differentiated somatic cells are reprogrammed to form complete plants without gamete fusion, reflecting the intrinsic totipotency of plant cells (Horstman et al., 2017). Through this pathway, somatic cells give rise to bipolar embryonic structures that can develop into whole plants, making SE one of the most distinctive regeneration mechanisms in plant biology. The induction of SE is governed by a complex interplay of endogenous and exogenous factors that promote extensive cellular reprogramming. At the cellular level, explant tissues respond to internal signalling cues that trigger modifications in gene expression and developmental fate (Méndez-Hernández et al., 2019). These responses are frequently stimulated by external factors, particularly the application of plant growth regulators and exposure to stress conditions

such as temperature extremes, osmotic shock, drought or heavy metal stress (Nic-Can et al., 2016). SE has been described as a morphogenic response to stress conditions (Arnholdt-Schmitt et al., 2016). Collectively, these stimuli activate intricate signalling networks that regulate morphological, physiological and biochemical changes during SE induction (Gulzar et al., 2020; Ding et al., 2024). Although SE represents one of the most extensively studied models of plant regeneration and a powerful tool in plant biotechnology, its underlying cellular mechanisms remain only partially understood. This limitation is particularly evident in woody perennial species such as *Olea europaea*, where SE research has been constrained by biological recalcitrance. To date, most studies have relied on juvenile tissues, especially zygotic embryos, as initial explants, since the induction of SE from adult material remains highly challenging (Mazri et al., 2020; Pires et al., 2020; Titouh et al., 2022).

Proteomic research has played a pivotal role in advancing the understanding of SE, with most studies focusing on tissue-level protein changes by comparing explants or cell lines displaying contrasting embryogenic potentials (Aguilar-Hernández & Loyola-Vargas, 2018; Quintana-Escobar et al., 2023). Early proteomic investigations into SE primarily relied on conventional gel-based techniques, including sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Griga et al., 2007; Mishra et al., 2012), two-dimensional electrophoresis (2-DE) (Lippert et al., 2005; Marsoni et al., 2008), and two-dimensional differential gel electrophoresis (2D-DIGE) (Guzmán-García et al., 2013). These approaches laid the foundation for understanding protein dynamics associated with embryogenic competence and developmental transitions (Zubair et al., 2022). Even with the emergence of advanced mass spectrometry-based platforms, gel-based methods remain relevant in SE proteomics, particularly for routine analyses and high-resolution profiling of proteoforms when combined with sensitive detection systems, advanced image analysis tools, and optimised sample preparation protocols (Santos et al., 2019).

In recent years, gel-free proteomic approaches such as liquid chromatography–tandem mass spectrometry (LC-MS/MS) and capillary electrophoresis–mass spectrometry (CE-MS) have become increasingly adopted (Birhanu, 2023). These mass spectrometry–based techniques bypass gel electrophoresis and enable the direct analysis of complex protein mixtures, offering high sensitivity and resolution and facilitating the detection of low-abundance proteins. LC-MS/MS is particularly distinguished for its high sensitivity in detecting and quantifying peptides and proteins, including those present at low abundance (Wang et al., 2008). This sensitivity supports the comprehensive profiling of proteins in SE tissues, as demonstrated in species such as *Elaeis guineensis* (Aroonluk et al., 2018), *Catharanthus roseus* (Gulzar et al., 2019), *Coffea canephora* (Quintana-Escobar et al., 2023), and *Larix olgensis* (Hou et al., 2023). Moreover, this technology allows the detection and analysis of proteins secreted into the culture medium, providing insights into extracellular protein dynamics and their potential roles in embryogenic competence in *Pinus nigra* (Pernis et al., 2023) and *O. europaea* (Pires

et al., 2025a). Despite the extensive adoption of proteomic approaches in SE research, methodological aspects of protein preparation and mass spectrometric acquisition remain underexplored, particularly in woody and recalcitrant species.

Sample preparation represents a critical determinant of proteomic DAP and data quality. In most cases, LC-MS/MS have been combined with conventional sample preparation workflows, including in-solution digestion, gel-based approaches, or filter-aided sample preparation (FASP) (Duong & Lee, 2023). These strategies have enabled the identification of substantial numbers of proteins in plant systems, such as 2,409 proteins in *Quercus ilex* seed cotyledons using a GeLC-MS/MS approach (Sghaier-Hammami, 2021), and 901 proteins in *P. radiata* seedlings using LC-MS/MS (Escandón et al., 2017). More recently, alternative sample preparation methods have been developed to improve protein recovery, digestion efficiency, and reproducibility, including SP3 and S-Trap protocols. Despite their increasing use in proteomics, their application in SE remains relatively limited. One of the earliest SE-related proteomic studies analysed embryogenic cell suspensions of *C. arabica*, identifying 1,052 proteins and reporting several stress-related enzymes, including peroxidases, ascorbate peroxidase, phosphoglycerate dehydrogenase, cold-shock proteins, and aldolases (Campos et al., 2016). In *E. guineensis* spp. (Ribeiro et al., 2019), approximately 4,600 proteins and more than 10,000 peptide sequences were quantified during embryogenic callus induction, including antioxidant-related proteins as well as cytoskeletal and cell wall-associated proteins involved in cell division and differentiation. More recently, in *C. canephora*, Quintana-Escobar et al. (2023) quantified 1,630 proteins during SE induction. However, these studies have typically relied on a single sample preparation strategy, preventing a systematic evaluation of how different workflows influence proteomic coverage and data quality in SE studies.

Recently, the Suspension-Trapping (S-Trap) method has gained increasing attention as an efficient and reproducible approach for protein digestion, particularly in samples enriched with contaminants (Wang et al., 2023). S-Trap workflow represents a recent advance in proteomics sample preparation, overcoming major limitations of classic approaches such as in-solution or gel-based digestion (Ludwig et al., 2018), particularly their sensitivity to detergents, incomplete digestion, and peptide losses (Wojtkiewicz et al., 2021). By enabling rapid detergent removal, efficient proteolysis, and high peptide recovery, S-Trap protocols improved proteome coverage and reproducibility and are compatible with partial automation (Wang et al., 2023). Recently, S-Trap was applied to the analysis of the extracellular proteome secreted by embryogenic cultures of *O. europaea* (Pires et al., 2025b), allowing the identification of 1,096 peptides corresponding to 526 protein groups. Notably, this dataset included proteins commonly associated with SE, such as kinases and peroxidases, supporting the suitability of this workflow for SE-related proteomic studies.

Another widely used strategy for proteomic sample preparation is the single-pot, solid-phase-enhanced sample-preparation (SP3) method (Hughes et al., 2019). This approach relies on the binding of proteins and peptides to carboxylate-coated paramagnetic beads under high concentrations of organic solvents, enabling efficient protein capture, clean-up and enzymatic digestion within the same workflow (Ciordia et al., 2024). Due to its simplicity, robustness and compatibility with high-throughput processing, SP3 has gained increasing attention in recent years. Moreover, the method is well-suited for automation using liquid-handling platforms, which improves reproducibility while reducing manual handling and processing time (Müller et al., 2020). Automated SP3-based workflows have been implemented on several robotic systems, including the Opentrons® OT-2 platform (Liu et al., 2021), facilitating standardised and scalable proteomic sample preparation. Recently, automated SP3 protocols implemented on the OT-2 platform have also been successfully applied to different biological matrices (Ciordia et al., 2024), including plant tissues such as *Arabidopsis thaliana*, demonstrating their suitability for proteomic analyses across diverse sample types.

In this context, the present study evaluates the performance of two complementary protein digestion strategies in proteome analyses of embryogenic tissues of *O. europaea* by LC–MS/MS analysis, including the SP3 implemented on an automated Opentrons® liquid-handling platform, and the S-Trap. Both SP3 (Ciordia et al., 2024) and S-trap (Ciordia et al., 2021) protocols used in this study were originally optimised for animal tissues and fluids. Furthermore, although both methods have previously been applied to plant tissues and cells (Ancín et al., 2024; Ciordia et al., 2024; Pires et al., 2025b), their performance has not been systematically compared, particularly in recalcitrant species such as *O. europaea*. Protein yield, identification efficiency, reproducibility and the functional representation of SE-related protein categories were assessed using three high-efficiency (HE) and three low-efficiency (LE) embryogenic lines obtained through cyclic SE. Overall, the results provide valuable insights into the importance of sample preparation workflows combined with high-resolution mass spectrometry, which can enhance proteome coverage and data quality, supporting their application in proteomic studies of SE in olive.

VII. 3. MATERIALS AND METHODS

VII. 3.1. Plant Material

Embryogenic cultures previously established using zygotic embryos of *O. europaea* cv. 'Arbequina' (for details on SE protocol establishment, see Pires et al., 2020) was used for the selection of embryogenic lines with different efficiency to differentiate somatic embryos (for methodology used on embryogenic lines see Pires et al. (Pires et al., 2025a). Three HE (A55, A140, A148) and three LE (A23, A157, A158) embryogenic lines were selected (Figure C.1) based on parameters described by

Pires et al. (2025a) and maintained through cyclic SE in liquid embryogenesis cyclic olive (ECO) culture medium. Each embryogenic line was propagated independently in liquid culture, and for each line, six independent Erlenmeyer flasks were established. After three subcultures of 30 days each, the embryogenic calli were collected from each Erlenmeyer and subsequently ground and homogenised in liquid nitrogen. Prior to downstream analysis, the six replicates of each line were pooled in a single sample to obtain a representative sample of each embryogenic line. Plant material was stored at -80°C until further analysis.

VII. 3.2. Analysis of the Proteome by LC-MS/MS

VII. 3.2.1. Protein digestion and tryptic peptides purification

Sample preparation and subsequent analysis were performed using LC-MS/MS analysis at the Proteomics Unit of the National Centre for Biotechnology (Madrid, Spain). Plant material was lysed with a buffer containing 5% sodium dodecyl sulfate (SDS), 25 mM triethylammonium bicarbonate (TEAB), 5 mM tris(2-carboxyethyl) phosphine (TCEP), and 10 mM chloroacetamide (CAA). Protein cysteine residues were reduced and alkylated at 60°C for 30 min, followed by sonication using a UP50H ultrasonic processor (Hielscher Ultrasonics) for 1 minute on ice (0.5 cycles, 100% amplitude). Samples were centrifuged at $18,400 \times g$ for 10 min. Total protein was measured using the Pierce 660 nm colourimetric assay (Thermo Scientific) supplemented with ionic Detergent Compatibility Reagent (Thermo Scientific).

Protein digestion was performed using SP3 and the S-Trap method according to previously established protocols (Ciordia et al., 2021, 2024). For SP3-based protein digestion, 50 μg of the sample type was automatically processed on the OT-2 platform, and the digestion was performed at 37°C for 16 h in a Jitterbug 4-plate incubated microplate shaker (Boekel Scientific). For S-trap digestion, 20 μg of sample were subjected to an overnight digestion process at 37°C on a S-Trap column (Protifi, Huntington, NY, USA) using 1 μg of trypsin (Ciordia et al., 2021). Following digestion, tryptic peptide-containing supernatants, obtained from both digestion approaches, were transferred to a new tube and acidified at a final concentration of 1% (v/v) formic acid (FA) (Ciordia et al., 2024). Tryptic peptides were cleaned up using in-home-made Stage-Tips prepared from Octadecyl C18 solid-phase extraction disks (EmporeTM, 66883-U), as previously described (Ciordia et al., 2022). Eluted peptides were dried in a speed vacuum and quantified by fluorimetry (QuBit) according to the manufacturer's instructions.

VII. 3.2.2. Peptide analysis through nano LC-ESI-MS/MS

For nano-liquid Chromatography coupled to Electrospray Ionization Tandem Mass Spectrometry (nanoLC-ESI-MS/MS) analysis, 1 μg of each sample was individually analysed using an

Ultimate 3000 nano HPLC system (Thermo Fisher Scientific) coupled online to an Orbitrap Exploris™ 240 mass spectrometer (Thermo Fisher Scientific). Each sample [1 µg in 5 µl of sample resuspended in mobile phase A: 0.1% formic acid (FA)] was loaded on a 50 cm × 75 µm Easy-spray PepMap C18 analytical column at 45°C. Tryptic peptides were separated at a flow rate of 250 nL/min using a 90-minute gradient ranging from 6% to 95 % mobile phase B (80 % acetonitrile (ACN) in 0.1% FA). To avoid carry-over, two 40-minute blank samples (mobile phase A) were systematically run between samples. Data acquisition was performed using a data-dependent top method, in full scan positive mode, scanning 375 to 1200 m/z. MS1 scans were acquired at an orbitrap resolution of 60,000 at m/z 200, with a normalised automatic gain control target of 300%, a radio frequency lens of 80%, and an automatic maximum injection time (IT). The top 20 most intense ions from each MS1 scan were isolated and fragmented with a Higher-energy collisional dissociation (HCD) value of 30%. Resolution for HCD spectra was set to 15,000 at m/z 200, with a normalised AGC target of 50% and an automatic maximum IT. The precursor isolation window 1.0 m/z and dynamic exclusion (45 s) were applied. Precursor ions with single, unassigned, or six or higher charge states from fragmentation selection were excluded.

VII. 3.2.3. Processing of *nanoLC-ESI-MS/MS* data

Raw data files were processed using the Proteome Discoverer 2.5.0.400 software (Thermo Scientific, Bremen, Germany), and database search was carried out using four search engines (Mascot (v2.8.0), MsAmanda (v2.4.0), MsFragger (v3.1.1), and Sequest HT) against an *O. europaea* UniProtKB database (19th December 2023) containing the most common laboratory contaminants (cRAP database with 69 sequences). Search parameters were set as follows: cysteine carbamidomethylation (+57.021464 Da); methionine oxidation (M) (+15.994915 Da), N-term acetylation (+42.010565 Da), and Gln→pyro-Glu (-17.026549 Da) as variable modifications. Precursor mass tolerances were set at 10 ppm, and the fragment mass tolerance at 0.02 Da, and trypsin/P was selected as a protease with a maximum of 2 missed cleavage sites. False discovery rate (FDR) was calculated using the processing node Percolator (maximum delta Cn 0.05; decoy database search target) and the validation of proteins, peptides, and peptide spectral matches (PSMs) with a FDR ≤ 1 %. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2025) partner repository with the dataset identifier PXD075995. “Minora” feature in the processing method and the “Feature Mapper” and “Precursor Ions Quantifier” nodes in the consensus step were applied for precursor ion quantitation. The Feature Mapper node in the consensus method was used to create features from unique peptide-specific peaks by applying chromatographic retention time alignment with a maximum shift of 10 min and a signal-to-noise threshold of 5. In the 'Precursor Ions Quantifier' node, the following parameters were set: unique+razor peptides were used for quantitation, precursor

abundance was based on intensity, and the normalisation mode was based on the total peptide amount. Protein abundance was calculated by summing the sample abundance of the connected peptide groups.

VII. 3.3. Identification of Differentially Expressed Proteins (DAPs) and Construction of Protein Co-Expression Networks

Protein abundance data obtained from LC–MS/MS analyses were processed and statistically analysed in R. Protein intensity values equal to zero were considered as non-detected proteins (missing values). Prior to differential abundance analysis, protein intensities were log₂-transformed and normalised across samples using median normalisation to minimise technical variation among biological replicates. Pearson correlation coefficients between samples were calculated using normalised log₂-transformed protein intensity values and visualised as clustered heatmaps in R using the pheatmap package. Correlation matrices were generated using Pearson's correlation coefficient to assess sample reproducibility and global proteomic similarity among extraction methods and embryogenic efficiency groups.

Differential abundance analyses were performed using the limma package in R. Linear models were fitted to evaluate the effects of protein extraction method (S-Trap and SP3) and embryogenic efficiency (HE and LE). The experimental design followed a 2×2 factorial structure with two factors: efficiency (HE, LE) and extraction method (SP3 and S-Trap), each comprising three biological replicates per condition. A two-way linear model including main and interaction effects ($\text{abundance} \sim \text{efficiency} + \text{method} + \text{efficiency} \times \text{method}$) was fitted to the log₂-transformed data. Protein-wise linear models were estimated using the lmFit function, and empirical Bayes moderation of standard errors was applied using eBayes to improve variance estimation and statistical power, given the limited number of replicates. Main effects of efficiency (HE vs LE) and extraction method (SP3 vs S-Trap), as well as their interaction term, were evaluated. In addition, method-specific contrasts were computed to assess HE vs LE differences within SP3 and S-Trap separately. Due to the limited number of biological replicates ($n = 3$ per group), differentially abundant proteins (DAPs) were defined using a nominal p -value < 0.05 combined with an absolute log₂ fold change ($|\log_2\text{FC}| > 1$). To further investigate qualitative differences in protein detection, presence/absence analyses were performed separately for the SP3 and S-Trap methods. Protein abundance matrices were converted into binary matrices, in which detected proteins were assigned a value of 1 and non-detected proteins a value of 0. Proteins were subsequently classified as HE-associated, LE-associated, or shared between both embryogenic conditions according to their occurrence across biological replicates. Data visualisation included volcano plots, Pearson correlation, and Venn diagrams generated in R using the ggplot2, pheatmap, and VennDiagram packages. The Gene Ontology (GO) enrichment analysis of DAPs in each protein-digestion method was performed using OmicsBox v2.0.36. software (BioBam Bioinformatics, Valencia, Spain) (Gotz et al., 2008).

To further capture coordinated protein expression patterns, a co-expression protein network related to the protein digestion method was constructed using the Weighted Gene Co-Expression Network Analysis (WGCNA) package (Ritchie et al., 2015) in R. After filtering proteins with >30% missing values and low variance, missing values were imputed using median substitution. To reduce noise, proteins in the lowest quartile of variance were excluded, and proteins with zero median absolute deviation (MAD) were removed. A signed network was constructed using biweight miscorrelation and a soft-thresholding power of 20. Modules were identified by hierarchical clustering of the topological overlap matrix (TOM) with a minimum module size of 30 and merged at a cut height of 0.25. Module–trait associations (protein digestion method or embryogenic efficiency) were assessed by correlating module eigengenes with traits. Selected modules were exported to Cytoscape (Shannon et al., 2003) for network visualisation, and the top 100 hubs were identified using maximal clique centrality (MCC) in the cytoHubba plugin. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses for the top 100 hub proteins were performed using OmicsBox v2.0.36. software.

VII. 4. RESULTS

VII. 4.1. Comparison of the SP3 and S-Trap Methods for Protein Identification and Quantification

The proteome of HE and LE embryogenic olive lines were analysed using a label-free quantitative (LFQ) strategy. For this purpose, two distinct sample preparation strategies were implemented and subsequently evaluated. Using these workflows, a total of 13,659 proteins (corresponding to 69,206 peptides) were identified with the SP3 method, and 13,567 proteins (corresponding to 69,694 peptides) using the S-Trap method ($\text{FDR} < 1\%$, $\text{PSM} \geq 2$). Of these proteins, 11,346 overlapped between methods, whereas only a small percentage were uniquely detected by either strategy (2221 for S-Trap vs 2313 for SP3). These identified proteins were further grouped into protein groups, defined as groups of proteins sharing the same set of identified peptides and therefore not unambiguously distinguishable based on peptide evidence. Following this criterion, 9,200 protein groups were identified for SP3 and 9,108 protein groups for the S-Trap method.

The performance of both methods was compared by evaluating the number of proteins and peptides identified in each biological sample, as well as digestion efficiency based on the number of missed cleavages. The average number of proteins identified per biological replicate was significantly higher with the SP3 method than with the S-Trap method ($12,661 \pm 89$ vs. $12,481 \pm 61$; $p = 0.0022$) (Figure C.2). However, both methods showed similarly low coefficients of variation ($\text{CV}=0.7\%$ for SP3 and $\text{CV}=0.5\%$ for S-Trap), indicating comparable reproducibility. A similar trend was observed for protein groups, with SP3 yielding a significantly higher number of identified protein groups compared

with S-Trap ($8,529 \pm 47$, CV=0.6% for SP3 and $8,411 \pm 40$, CV=0.5% for S-trap; $p = 0.0009$) (Figure C.3).

Regarding peptide identification, no significant differences were observed between methods in the total number of identified peptides. Although S-Trap showed a slightly higher average number of identified peptides than SP3, both methods displayed similar variability across samples ($44,454 \pm 1,625$, CV=3.7% for S-trap and $43,288 \pm 1,417$, CV=3.3% for SP3). Differences in digestion efficiency between methods were also assessed based on the number of missed cleavages. The number of peptides identified without missed cleavages was nearly identical across techniques ($39,965 \pm 1479$ for SP3 vs. $39,414 \pm 1372$ in S-Trap; $p\text{-value}=0.5189$). Therefore, the slightly higher number of peptides identified with S-Trap appeared to be mainly attributable to an increased detection of peptides containing one or two missed cleavages. Indeed, the number and percentage of peptides with missed cleavages were significantly higher in the S-Trap method than in the SP3 method. In S-Trap, 10.69% ($4,753 \pm 382$) of peptides presented one missed cleavage, and 0.64% (286 ± 50) presented two missed cleavages, compared with 7.34% ($3,174 \pm 259$) and 0.35% (149 ± 22), respectively, in SP3 ($p\text{-value}<0.0001$ for both comparisons). These results suggest less efficient digestion with S-Trap, which may explain why SP3 yielded a higher number of identified proteins and protein groups, despite the slightly higher total number of peptides detected with S-Trap.

For protein quantitation analysis, only high-confidence proteins (protein FDR $q\text{-value} \leq 1\%$) supported by >2 PSMs, containing at least one unique peptide, and consistently quantified across all three biological replicates of either the HE or LE lines, were retained as criteria for quantification analysis. Therefore, a total of 8,454 and 8,365 protein groups were able to be quantified using the SP3 and S-Trap workflows, respectively, with 7,075 protein groups identified in common (Table C.1).

To explore the relationships among samples based on overall proteomic variation, an unsupervised principal component analysis (PCA) was performed on $\log_2$ -transformed protein abundance values. The first principal component (PC1), which explains 29.8% of the total variance, clearly separated samples by protein extraction method, distinguishing the SP3 and S-Trap datasets (Figure VII.1A). Within each method, samples were further segregated according to embryogenic competence, although some intra-group variability was observed, particularly for HE samples along PC2, which explains 13.6% of the total variance. Pearson correlation analysis further confirmed the high reproducibility of the datasets (Figure VII.1B). Samples belonging to the same extraction method exhibited strong correlations, with coefficients generally ranging from 0.93 to 0.96, whereas correlations between methods were lower, ranging from approximately 0.80 to 0.86. Hierarchical clustering grouped samples primarily according to extraction methodology rather than embryogenic efficiency, reinforcing the major influence of protein extraction strategy on the resulting proteomic profiles (Figure VII.1B).

Differential abundance analysis using a two-way linear model identified a large effect for extraction method, with 2,155 proteins differing between SP3 and S-Trap (FDR < 0.05), irrespective of SE efficiency (Supplementary Table S2). From these, 1170 proteins were found to be related to SP3 and 985 were significantly associated with the S-Trap method (Table C.2).

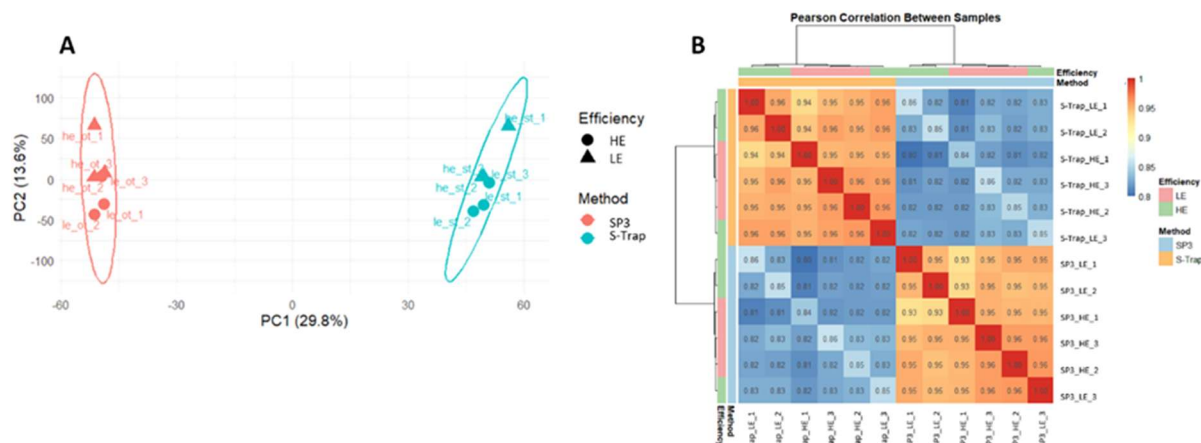


Figure VII.1. (A) Principal component analysis (PCA) of normalised protein abundance data showing sample clustering according to extraction method and embryogenic efficiency. PC1 explained 29.8% of the total variance, while PC2 explained 13.6%. Colours represent extraction methods (SP3 and S-Trap), and symbols represent embryogenic efficiency classes (high efficiency, HE; low efficiency, LE). Ellipses indicate sample grouping patterns. (B) Pearson correlation heatmap and hierarchical clustering of all samples based on normalised protein abundances. Correlation coefficients are indicated within each cell. Sample annotations represent embryogenic efficiency (HE and LE) and extraction method (SP3 and S-Trap).

VII. 4.2. Identification of a Proteome Signature Associated with Embryogenic Efficiency using SP3 and S-Trap Methods

In addition to comparing the SP3 and S-Trap extraction methods for protein identification and quantification, we performed differential abundance analysis between HE and LE embryogenic lines. Three HE lines (A55, A140, and A148) and three LE lines (A23, A157, and A158) were analysed with each method. This design enabled evaluation of the ability of both extraction strategies to detect key molecular pathways associated with SE efficiency in *O. europaea*.

To have a broader understanding of the protein regulation between HE and LE lines, regardless of a strict FDR, the top-regulated proteins between HE and LE lines identified by a two-way linear model were further identified. Figure VII.2 summarises the differential proteomic responses between HE and LE lines within the SP3 and S-Trap extraction methods. Volcano plots identified subsets of proteins significantly associated with embryogenic efficiency using a nominal *p-value* < 0.05 and

$|\log_2\text{FC}| > 1$ (Figure VII.2A). Most detected proteins were not significantly differentially abundant, reflecting the overall similarity between the embryogenic lines, although distinct subsets of efficiency-associated proteins were observed for both methods (Figure VII.2A, Table C.3). We found 122 proteins associated with HE and 106 associated with LE in the SP3 method, and 120 proteins associated with HE and 141 associated with LE in the S-Trap method (Figure VII.2A, Table C.3). To further explore abundance patterns, a combined heatmap was generated using all proteins identified by $p\text{-value} < 0.05$ and $|\log_2\text{FC}| > 1$ in either extraction method (Figure VII.2B). Hierarchical clustering based on normalised and scaled protein abundances clearly separated samples according to extraction methodology, confirming the strong influence of protein extraction strategy on the overall proteomic profile. Within each extraction method, subsets of proteins displayed contrasting abundance patterns between HE and LE lines, indicating that embryogenic competence is associated with coordinated alterations in specific protein groups (Figure VII.2B).

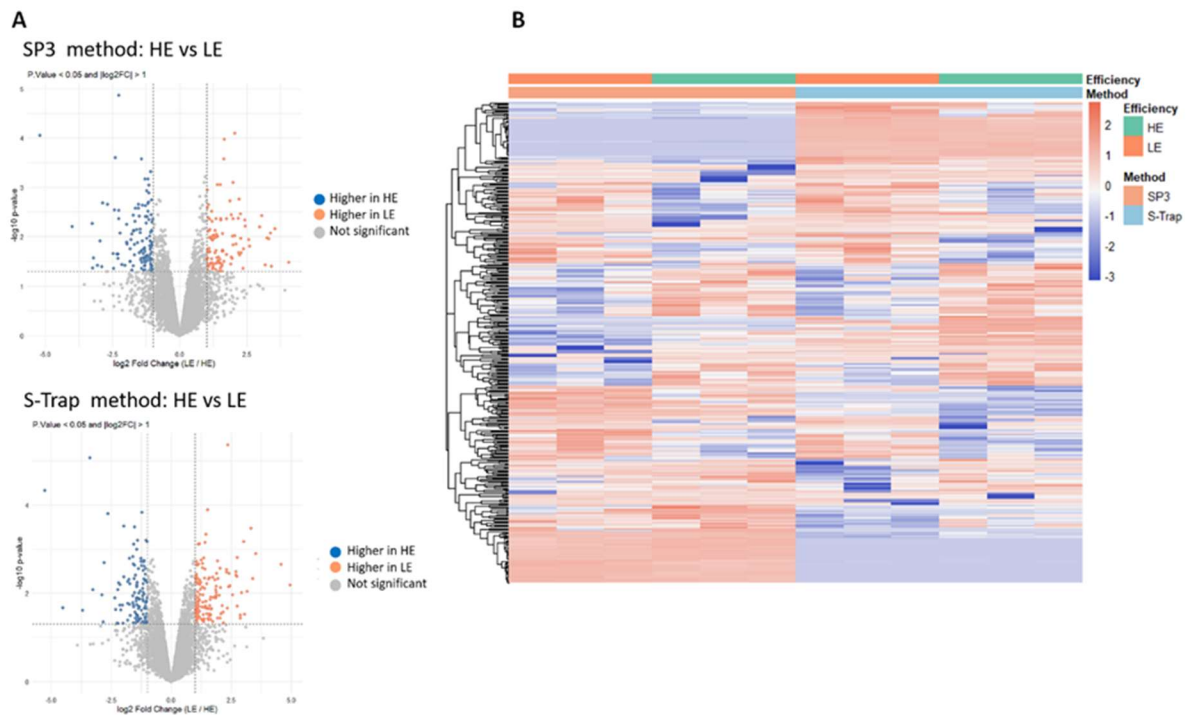


Figure VII.2. Differential proteomic analysis of high-efficiency (HE) and low-efficiency (LE) somatic embryogenic lines. (A) Volcano plots showing differentially abundant proteins identified between HE and LE lines within the SP3 and S-Trap methods using $p\text{-value} < 0.05$ and $|\log_2\text{FC}| > 1$. Proteins enriched in HE are shown in blue, proteins enriched in LE in orange, and non-significant proteins in grey. Dashed lines indicate fold-change and significance thresholds. (B) Heatmap of all differentially abundant proteins identified in SP3 and S-Trap comparisons. Values correspond to row Z-score-scaled $\log_2$ -transformed normalised protein abundances. Sample annotations indicate embryogenic efficiency (HE and LE) and extraction method (SP3 and S-Trap). Red indicates relatively higher abundance, and blue indicates relatively lower abundance for each protein across samples.

Presence/absence analyses revealed that most proteins were shared between HE and LE somatic embryogenic lines in both extraction methods. In the SP3 dataset, 8,353 proteins were commonly detected between HE and LE samples, whereas 52 proteins were exclusively detected in LE lines and 50 proteins were unique to HE lines (Figure VII.3, Table C.4). Similarly, the S-Trap dataset contained 8,235 shared proteins, with 68 proteins uniquely detected in LE samples and 62 proteins specific to HE samples (Figure VII.3).

Functional classification of proteins identified exclusively in each embryogenic condition revealed distinct biological tendencies. In the SP3 method, HE-specific proteins were mainly associated with catabolic processes, reorganisation, vesicle-mediated reorganisation, vesicle-mediated transport, transmembrane transport, and phosphate-containing compound metabolic processes, and included ABC transporters, heat shock protein 70, calcium-transporting ATPase, DOG1-like protein, late embryogenesis abundant protein, small ubiquitin-related modifier 1-like and xyloglucan endotransglucosylase/hydrolase proteins (Table C.4). In contrast, LE-specific proteins were mainly associated with carbohydrate metabolic processes, transmembrane transport, response to stress, and protein metabolism (Figure C.3). These proteins included aquaporin PIP2-2-like, glutathione S-transferase, plasma membrane ATPase 4, alcohol dehydrogenase 1, cytochrome P450 proteins, berberine bridge enzyme-like 22, receptor-like kinases, and callose synthase 5 (Table C.4).

Within the S-Trap dataset, HE-specific proteins were primarily associated with carbohydrate metabolism, defence response, response to other organisms, and protein metabolic processes (Figure VII. 3). Specific proteins included ABC transporters, thaumatin-like protein, glutathione S-transferase, phospholipase C2, receptor-like kinases, heat shock proteins, vacuolar-sorting receptor 3-like, ROOT HAIR DEFECTIVE 3-like, and glucan endo-1,3-beta-glucosidase (Table C.4). S-Trap LE-specific proteins were mainly enriched in defence response, protein metabolic processes, gene expression, and response to chemical stimuli (Figure VII.3). Proteins such as catalase isozyme 1, glutaredoxin-C4, thioredoxin-like protein HCF164, defensin J1-2-like, ubiquitin-related proteins, aquaporin PIP2-2-like, and multiple cytochrome P450 family members were exclusively detected in S-Trap LE lines (Table C.4).

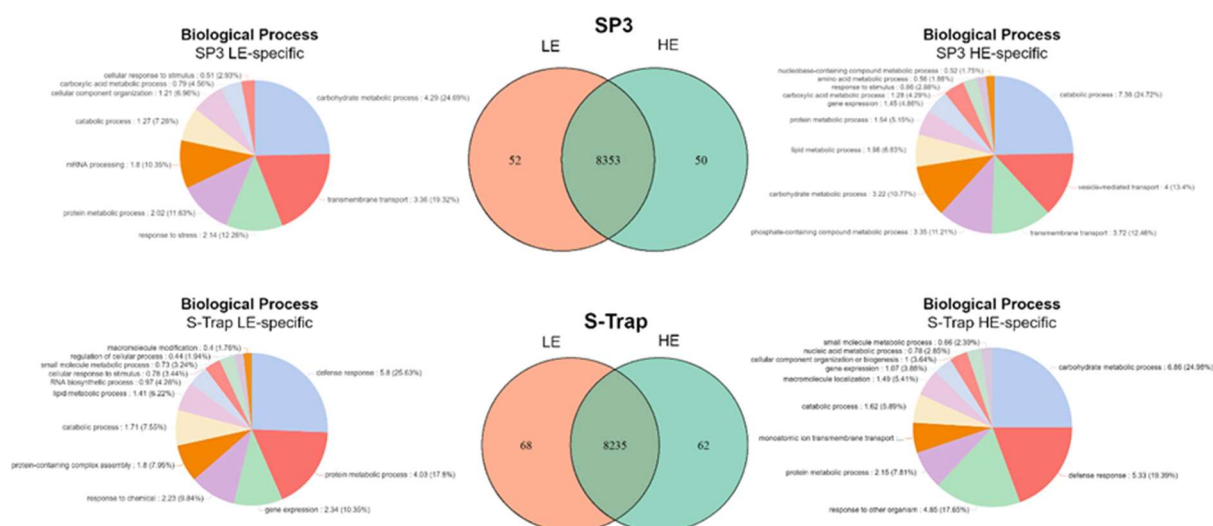


Figure VII.3. Gene Ontology (GO) enrichment analysis of the differentially expressed proteins (DEPs) between HE and LE identified using the SP3-OT-2 (A) and S-Trap (B) methods. Pie charts represent GO level 3 terms for the Biological Process (BP) and Cellular Component (CC) categories. The size of each sector corresponds to the relative proportion of DAPs assigned to each GO term within each dataset, highlighting both shared and workflow-specific functional distributions.

VII. 4.3. Weighted Gene Co-Expression Network Analysis (WGCNA)

To get insight into the influence of the method used for sample preparation on the understanding of the biological network involved in SE, a WGCNA analysis was performed, and further identification of proteins significantly correlated with modules was performed. Protein co-expression module eigengenes (MEs) showed that ME1 was significantly correlated with the SP3 method (downregulated) ($p < 0.001$), whereas ME2 was tightly correlated with the S-Trap method (upregulated) ($p < 0.001$) (Figure C.3). A total of 2337 proteins were found related to the SP3 method, and 2066 to the S-Trap method.

The top hub 100 proteins from the selected co-expression modules associated with the SP3 (Module 1) and S-Trap (Module 2) workflows (Figure C.4) were functionally characterised using GO and KEGG pathway enrichment analyses (Figures VII.4 and VII.5).

GO enrichment analysis of proteins showed that the top GO terms were common between SP3 and S-Trap (Figure VII.4A and VII.4B). In both datasets, biological processes such as translation, proteolysis, protein transport, and vesicle-mediated transport were prominently represented, underscoring the importance of coordinated protein synthesis and intracellular trafficking during embryogenic calli proliferation. Despite these common features, differences in the relative contribution of specific biological processes were evident. SP3 revealed a broader distribution of transport- and

metabolism-related processes, notably intracellular transport, ribosome biogenesis, and proteolysis (Figure VII.4A). In contrast, S-Trap-associated proteins showed a higher representation of processes linked to vesicle-mediated transport, translation, and RNA processing, including mRNA metabolic processes, suggesting a functional emphasis on protein biosynthesis and post-transcriptional regulation (Figure VII.4B). Analysis of the Cellular component enrichment indicated a predominance of intracellular organelles and protein-containing complexes in both modules. Commonly enriched components included the Golgi apparatus, nuclear protein-containing complexes, organellar membranes, and ribosomal subunits, highlighting the importance of endomembrane trafficking, nuclear regulation, and translational machinery. However, the relative distribution of cellular components differed between workflows. The S-Trap dataset exhibited a higher proportion of proteins associated with the Golgi apparatus, cytosolic ribosomes, and cell periphery, consistent with the biological process enrichment related to intracellular trafficking and protein synthesis. Conversely, proteins from the SP3 were more broadly distributed across organelle-specific compartments, including mitochondria, plastid-associated compartments, and intracellular protein-containing complexes, suggesting greater representation of proteins involved in energy metabolism, plastid-associated functions, and organelle-specific regulation.

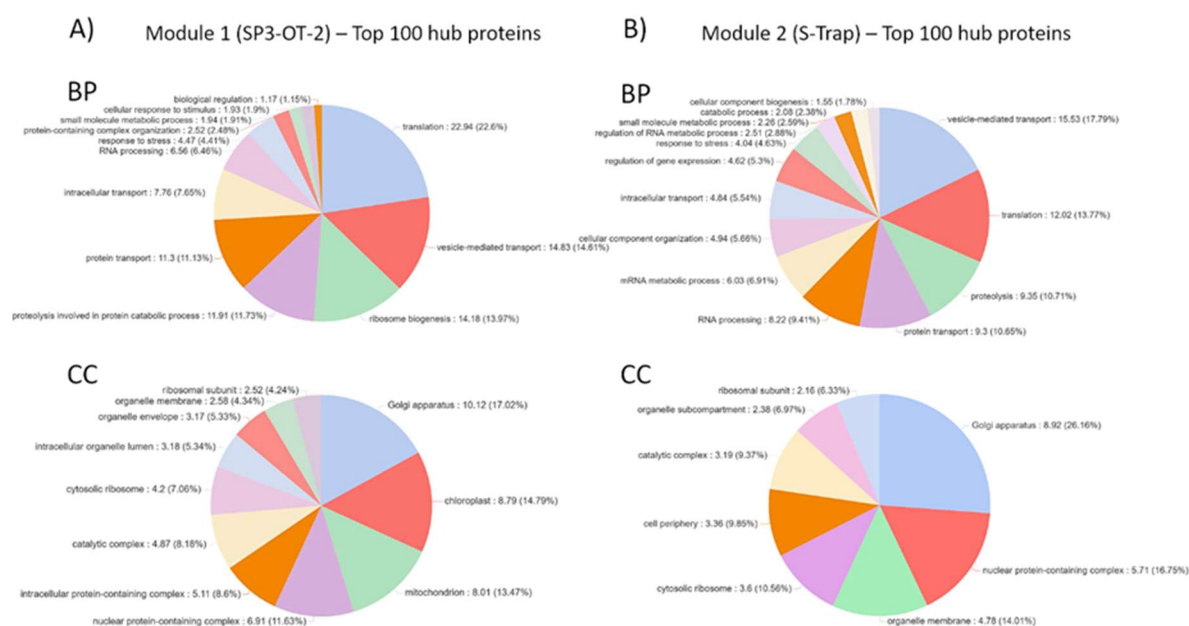


Figure VII.4. GO of top 100 hub proteins in module 1 (A) and 2 (B), identified through WGCNA analysis (see Figure C.4) and associated with the SP3 and S-Trap methods, respectively. Pie charts illustrate the biological processes (BP) and cellular components (CC) terms associated with these proteins.

KEGG pathway mapping further showed that both the number and the general pattern of enriched pathways were comparable between the SP3 and S-Trap approaches (Figure VII.5A and VII.5B). In both modules, pathways related to purine metabolism, thiamine metabolism, and ribosome were among the most enriched, emphasising the central role of nucleotide metabolism and protein biosynthesis. Distinct enrichment patterns were also observed between workflows. The SP3-associated module was characterised by enrichment of pathways linked to oxidative phosphorylation and other mitochondrial-associated processes, together with plastid-related pathways and the Ras signalling pathway (Figure VII.5A). In contrast, the S-Trap-associated module showed significant enrichment of KEGG pathways related to endocytosis, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) interactions in vesicular transport, and protein processing in the endoplasmic reticulum, reflecting the over-representation of proteins annotated to intracellular trafficking and endomembrane-associated pathways within this module (Figure VII.5B). The S-Trap approach preferentially captures coordinated networks associated with protein synthesis, processing, and vesicular trafficking, whereas the SP3 workflow highlights broader organelle-specific pathways, including mitochondrial- and plastid-associated processes related to energy metabolism, stress response, and cellular homeostasis. Overall, while both workflows converge on fundamental protein metabolic and organisational processes underpinning SE, they differ in their functional emphasis.

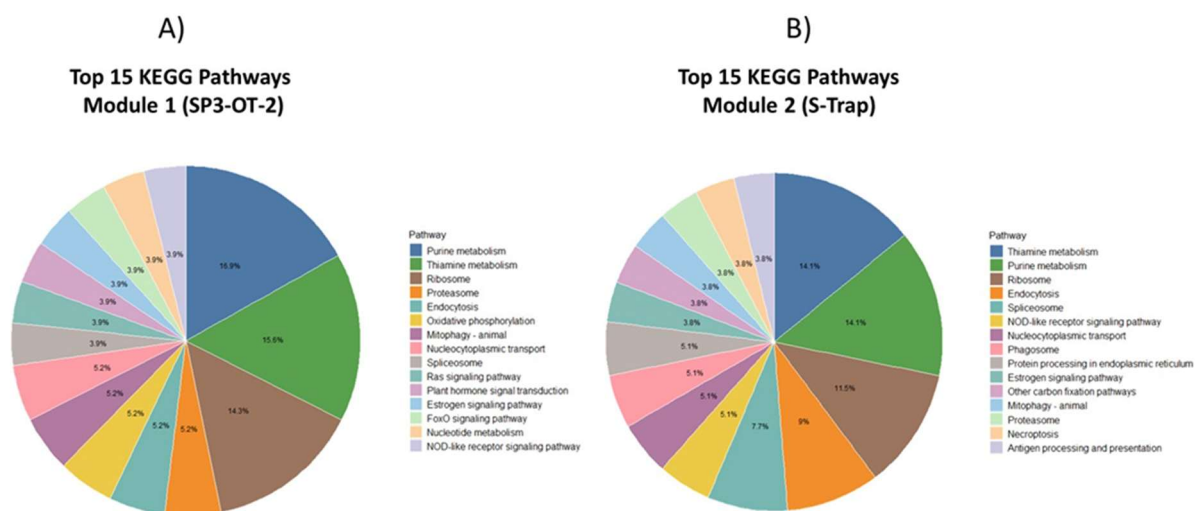


Figure VII.5. Top 15 enriched KEGG pathways for (A) module 1, related to the SP3 method, and (B) for module 2, related to the S-Trap method.

VII. 5. DISCUSSION

This study presents, for the first time, a comprehensive proteomic analysis of embryogenic tissues from *O. europaea* exhibiting a differential efficiency-related behaviour. Analysis of HE and LE lines across cyclic SE identified a consistent set of DAPs, supporting the view that embryogenic

competence in olive involves extensive and coordinated proteomic reprogramming. Proteomics generally exhibits lower sensitivity than transcriptomic approaches, as it is inherently biased toward highly abundant proteins and often has limited capacity to detect low-abundance species (Martínez-Esteso et al., 2024). Therefore, methodological choice is of utmost importance when analysing plant proteomes. To assess the impact of sample preparation on proteome coverage and SE-associated protein detection, LC–MS/MS analyses were performed using two widely applied digestion workflows: SP3 and S-Trap. Both SP3 (Ciordia et al., 2024) and S-trap (Ciordia et al., 2021, 2024) protocols used in this study were originally optimised for animal tissues and fluids; therefore, their performance has not been systematically compared to date, particularly in recalcitrant species such as *O. europaea*. Indeed, comparative evaluations in non-plant systems indicate that the performance of proteomic digestion workflows may be sample-dependent rather than universally superior (Araújo et al., 2021). For example, differences between SP3, FASP, and S-Trap-based approaches have been reported in animal tissues and cell lysates, with variability in protein recovery and coverage depending on matrix complexity (Sielaff et al., 2017; Araújo et al., 2021). In the present study, we also leveraged the implementation of SP3 on an automated OT-2 platform (Ciordia et al., 2024), providing additional technical standardisation. The implementation of SP3 on the automated OT-2 platform further minimised technical variability (Johnston et al., 2022; Ciordia et al., 2024), as reflected in the lower dispersion of HE replicates compared with S-Trap. This observation highlights how both digestion methods contribute to the resolution of embryogenic competence-associated proteomic differences. Notably, the number of proteins identified in this work is comparable to or higher than those reported in most previous SE-focused proteomic studies, likely reflecting advances in sample preparation workflows, analytical sensitivity and data acquisition strategies. For example, Orbitrap-based proteomic analyses in different plant species have reported variable protein numbers depending on the biological material and developmental stage analysed. In *E. guineensis*, approximately 4,600 proteins were identified in embryogenic calli derived from leaf explants during the induction phase, with several hundred showing differential accumulation throughout embryogenic development (Ribeiro et al., 2019). In *C. arabica*, 1,052 proteins were detected in embryogenic cell suspension cultures (Campos et al., 2016), whereas in *C. canephora*, 1,630 proteins were quantified in embryogenic calli during induction in liquid medium from *in vitro*-germinated leaf explants (Quintana-Escobar et al., 2023). These differences likely reflect not only technical aspects of the proteomic method but also species-specific characteristics, the type of explant, culture system, and the specific stage of SE. In parallel, the S-Trap method has been widely adopted due to its high digestion efficiency and effective removal of contaminants such as SDS that can interfere with mass spectrometric performance (Wang et al., 2023). In another study focusing on the extracellular secretome of *O. europaea*, Pires et al. (2025b) applied the S-Trap workflow to culture medium, identifying 1096 proteins (526 protein groups), including several biomarkers previously associated with SE. The reduced proteome complexity reported in that study likely reflects the analysis

of secreted proteins rather than plant tissues, emphasising the strong influence of the biological matrix on proteomic outcomes. In contrast, the application of S-Trap to embryogenic calli tissue in the current study enabled a broad proteomic coverage, with 13,567 proteins identified (9108 protein groups), reflecting its suitability for analysing more complex plant tissues, which are characterised by high levels of polysaccharides, phenolic compounds, and other interfering molecules (Wojtkiewicz et al., 2021; Wang et al., 2023). The SP3 workflow performed similarly, identifying 13,659 proteins (9200 protein groups), demonstrating comparable effectiveness in handling tissue-derived proteomes. Both values substantially exceed those obtained from culture medium, which primarily contains secreted proteins, reflecting the higher complexity of tissue-derived proteomes.

Heatmap expression patterns and GO enrichment analyses of DAPs revealed clear molecular differences between embryogenic lines. In the SP3 method, HE-specific proteins were mainly associated with catabolic processes, vesicle-mediated reorganisation, vesicle-mediated transport, transmembrane transport, and phosphate-containing compound metabolic processes. These proteins included heat shock proteins, calcium-transporting ATPase, DOG1-like protein, late embryogenesis abundant protein, and small ubiquitin-related modifier 1-like, several of which have previously been reported as markers associated with SE competence and stress-responsive developmental reprogramming (Neves et al., 2021; Quintana-Escobar et al., 2023). In particular, heat shock proteins and late embryogenesis abundant proteins are frequently linked to embryogenic competence and protection against cellular stress during embryo development (Dirk et al., 2020; Juarez-Escobar et al., 2021). Similar trends were reported in *Medicago truncatula*, where highly embryogenic lines displayed increased levels of peroxidases, aminotransferases, and ATP-related enzymes (Almeida et al., 2012). These functions were also enriched in HE lines in this study using the S-Trap method. Overall, these proteins are associated with stress regulation, redox homeostasis, intracellular transport, and metabolic reprogramming, processes considered essential for SE induction and progression (Zhou et al., 2016; Chen et al., 2026).

Our proteomics comparative analysis showed that SP3 displayed a wider range of GO terms than the S-Trap method, both for Biological Process and Cellular Component. These categories collectively indicate a greater representation of regulatory and adaptive proteins. This broader functional diversity may reflect differences in protein binding and cleanup efficiency inherent to the paramagnetic bead-based SP3 workflow, potentially enabling the retention of lower-abundance or regulatory proteins that are more susceptible to loss during harsher extraction conditions. Similar results were observed in previous studies in *A. thaliana* lysates. By comparing both methodologies, a higher peptide recovery was reported with SP3 (carboxylated beads) versus several other methods, including S-Trap, supporting the point that SP3's binding/cleanup properties can give broader functional coverage and better retention of certain protein classes in plant tissues (Mikulášek et al., 2021). In addition, the SP3 approach may differentially support the recovery of proteins based on their physicochemical properties, including

hydrophobicity, compared to the S-Trap method. This is particularly relevant for membrane- and organelle-associated proteins involved in developmental reprogramming (Conforti et al., 2024), as reflected by the enrichment of vesicle trafficking-, transport-, and membrane-associated proteins detected in the present study.

From the differential expression analysis, vesicle-mediated transport, along with signalling and cell communication processes, was uniquely captured by SP3, whereas the S-Trap dataset was predominantly associated with transmembrane transport. While vesicle trafficking is well recognised as fundamental for plant development, its specific contribution to embryogenic competence remains comparatively underexplored. Nevertheless, proteomic studies in *Dimocarpus longan* (Fang et al., 2014) and *C. papaya* (Botini et al., 2021) have reported the accumulation of Ras-related RAB family proteins during early somatic embryo development, supporting a conserved role for vesicle trafficking and membrane reorganisation during SE induction.

To further explore coordinated biological behaviour beyond individual protein changes, protein co-expression networks were analysed using WGCNA. Co-expression modules were predominantly enriched in processes related to translation, ribosome biogenesis, vesicle-mediated transport, protein transport, RNA processing, and proteolysis. These categories represent fundamental cellular programs governing protein synthesis, intracellular trafficking, and protein turnover. The prominence of translation and ribosome-associated modules suggests that the core biosynthetic machinery constitutes a highly coordinated proteomic support that is robust to differences in extraction methodology. Similarly, the enrichment of vesicle-mediated transport and protein trafficking modules highlights the central role of endomembrane dynamics in maintaining cellular homeostasis, particularly in plant tissues where compartmentalisation is extensive (Aniento et al., 2022). At the organelle level, method-specific patterns were also evident. The S-Trap module showed strong associations with the Golgi apparatus and nuclear protein-containing complexes, whereas SP3 modules exhibited a more distributed representation across organelles such as chloroplasts and mitochondria. Among these signals, mitochondrial- and redox-related proteins were particularly informative in distinguishing differences between approaches. The SP3 method showed enrichment of proteins associated with mitochondrial homeostasis, oxidative stress regulation, and intracellular trafficking, including heat shock proteins, small ubiquitin-related modifiers, and vesicle trafficking-associated proteins. These functions are closely linked to cellular reprogramming and stress adaptation during SE. In this context, alternative oxidase (AOX)-mediated pathways are especially relevant, as AOX is known to regulate mitochondrial redox balance and limit reactive oxygen species (ROS) accumulation during developmental and environmental stress responses (Igamberdiev & Eprintsev, 2016; Vishwakarma et al., 2018). Although AOX itself was not directly identified in the present dataset, the enrichment of proteins related to redox regulation and mitochondrial activity may reflect similar regulatory mechanisms associated with embryogenic competence, consistent with

previous observations in highly embryogenic lines (Vale et al., 2022). Notably, these proteins were not detected using the S-Trap method, suggesting that methodological choice can influence the representation of membrane-associated and stress-responsive mitochondrial proteins. This may indicate subtle differences in subcellular protein recovery that are not fully captured by differential abundance analysis alone but become apparent when examining coordinated protein networks. In fact, the use of WGCNA to identify clusters of coordinated patterns has been previously used in plant proteomics, improving the detection of groups that may act together, even if individual genes show only moderate or non-significant changes (Roy et al., 2023; Xu et al., 2025).

Together, these findings indicate that embryogenic competence is regulated by interconnected protein networks integrating redox homeostasis, vesicle trafficking, and extracellular signalling. Although SP3 and S-Trap workflows provide complementary perspectives, SP3 captured a broader range of functional processes, particularly proteins associated with membranes and secretion, which were less represented in the S-Trap dataset. This comparison illustrates that methodological choice can influence the aspects of the proteome revealed in SE studies and underscores the importance of selecting appropriate methods for the features under investigation.

VII. 6. CONCLUSION

This study provides a comprehensive proteomic characterisation of cyclic SE in olive, integrating HE and LE lines to elucidate molecular determinants of embryogenic competence. Both digestion techniques achieved comprehensive proteome coverage in embryogenic calli tissue, with SP3 identifying a slightly greater number of proteins than S-Trap and revealing an additional functional range. While both approaches consistently captured key biological processes related to metabolism and intracellular organisation, SP3 showed enhanced representation of mitochondrial components, membrane-associated proteins, vesicle trafficking, and stress-responsive pathways. In contrast, S-Trap provided complementary, although comparatively more restricted, functional coverage. The results presented here collectively indicate that embryogenic competence in olive is linked to coordinated stress-adaptive and redox-regulated networks that involve mitochondrial activity, extracellular ROS homeostasis, and vesicle trafficking. By combining comparative proteomic workflows with the analysis of HE and LE embryogenic lines, this study refines the molecular framework underlying SE in olive and provides a validated basis for future mechanistic and applied studies in recalcitrant species.

VII. 7. REFERENCES

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CHAPTER VIII

Integrated Proteomic and Secretomic Profiling Identifies Class III Peroxidases as Regulators of Embryogenic Competence in Olive

The work presented in this chapter will be submitted to *BMC Plant Science*:

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VIII. 1. ABSTRACT

Somatic embryogenesis (SE) is an *in vitro* system that could be used for mass propagation of elite olive (*Olea europaea* L.) genotypes, and that could assist plant breeding techniques based on genetic engineering approaches. Nevertheless, the embryogenic competence is highly dependent on genotypes, tissue and even cell line. To investigate the molecular mechanisms underlying this variability, comparative proteomic and secretomic analyses were performed using embryogenic lines with high (HE) and low embryogenic efficiency (LE). Differential protein accumulation revealed a clear separation between high- (HE) and low-efficiency (LE) embryogenic lines. The HE tissues were enriched in proteins associated with redox regulation, signalling, stress responses, and secondary metabolism. In contrast, LE lines exhibited a greater abundance of proteins associated with primary metabolism, translation, and mitochondrial function. Secretome analysis demonstrated a marked enrichment of extracellular class III peroxidases (PODs) in HE lines, indicating that differential secretion rather than intracellular accumulation is a major feature associated with embryogenic competence. Biochemical and immunodetection analyses further supported these findings, showing higher POD activity and stronger accumulation of diverse POD isoforms in HE tissues. To characterise the olive POD family, an *in silico* genome-wide analysis was performed and 54 genes were identified as belonging to class III POD family, displaying conserved structural features but substantial variation in exon–intron organisation and conserved motifs, suggesting functional diversification. Consistent with their extracellular role, class III PODs are implicated in apoplastic redox regulation and cell wall remodelling during SE. Gene expression analysis confirmed differential regulation of selected OePODs between HE and LE lines. Collectively, these results demonstrate that coordinated intracellular and extracellular redox-related processes are closely associated with embryogenic competence in olive and highlight class III PODs as promising molecular markers and regulatory components of SE.

VIII. 2. INTRODUCTION

SE is a non-sexual propagation process in which somatic cells are induced to reprogram and develop into embryos that are structurally and developmentally similar to zygotic embryos (Cordeiro et al., 2025). This morphogenetic pathway is widely used in plant biotechnology and tissue culture systems (Martínez & Corredoira, 2024) and is markedly influenced by genotype, explant type, and culture conditions (Sánchez-Romero, 2021). Although SE holds considerable potential, regeneration in woody species tends to decline as plants lose their juvenile characteristics, making *de novo* embryo formation challenging (Long et al., 2022). Although significant advances have been achieved in model and highly responsive species, the induction and maintenance of embryogenic competence remain strongly genotype-dependent in many woody and recalcitrant plant species. In gymnosperms, including *Pinus*

elliottii (Xu et al., 2025) and *P. roxburghii* (Kousar et al., 2025), SE protocols have been successfully optimised, enabling efficient embryo induction and plant regeneration. In contrast, other species still exhibit low and heterogeneous embryogenic responses under in vitro conditions, limiting the large-scale application of SE technologies.

SE has been described as stress response (Arnholdt-Schmitt et al., 2016) and its competence is governed by a complex and tightly coordinated network of stress perception (Fehér, 2015), hormonal signalling (Pacheco et al., 2026), metabolic reprogramming (Miao et al., 2026), and redox regulation (Zhou et al., 2016). Understanding the molecular pathways associated with embryogenic competence is therefore essential for improving regeneration protocols and identifying reliable biomarkers linked to embryogenic efficiency. During the early stages of SE induction, explants are exposed to multiple stress factors, including wounding, osmotic imbalance, auxin accumulation, and oxidative stress (Hazubska-Przybył et al., 2020; Salaün et al., 2021), which collectively trigger the transition from a differentiated somatic state toward an embryogenic developmental program. Increasing evidence indicates that reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), play a central role in this process by acting as signalling molecules that regulate cell dedifferentiation, cell cycle activation, gene expression, and embryogenic pathway induction (Elhiti & Stasolla, 2022; Nigam et al., 2025). Although excessive ROS accumulation can compromise cellular integrity and impair embryo development, transient and spatially controlled ROS production is considered essential for successful embryogenic induction (Zhou et al., 2016). Therefore, the establishment of a tightly regulated intracellular redox environment appears to be a critical determinant of embryogenic competence (Chen et al., 2026).

Recent advances in plant proteomics have considerably improved our understanding of the molecular basis of SE by enabling the identification of proteins and pathways associated with embryogenic competence. Antioxidant enzymes such as catalases, ascorbate peroxidases (APX), glutathione peroxidases (GPX), and superoxide dismutase (SOD) have been consistently associated with SE induction and development in several plant species (Ufer, 2011; Kumaravel et al., 2020; Polesi et al., 2023). Additionally, recent research indicates that embryogenic tissues have higher metabolic flexibility and enhanced antioxidant capacity than non-embryogenic tissues (Cui et al., 2021; Kumaravel et al., 2020; Zein El Din et al., 2022), supporting the idea that effective oxidative stress management is tightly linked to embryogenic efficiency. In particular, class III peroxidases (PODs) have attracted increasing attention due to their multifunctional role in regulating hydrogen peroxide turnover during SE.

Class III PODs (EC 1.11.1.7), also known as guaiacol PODs, constitute a plant-specific group of haem oxidoreductases that occur as large multigene families in higher plants (Tognolli et al., 2002). These enzymes, also referred to as secretory PODs (Hiraga et al., 2000), belong to multigene families, with each species harbouring dozens of gene members (Fawal et al., 2012), and contain a haem prosthetic group composed of protoporphyrin IX coordinated with iron (III) (Freitas et al., 2024). To

date, POD enzymes have been suggested to play a regulatory role in SE and to serve as potential molecular markers of embryogenic competence (Petric et al., 2017). Nonetheless, despite their relevance, the molecular identity and specific roles of PODs during SE remain largely unexplored. Differential POD activity between embryogenic and non-embryogenic calli tissues has been frequently reported in studies of SE. Such correlations have been documented in several plant species, including *Phoenix dactylifera* (Din et al., 2021), *Cannabis sativa* (Hesami et al., 2023), *Picea mongolica* (Wang et al., 2023) and *Glycyrrhiza glabra* (Sarkar, 2024). In these studies, embryogenic calli exhibit higher total POD activity and distinct isoenzyme profiles compared with non-embryogenic tissues, suggesting a close association between POD-mediated redox regulation and embryogenic competence. In *P. eliottii* (Cheng et al., 2025), analysis of POD isoforms during long-term proliferation of embryogenic calli revealed that the proliferation coefficient was closely linked to the activity of redox-related enzymes. Similarly, in *Medicago arborea* (Gallego et al., 2014), an increase in POD activity observed during the third subculture coincided with a higher frequency of somatic embryo formation. Both soluble and cell wall-bound POD fractions appear to be involved in this process. In *P. dactylifera* (Din et al., 2021), embryogenic calli displayed elevated activities of both soluble and ionically bound cell wall PODs compared with non-embryogenic tissues. Comparable observations were reported in *Asparagus officinalis* (Takeda et al., 2003), where a cell wall-bound POD was particularly abundant during early stages of SE and catalysed the oxidation of coniferyl alcohol derivatives, indicating a functional role in cell wall modification during embryogenic differentiation. In *Fritillaria meleagris* (Petrić et al., 2017), dynamic changes in POD isoenzyme patterns were also observed throughout the early phases of somatic embryo development. In olive, the limited available evidence further supports this association. Oubli et al. (2021) demonstrated that embryogenic calli derived from zygotic embryos of cv. ‘Dahbia’ and from leaf explants of cv. ‘Picholine Marocaine’ were generally enriched in both soluble and ionically bound POD isoforms, as revealed by electrophoretic analysis. Secretome analyses performed in different plant species have revealed the preferential accumulation of POD isoforms in CM derived from embryogenic lines with enhanced embryogenic performance, suggesting an active role of these enzymes within the extracellular embryogenic microenvironment. In *P. nigra*, specific POD isoforms were found to be enriched in the secretome of highly embryogenic cell lines (Pernis et al., 2023), while similar trends were subsequently reported in *Sorghum bicolor* (Ngwenya et al., 2024) and *O. europaea* (Pires et al., 2025a). The recurrent identification of extracellular PODs across distinct species supports the hypothesis that these enzymes may represent conserved molecular signatures associated with embryogenic competence. The presence of PODs in conditioned medium (CM) also suggests potential involvement in extracellular redox regulation, cell wall remodelling, and intercellular signalling processes required for somatic embryo development. CM refers to culture medium enriched with secreted biomolecules, including proteins, metabolites, and signalling compounds released by cultured tissues (Pires et al., 2026a). However, despite these biochemical observations, the POD gene family has not yet been

comprehensively identified in *O. europaea*, and its specific roles in olive development remain largely unexplored.

Using embryogenic lines with varying embryogenic efficiencies, the current work investigates the role of POD-associated redox regulation during SE in *O. europaea*. To explore the molecular basis underlying embryogenic competence, comparative analyses were performed on both embryogenic tissues and their corresponding CM derived from high-efficiency (HE) and low-efficiency (LE) embryogenic lines established through secondary SE from zygotic embryos of cv. ‘Arbequina’. Given the central role of PODs in ROS homeostasis, stress signalling, and cell wall dynamics, POD-related responses were further characterised to identify redox-associated mechanisms linked to embryogenic performance. In addition, an *in silico* analysis of olive PODs were conducted to provide insights into their structural organisation and potential functional diversification. Overall, this study provides new insights into the redox-regulatory networks associated with embryogenic competence in olive and contributes to the identification of potential molecular markers for improving SE efficiency.

VIII. 3. MATERIAL AND METHODS

VIII. 3.1. Plant Material

Embryogenic cultures of *O. europaea* L. cv. ‘Arbequina’, previously established from zygotic embryos using the protocol described by Pires et al. (2020, 2025b), was used to select embryogenic lines exhibiting contrasting embryogenic efficiencies. Based on the criteria previously defined by Pires et al. (2025b), three HE (A55, A140, and A148) and three LE (A23, A157, and A158) embryogenic lines were selected and maintained through cyclic somatic embryogenesis in liquid ECO (Embryogenesis Cyclic Olive) culture medium. Following three consecutive subcultures of 30 days each, embryogenic tissues (HE and LE) were harvested, immediately frozen in liquid nitrogen, finely homogenised, and stored at -80°C until further analyses. CM from the harvested embryogenic lines was collected and further processed (see below 2.2).

VIII. 3.2. Proteome and Secretome Sample Preparation and Analysis by LC-MS/MS

Proteome isolation from embryogenic lines was performed according to Pires et al. (2026b). Secretome isolation from the CM collected from the HE and LE lines was performed according to the protocol previously described by Pires et al. (2025a). For LC-MS/MS analysis at the Proteomics Unit of the National Centre for Biotechnology (Madrid, Spain), proteome and CM-derived secretome samples were subjected to different digestion workflows due to their distinct biochemical characteristics,

in order to optimise protein recovery and peptide generation efficiency. Embryogenic calli, characterised by a complex intracellular protein matrix and high detergent content, were processed using SP3-OT-2 and S-Trap-based workflows according to previously established protocols (Ciordia et al., 2022, 2024). Briefly, embryogenic tissues were lysed in a buffer containing 5% sodium dodecyl sulfate (SDS), 25 mM triethylammonium bicarbonate (TEAB), 5 mM tris(2-carboxyethyl) phosphine (TCEP), and 10 mM chloroacetamide (CAA). Samples were incubated at 60 °C for 30 min for cysteine reduction and alkylation, followed by sonication and centrifugation. Protein concentration was determined using the Pierce 660 nm assay supplemented with ionic detergent compatibility reagent (Thermo Scientific). Subsequently, for the SP3-based digestion workflow, 50 µg of protein were automatically processed on the OT-2 platform and digested overnight at 37°C.

In contrast, CM-derived secretome samples, characterised by lower protein abundance and reduced sample complexity, were processed using S-Trap columns (Protifi, Huntington, NY, USA) following previously described protocols (Ciordia et al., 2021, 2024). Protein reduction and alkylation were performed under the same buffer conditions described above, followed by overnight tryptic digestion at 37 °C. Peptides were subsequently desalted using homemade C18 StageTips, dried under vacuum, and quantified by fluorimetry (Qubit) according to the manufacturer's instructions. Proteomic analyses of embryogenic tissues and CM-derived secretome samples were performed by nano-liquid Chromatography coupled to Electrospray Ionization Tandem Mass Spectrometry (nanoLC-ESI-MS/MS) following the workflow previously described by Pires et al. (2026b). Briefly, 1 µg of each sample was analysed using an Ultimate 3000 nano HPLC system (Thermo Fisher Scientific) coupled online to an Orbitrap Exploris™ 240 mass spectrometer (Thermo Fisher Scientific).

Proteomic datasets used in this study were previously generated and processed as described by Pires et al. (2026b). Briefly, raw MS files from embryogenic tissue and CM-derived secretome samples were analysed using Proteome Discoverer v2.5.0.400 (Thermo Fisher Scientific) with Mascot, MsAmanda, MsFragger, and Sequest HT search engines against the *O. europaea* UniProtKB database supplemented with common contaminant sequences (cRAP database). Protein identification was validated using a false discovery rate (FDR) threshold of 1% at peptide spectrum match (PSM), peptide, and protein levels. Label-free quantification was performed within the Proteome Discoverer environment using precursor ion intensity-based quantification workflows. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2025) partner repository with the dataset identifiers PXD075995 and PXD079057.

VIII. 3.3. Integrated Proteome–Secretome Analysis

Proteomic (tissue; PROT) and secretomic (secreted to culture medium; SEC) datasets were obtained from HE and LE *O. europaea* SE lines. Protein abundance matrices from both compartments were merged based on shared protein identifiers to generate an integrated dataset.

Raw intensity values were log₂-transformed, and zero values were treated as missing. To ensure comparability across samples, data were normalised using quantile normalisation implemented in the limma package in R (Ritchie et al., 2015a). To assess differences between HE and LE lines, linear models were fitted separately for proteome and secretome datasets using empirical Bayes moderation. Log₂ fold changes (HE vs LE) were calculated independently for each compartment. Thresholds were applied to define regulation patterns, including concordant upregulation in both compartments (High_High), concordant downregulation (Low_Low), and discordant patterns where proteins showed opposite trends between intracellular abundance and secretion (HighProt_LowSec and LowProt_HighSec). Proteins not meeting these criteria were classified as intermediate. This approach enabled discrimination between global protein abundance changes and compartment-specific regulation.

VIII. 3.4. Differential Expression Analysis of the Proteome Dataset, Functional Annotation and Pathway Analysis

The identification of differentially expressed proteins (DAPs) was performed at the proteome level by comparing HE and LE datasets. Protein abundance values were log₂-transformed, and zero values were treated as missing. To ensure comparability across samples, data were normalised using quantile normalisation implemented in the limma package in R (Ritchie et al., 2015b). Differential protein abundance between HE and LE lines was assessed using linear modelling with empirical Bayes moderation. A design matrix was constructed with embryogenic lines (HE vs LE) as the main factor, and statistical significance was evaluated using moderated t-tests. Resulting p-values were adjusted for multiple testing using the Benjamini–Hochberg FDR correction. Proteins were selected for downstream visualisation using a threshold (FDR < 0.1) or by ranking based on adjusted *p*-values. A heatmap was generated using the top 50 ranked proteins using the pheatmap package in R.

Differentially abundant proteins between HE and LE lines were functionally annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Plant Reactome databases implemented in OmicsBox (Gupta et al., 2024). KEGG provided broad metabolic and cellular pathway annotations, while Plant Reactome enabled the identification of plant-specific biological processes, including hormone biosynthesis, developmental pathways, and stress responses. Pathways were merged into higher-level functional categories to reduce redundancy. To enable direct comparison between lines,

category counts were normalised to relative proportions within each dataset. Functional distributions were visualised using stacked bar plots generated in R with the ggplot2 package.

VIII. 3.5. Functional Analysis of Peroxidases

Class III PODs proteins were analysed as a representative functional group to explore biologically relevant regulation patterns in *O. europaea* SE lines. Changes in abundance between HE and LE lines were evaluated separately for proteome and secretome fractions. Distribution of fold changes among POD family members were visualised, using boxplots, allowing comparison of intracellular accumulation and extracellular secretion.

VIII. 3.5.1. Classification of peroxidases and comparative analysis among compartments

To distinguish secreted PODs from intracellular PODs, signal peptide prediction was performed using SignalP online server (Teufel et al., 2022a). Proteins containing a predicted signal peptide were classified as secreted, while those lacking a signal peptide were classified as non-secreted. Differential protein abundance between HE and LE lines was evaluated separately for proteome and secretome fractions, and log₂ fold changes were calculated as described above. To assess whether secretion status influences protein regulation, distributions of log₂ fold changes were compared between secreted and non-secreted proteins in both compartments.

VIII. 3.5.2. Identification of the class III peroxidase gene family members in *Olea europaea* subsp. *europaea*

The genome and gene annotation files of *O. europaea* subsp. *europaea* cv. “Farga” (Genome assembly OLEA9) were retrieved from Ensembl Plants (release 62), and Class III POD genes were identified through BlastX against Viridiplantae protein sequences, using the software OmicsBox 3.3.2. (BioBam Informatics, Valencia, Spain). The key parameters were set as default, and the cutoff value was set as 1E-3. Candidate Class III peroxidase proteins were further validated by confirming the presence of conserved domains using Pfam (<https://www.ebi.ac.uk/interpro/entry/pfam>), InterProScan (Kozomara & Griffiths-Jones, 2014), and HMMER (<https://www.ebi.ac.uk/Tools/hmmer/search/hmmsearch>). Only sequences with complete conserved domains were retained.

VIII. 3.5.3. Physicochemical characterisation of class III peroxidase proteins and prediction of subcellular location

The gene ID, chromosomal location, coding sequence and putative amino acid sequence length of *O. europaea* class III peroxidases were retrieved from the EnsemblPlants database. The predicted physicochemical properties of the encoded peptides were estimated using the ProtParam tool of ExPASy Server (<https://web.expasy.org/protparam/>), including protein molecular weight (MW) and isoelectric point (pI) values. Subcellular localisation of proteins was predicted using DeepLoc (Thummuluri et al., 2022). Protein amino acid sequences were used as input, and localisation probabilities were assigned across multiple cellular compartments based on deep learning models.

VIII. 3.5.4. Gene organisation, chromosomal location and duplication events analysis

The exon–intron organization of class III POD genes was visualised using TBtools–II (Chen et al., 2023a), according to their genome annotation files. Motifs and domains were visualised using TBtools, using the output of MEME Suite (maximum –20 conserved motifs and a motif width of 10 to 50 amino acids) (Bailey et al., 2015) and CDD database at NCBI (Wang et al., 2023) (expect value threshold = 0.01, composition–corrected scoring), respectively.

Syntenic relationships of class III POD genes between *O. europaea* subsp. *europaea*, *O. europaea* subsp. *europaea* var. *sylvestris* and *Abeliophyllum distichum* (Oleaceae family) (Teufel et al., 2022b) were visualised using the Multiple Synteny Plot in TBtools. Duplicated chitinase gene pairs in *O. europaea* were identified using TBtools by conducting an all–versus–all BLASTP search (E -value $\leq 1e-5$) followed by MCScanX collinearity analysis (Wang et al., 2012) with genome annotation files. Segmental duplications were defined based on syntenic blocks, and tandem duplications as adjacent homologous genes separated by 5 or fewer genes within a 100 kb region on the same chromosome (Zhao et al., 2019).

VIII. 3.6. RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR (qPCR)

VIII. 3.6.1. RNA extraction and cDNA synthesis

Total RNA was isolated with the Maxwell 16 LEV simplyRNA purification kit (Promega, Madison, WI, USA) on a Maxwell 16 Instrument (Promega, Madison, WI, USA) according to the supplier's instructions and eluted in 50 μ L of RNase-free water. The concentration of total RNA was determined with a NanoDrop-2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Total RNA purity was assessed based on the A260/A280 and A260/A230 absorbance ratios, with values close to 2.0 considered indicative of high-quality RNA (Thermo Scientific, 2009). RNA integrity was evaluated by electrophoresis on a 1.2% (w/v) agarose gel prepared in Tris–acetate–EDTA (TAE) buffer. The gel was supplemented with GreenSafe nucleic acid dye (2 µL per 100 mL of agarose) to visualise nucleic acids under UV light. For each sample, 5 µL of RNA was mixed with 4 µL of RNA loading buffer (Thermo Scientific) and loaded onto the gel. Migration of the RNA bands was monitored using the NZYDNA Ladder VII molecular marker (NZYTech). Gel images were recorded and assessed using a UV transilluminator (GENE FLASH, Syngene Bio Imaging).

cDNA was synthesised from 1 µg of total RNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, Germany) in a T100™ Thermal Cycler (Bio-Rad®), following the manufacturer's instructions. The reaction mixture was prepared as a master mix for all samples, to which the appropriate volume of RNA and nuclease-free water was added to a final reaction volume of 20 µL per sample. Reverse transcription was performed under the following thermal conditions: 25°C for 10 min for primer annealing, followed by 42°C for 15 min, then 48°C for 15 min. A final enzyme inactivation step was performed at 85°C for 5 min. The resulting cDNA was diluted 1:100 in nuclease-free water and divided into 20 µL aliquots for downstream applications.

VIII. 3.6.2. Gene expression analysis through reverse transcription quantitative PCR (RT-qPCR)

For target and reference genes amplification gene-specific primers for *O. europaea* POD genes and putative reference genes were designed using Primer-BLAST (NCBI; <https://www.ncbi.nlm.nih.gov/tools/primer-blast>) based on exon sequences retrieved from EnsemblPlants (<https://plants.ensembl.org> (accessed on 21 October 2025)). Expression stability of Elongation factor-1 α (OeEF1a), Ubiquitin-conjugating enzyme E2 (OeUBI), Iron superoxide dismutase 1 (OeFeSOD1), and Vacuolar fusion SAND family protein (OeSAND) were tested. From those, OeEF1a and OeUBI were selected based on Genorm algorithm for target gene expression data normalisation.

Real-time PCR was performed with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in 96-well plates. All the reactions were prepared to a final volume of 16 µL, each containing 1x SYBR Green qPCR Master Mix (Bioline, Germany), 0.2 or 0.3 µM of each specific primer pair (gene specificities described in Table 1), and 5 µL of cDNA template (1:5). Three technical replicates were considered for each biological replicate. To discard the presence of contamination, non-template controls (NTCs) were performed for each primer pair. Three technical replicates were considered for each biological replicate. The amplification was carried out considering

an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 s, and a combined annealing/extension phase at 60-64°C for 30 s (see details in Table VIII.1).

The presence of primer-dimers or non-specific amplicons was evaluated through the analysis of the dissociation (melting) curve, obtained at the end of each reaction by subjecting the samples to a temperature gradient from 60 to 95°C. To assess the amplification efficiency (*E*) of the RT-qPCR reaction, a six-point standard curve of a two-fold dilution series (1:1 to 1:32) was prepared from a single cDNA sample achieved by pooling 2 µL of each cDNA aliquot. *E* (%) was determined based on the slope of each standard curve according to the formula of (Rebrikov & Trofimov, 2006). *E* values in the range of 90-110% were defined as acceptable (Rogers-Broadway & Karteris, 2015).

For transcript expression levels normalisation, Cq values were converted into relative quantities (RQ) by the delta-Ct method described by Vandesompele et al. (2002). The normalisation factor was determined by the GeNorm algorithm (Vandesompele et al., 2002), which corresponds to the geometric mean between the RQ of the selected reference genes for each sample. Finally, calculating the ratio between the RQ for each sample and the corresponding.

Table VIII.1. Primers designed for target and candidate reference genes, the respective concentration (nM), expected amplicon size (AS), and annealing temperature (Ta) for the RT-qPCR reaction.

Gene	Locus id	Primer Sequences 5'-3'	nM	AS (bp)	Ta (°C)
OePOD	OE9A030280	Fw: CAGTGGTTCTAAGTGGAGGA Rv: TGCATTGGCTCTTGGACTTG	200	113	61
OePOD	OE9A000398	Fw: GGTCGGCTTGCTTGGAGGA Rv: CATCCCTCCTGCCTAGTGGT	200	53	61
OePOD	OE9A064890	Fw: TGTGTCTTCATGAGAGAGAATTCAG Rv: GCACAATTTTCGAGCCAAAGAC	200	52	61
OePOD	OE9A073614	Fw: TGACCCTTCCATTGACACCG Rv: CTCTTGGAAGAGTCGCCGTT	200	75	62
OeEF-1α	XM_023019313.1	Fw: TTTTGAGGGTGACAACATGAT Rv: CAGGGTTGGGCCCTTGATC	300	76	61
OeUBI	XM_022996901.1	Fw: TGATGTTGCGGCTATTCTTACTTC Rv: GAACATCCGAGCAGCTTCAGA	300	71	61

VIII. 3.7. Determination of Protein Expression

VIII. 3.7.1. Protein extraction, solubilization and quantification

Proteins from HE (n=3) and LE (n=3) embryogenic lines were extracted using TCA/acetone precipitation, following the method of Méchin et al. (2007) with minor modifications, according to Pires

et al. (2025a). To improve solubilization, samples were incubated for 45 min on a multi-tube vortexer. Insoluble material was removed by centrifugation ($5000 \times g$, 5 min), and the supernatants from two tubes corresponding to the same embryogenic line were combined and transferred to a new Eppendorf tube. Total protein concentration was determined using the Pierce 660 nm Protein Assay Reagent Kit (Thermo Scientific™) according to the manufacturer's instructions.

VIII. 3.7.2. Protein analysis by western blot

A total of 20 µg protein of each sample was solubilised in Laemmli loading buffer and heated at 37 °C for 10 min. Samples and the molecular mass standard (Bio-Rad Precision Plus Protein™ Dual Color 161-0394) were loaded in a 14% polyacrylamide mini-gel (8 cm x 10 cm x 0.75 mm). SDS-PAGE was performed at 130 V on a Mini-PROTEAN System (Bio-Rad) until the dye front reached the end of the gel (Laemmli, 1970). Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham™ Hybond™) at 25 V, 1 A for 50 min using a Semi-dry Trans-Blot Turbo Transfer System (Bio-Rad). After 2 h blocking with 5% non-fat dry milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20), membrane was incubated overnight at 4 °C with primary antibody Anti-HRP (Agrisera AS16 3951, dilution 1:1000). Thereafter, the membrane was washed and incubated 2 h at room temperature with an alkaline phosphatase-linked secondary antibody (anti-rabbit IgG, Agrisera AS09607, dilution 1:10000). PODs bands were detected using an ECF Plus Western Blotting Detection Reagents (GE, Healthcare) on a Bio-Rad Gel-doc system. Semi-quantitative analysis of band expression was carried out using the software Bio-Rad Image Lab 5.2.1.

VIII. 3.7.3. Determination of hydrogen peroxide levels

The quantification of H₂O₂ was performed by spectrophotometry, following the protocol described by Alexieva et al. (2001), with minor modifications. The procedure was applied to three HE and three LE embryogenic lines, each analysed in triplicate. Briefly, 200 mg of ground plant material were homogenised in 1,2 µL of 0.1% (w/v) trichloroacetic acid (TCA), followed by vortexing and centrifugation for 15 minutes ($16,000 \times g$; 4 °C). The resulting supernatant from each sample was collected, while the corresponding pellet was retained for total protein quantification and enzymatic expression analysis via Western blot.

From the recovered supernatant, 50 µL were transferred to the respective wells of a microplate. Subsequently, 50 µL of phosphate buffer (100 mM, pH 7.0) and 200 µL of 1 M potassium iodide were added. The microplate was incubated in the dark at room temperature for 1 hour, after which the absorbance was measured spectrophotometrically at 405 nm. (Multiskan™ FC; ThermoScientific). The H₂O₂ content was determined by interpolation against a standard curve prepared using known

concentrations of H₂O₂ (30%) at 0, 37,5, 75, 150, 300, 600, 1200, 2500, and 5000 µM/mL. H₂O₂ levels were quantified for all HE and LE embryogenic lines, with three technical replicates analysed per line.

VIII. 3.8. Statistical Analysis

Statistical analyses were performed by SPSS version 28.0 (SPSS Inc., Chicago, IL, USA). Normality and homogeneity of variances were assessed using the Shapiro-Wilk and Levene's tests, respectively. Comparisons between HE and LE embryogenic lines were performed using one-way ANOVA (followed by Tukey's HSD test) or the Kruskal–Walli's test when parametric assumptions were not met. Statistical significance was set at $p < 0.05$.

VIII. 4. RESULTS

VIII. 4.1. Integrated Analysis of Proteome and Secretome

The integrated analysis of proteome and secretome data revealed distinct patterns of protein regulation between HE and LE embryogenic lines (Figure VIII.1). Most proteins clustered around the centre of the plot, indicating relatively small changes in abundance across both compartments. However, several subsets of proteins exhibited clear differential regulation (Table D.1).

A group of proteins showed concordant upregulation in both the proteome and secretome (High_High) (Table D.2), suggesting coordinated increases in intracellular abundance and secretion in HE lines. Conversely, a smaller set of proteins displayed concordant downregulation (Low_Low) (Table D.3), indicating reduced abundance in both compartments. Importantly, several proteins exhibited discordant regulation patterns. Proteins classified as HighProt_LowSec (Table D.4) were more abundant in the proteome, suggesting limited secretion or intracellular retention. In contrast, proteins in the LowProt_HighSec (Table D.5) group showed increased abundance in the secretome despite low intracellular levels, indicating preferential secretion or accumulation in the extracellular space.

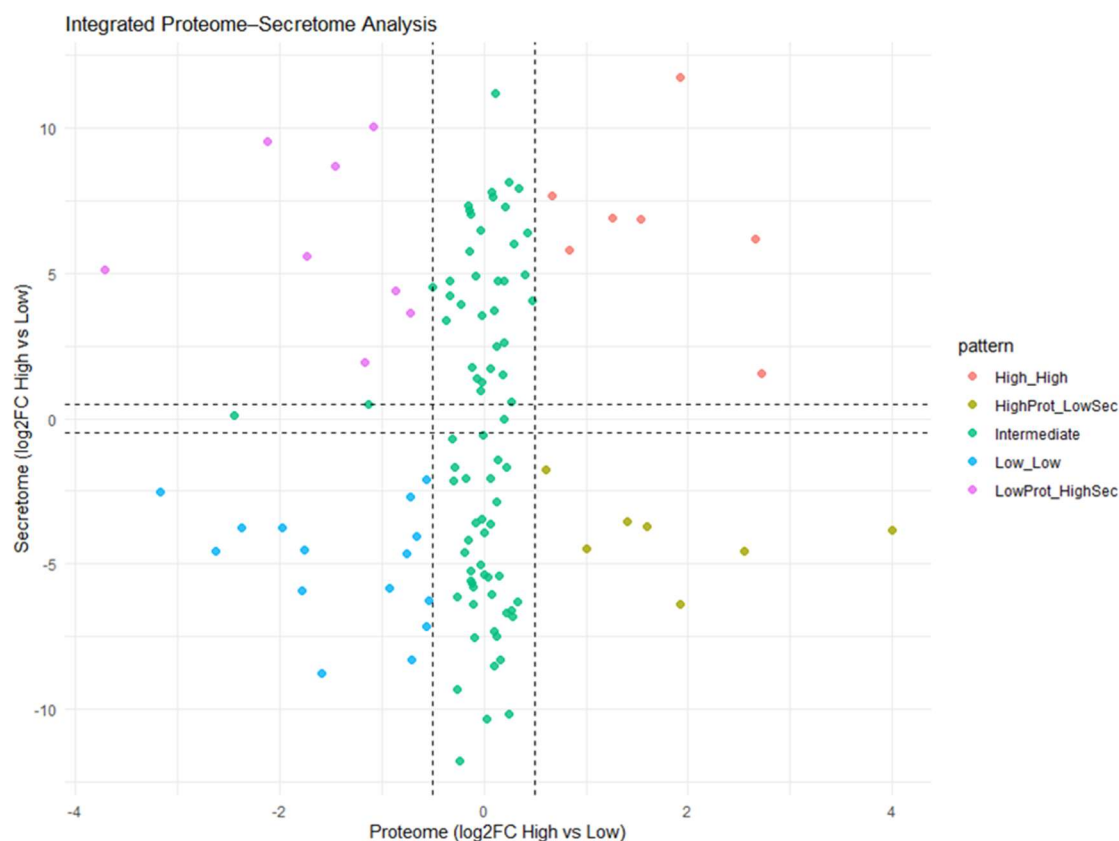


Figure VIII.1. Integrated proteome–secretome analysis of differential protein abundance between high-efficiency (HE) and low-efficiency (LE) lines. Scatter plot comparing log₂ fold change (HE vs LE) in the proteome (x-axis) and secretome (y-axis). Each point represents a protein. Dashed lines indicate thresholds for differential regulation. Proteins are classified according to their regulation patterns: concordant upregulation in both compartments (High_High), concordant downregulation (Low_Low), discordant patterns between proteome and secretome (HighProt_LowSec and LowProt_HighSec), and intermediate responses.

VIII. 4.2. Comparative Proteome Profiling of HE and LE Embryogenic Lines

Analysis of the most differentially abundant proteins from the proteome dataset revealed a clear separation HE and LE embryogenic lines (Figure VIII.2A), with distinct clustering of samples according to embryogenic competence. Proteins enriched in HE predominantly included enzymes and regulatory proteins associated with metabolic reprogramming, such as peroxidase P7-like, thioredoxin reductase NTRB-like, and putative thiol-disulfide oxidoreductase, as well as proteins involved in secondary metabolism, including premnaspirodiene oxygenase-like and probable linoleate 9S-lipoxygenase 5 (Figure VIII.2A). These proteins suggest an active redox and signalling environment in HE tissues. In contrast, proteins more abundant in LE were associated with a broader range of cellular functions, including protein synthesis and mitochondrial activity, such as 40S ribosomal protein S16-like,

elongation factor Tu, mitochondrial-like, and NADH dehydrogenase (ubiquinone) iron-sulfur protein 4, as well as enzymes involved in primary metabolism (Figure VIII.2A).

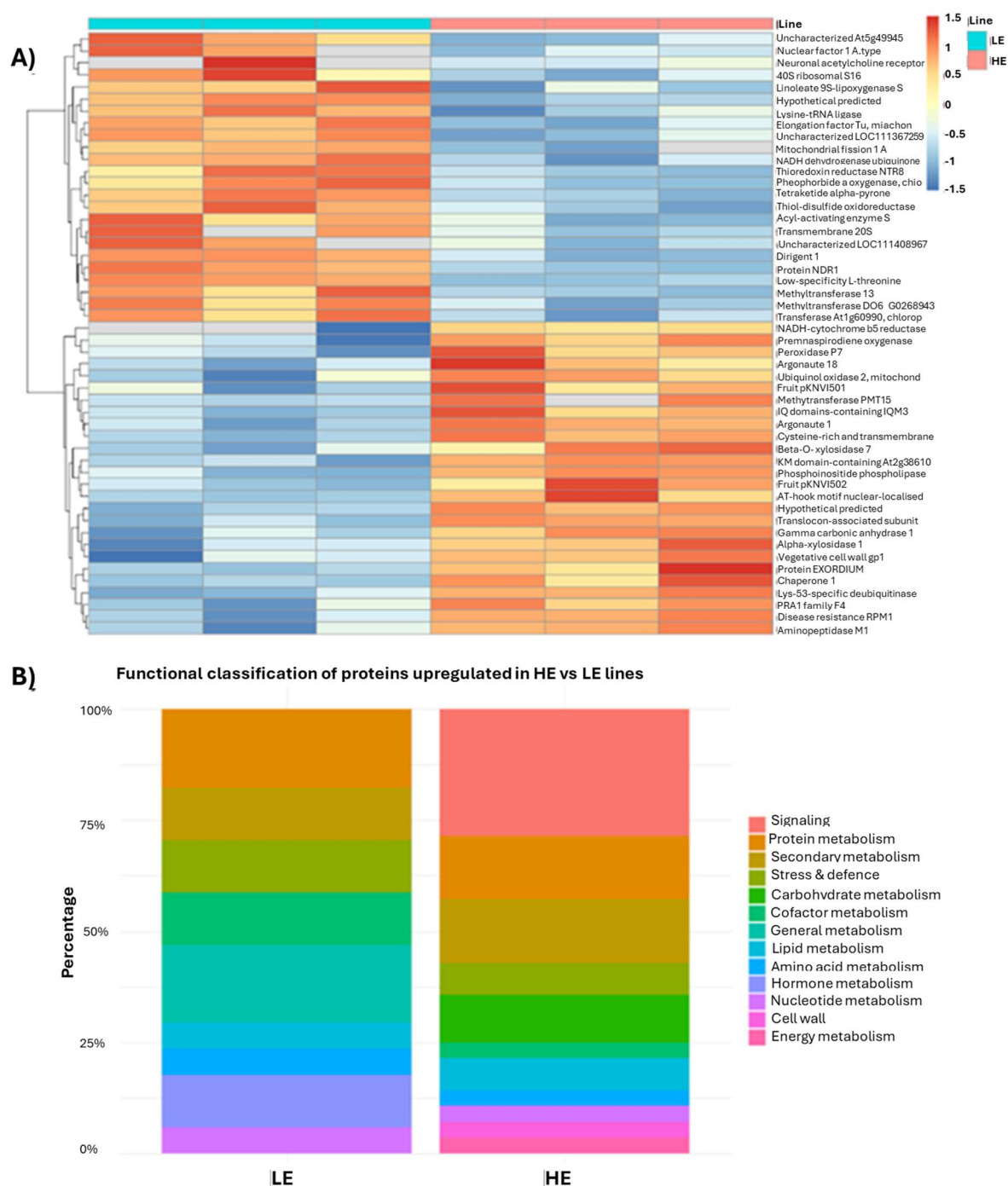


Figure VIII.2. A) Heatmap of the top 50 differentially abundant proteins between high-efficiency (HE) and low-efficiency (LE) embryogenic lines in the proteome dataset. Red denotes higher abundance and blue indicates lower abundance. B) Functional classification of proteins upregulated in HE and LE lines. Proteins were annotated using KEGG and Plant Reactome databases and grouped into major functional categories. Stacked bars represent the percentage of proteins in each category.

Functional classification of proteins upregulated in each condition, as obtained by KEGG and Plant Reactome databases, further emphasised the differences between lines with different embryogenic competence (HE and LE) (Figure VIII.2B). In HE lines, proteins were predominantly associated with signalling, secondary and protein metabolism, which together represented the largest proportion of functional categories, suggesting active regulation and adaptation mechanisms in HE lines. In contrast, LE lines showed a more homogeneous distribution of functional categories, with a slightly higher relative contribution of protein metabolism and general metabolism. Categories such as amino acid, hormone and nucleotide metabolism were slightly more represented in LE compared to HE (Figure VIII.2B).

VIII. 4.3. Functional Analysis of Proteome and Secretome Peroxidases

Differential protein abundance between HE and LE lines was evaluated separately for proteome and secretome fractions, enabling evaluation of differences in intracellular accumulation and extracellular enrichment between PODs. Plant PODs comprise multiple classes with distinct cellular localisations and biological functions, including intracellular antioxidant enzymes and secreted class III PODs involved in cell wall-related processes.

POD proteins displayed distinct regulatory patterns between HE and LE lines (Figure VIII.3A). In the proteome, most non-secreted POD classes (no peptide signal), including APX, and peroxiredoxins, showed relatively small changes between conditions, with values centered around zero, and GPX showed a slight increase in HE vs LE. Class III PODs (with peptide signal) exhibited a clear tendency toward increased abundance in HE, although variability was observed among individual proteins. A stronger effect was evident in the secretome, where secreted class III PODs showed a pronounced increase in HE compared to LE, with consistently high positive fold-change values (Figure VIII.3A). This pattern indicates that differential secretion, rather than intracellular abundance, is a key feature of POD regulation in somatic embryogenic tissues.

Subcellular localisation analysis further supported functional differences among peroxidase classes (Figure 3B). Non-secreted peroxidases were distributed across multiple compartments, including cytoplasm, mitochondria, and plastids, reflecting their roles in intracellular redox homeostasis. In contrast, secreted class III PODs were predominantly associated with extracellular and vacuole compartments (Figure VIII.3B).

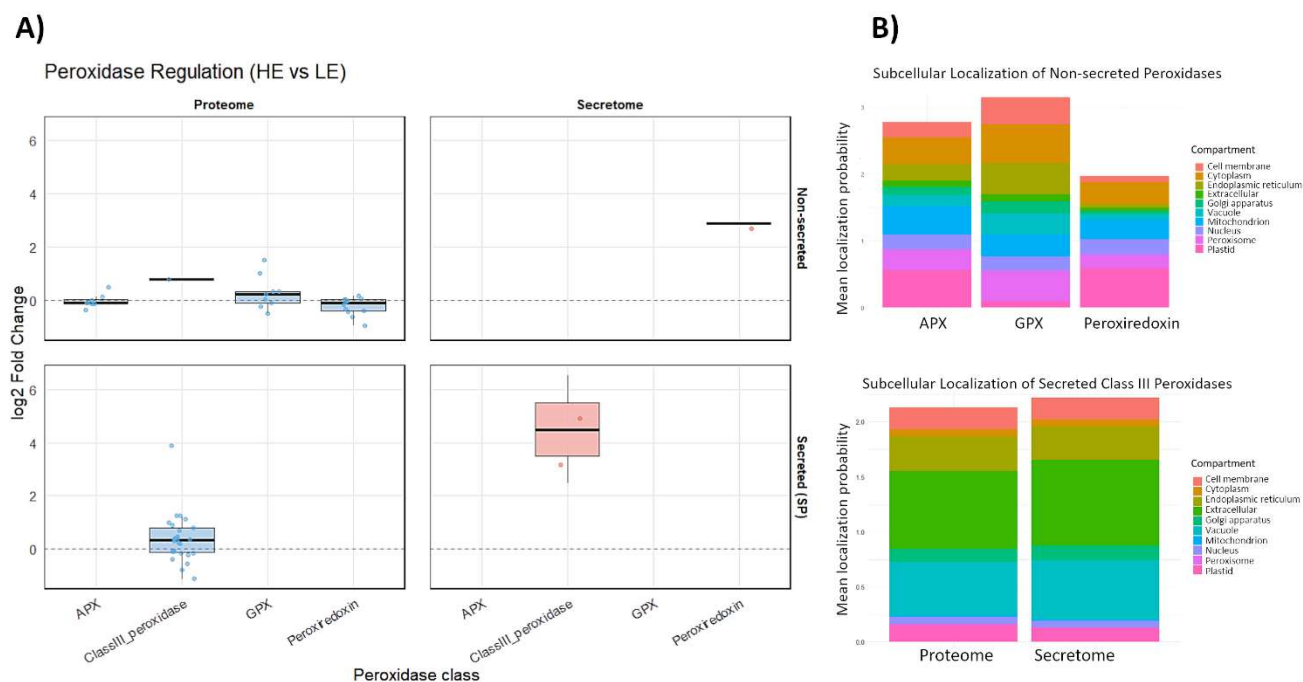


Figure VIII.3. Regulation and subcellular localisation of peroxidase classes in HE and LE lines. (A) Log2 fold change of peroxidase (POD) classes between high efficiency (HE) and low efficiency (LE) somatic embryogenic lines in the proteome and secretome compartments. Proteins are grouped into non-secreted and secreted (signal peptide, SP) fractions. Points represent individual proteins and boxplots summarise their distribution. B) Subcellular localisation of peroxidases as predicted by DeepLoc. Stacked bar plot shows the mean localisation probability of ascorbate peroxidases (APX), glutathione peroxidases (GPX), and peroxiredoxins lacking signal peptides, and the mean localisation probability of Class III PODs with signal peptides in secretome and proteome datasets. For each POD class, DeepLoc prediction scores were averaged across proteins for each subcellular compartment, and the resulting mean probabilities are displayed as stacked proportions. Higher values indicate a stronger predicted association with a given compartment. Stacked bars represent the overall distribution of localisation tendencies rather than exclusive assignments. Colours represent different cellular compartments.

VIII. 4.4. Genome-wide Identification and Expansion Patterns of Class III Peroxidase Genes in *Olea europaea* subsp. *europaea*

A total of 54 *O. europaea* Class III POD genes were accurately identified (named *OePOD*). The predicted protein molecular weights ranged from 14.78 kDa (OE9A011975) to 66.58 kDa (OE9A099911), although the majority of proteins clustered within ~34–40 kDa, consistent with typical plant class III peroxidases. The presence of a few smaller or larger proteins suggests potential structural

divergence (Table D.6). The isoelectric point (pI) values varied from 4.56 to 9.50. Most proteins exhibited alkaline pI values ($pI > 7$), suggesting that the majority of *O. europaea* class III PODs are likely cationic, while a smaller subset displayed acidic characteristics (Table D.6).

Among the 54 class III PODs, 26 genes could be located on chromosomes, while the remaining 28 genes are specified in their contig (Table D.6, Figure VIII.4A, B). MCScanX tool was employed to identify duplication events in *O. europaea* subsp. *europaea* class III PODs. Segmentally duplicated genes were identified by collinearity analysis (Figure D.1). There were found tandem duplications in chromosomes 2 and 9 (Figure VIII.4A) and two segmental duplications (chromosomes 2 and 15 and chromosomes 4 and 5) (Figure VIII.4B). Multiple synteny analysis showed 24 collinear pairs between *O. europaea* and its wild relative and/or *Abeliophyllum distichum* (*Oleaceae* family) (Figure VIII.4B).

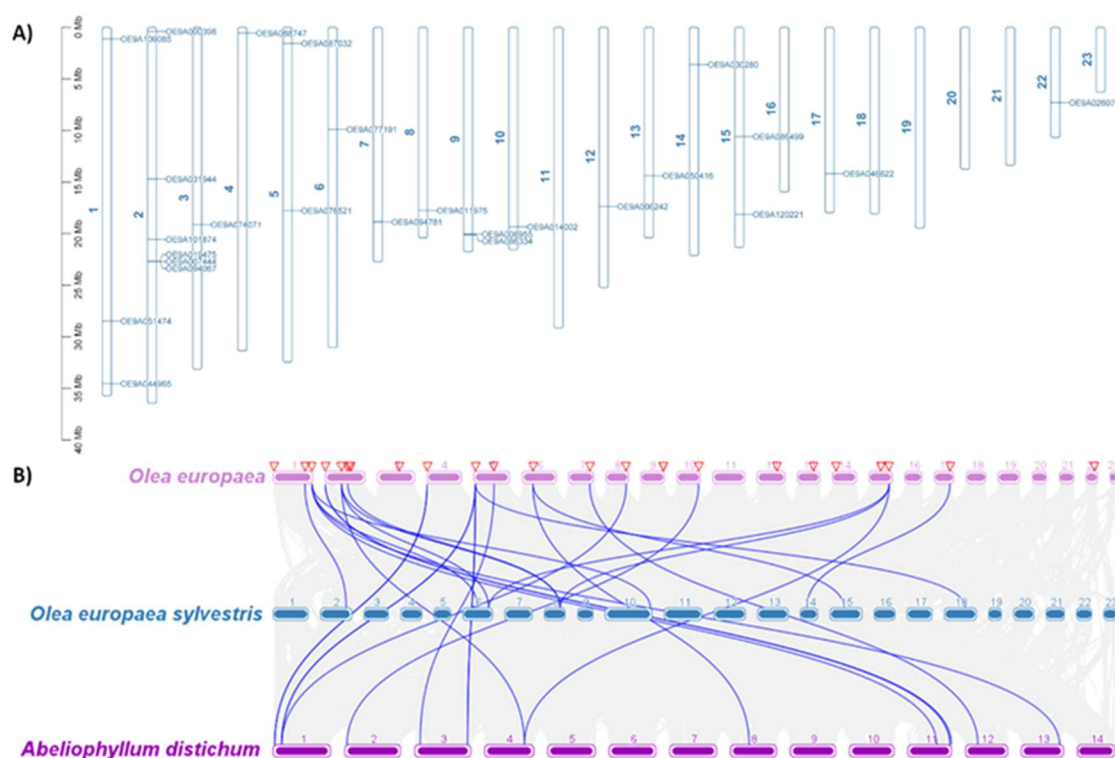


Figure VIII.4. A) Chromosomal localisation of Class III peroxidase (POD) genes across the 23 chromosomes of *O. europaea*, showing their physical positions along each chromosome. The scale of the chromosomes on the left is in megabases (Mb). B) The homology analysis of class III POD genes from *O. europaea* subsp. *europaea*, *O. europaea* subsp. *europaea* var. *sylvestris* and *Abeliophyllum distichum* genomes. The grey lines in the background indicate the homologous relationship within *O. europaea* subsp. *europaea* and the other two plant genomes, while the blue lines highlight the members of the chitinase gene family that have a homologous relationship between the three genomes. Numbers indicate chromosome number, and triangles indicate the location of the genes.

VIII. 4.5. Gene Structure, Conserved Motifs and Domain Organisation of Class III Peroxidases in *Olea europaea*

The 54 predicted *O. europaea* class III POD genes were analysed for structure, conserved motifs, and domain organisation (Figure VIII.5). The phylogenetic tree grouped the proteins into several clades, within which members generally exhibited similar structural and motif patterns.

A total of 20 conserved motifs (Motifs 1–20) were identified among the *O. europaea* class III POD proteins (Figure VIII.5A). Overall, most members exhibited a highly conserved motif composition and arrangement, particularly within the central region of the proteins. Several motifs were widely distributed across nearly all sequences, including motifs 1, 2, 3, 4, 6, and 7. These motifs were consistently arranged in a similar order, suggesting their importance for maintaining the structural and catalytic integrity of peroxidases. In contrast, other motifs showed variable distribution patterns. Motifs such as motif 5, 8, 10, and 11 were present in most but not all proteins, indicating moderate conservation and possible roles in functional specialisation. A subset of motifs, including motifs 12, 15, 16 and 20, exhibited restricted occurrence and were primarily located toward the N-terminal or C-terminal regions.

Most proteins contained a conserved class III POD domain (secretory peroxidase), spanning nearly the entire protein length (Figure VIII.5B). This domain showed a highly conserved position and size across the majority of genes, indicating strong evolutionary conservation of catalytic function. A small subset of genes displayed only plant POD-like superfamily (OE9A011083 and OE9A040599) or PLN03030 (cationic POD) domains (OE9A073614, OE9A026076 and OE9A037501), lacking the specific secretory peroxidase annotation (Figure VIII.5B). These proteins also tended to exhibit shorter or slightly shifted domain regions.

Gene structure analysis revealed that most *O. europaea* POD genes shared a similar exon–intron organisation. Nevertheless, some genes displayed structural variation, including differences in exon number and length, as observed for gene OE9A094781, which revealed an exceptionally large intron (Figure VIII.5C). These variations were particularly evident in the divergent genes identified by domain analysis, indicating that structural changes may accompany functional divergence.

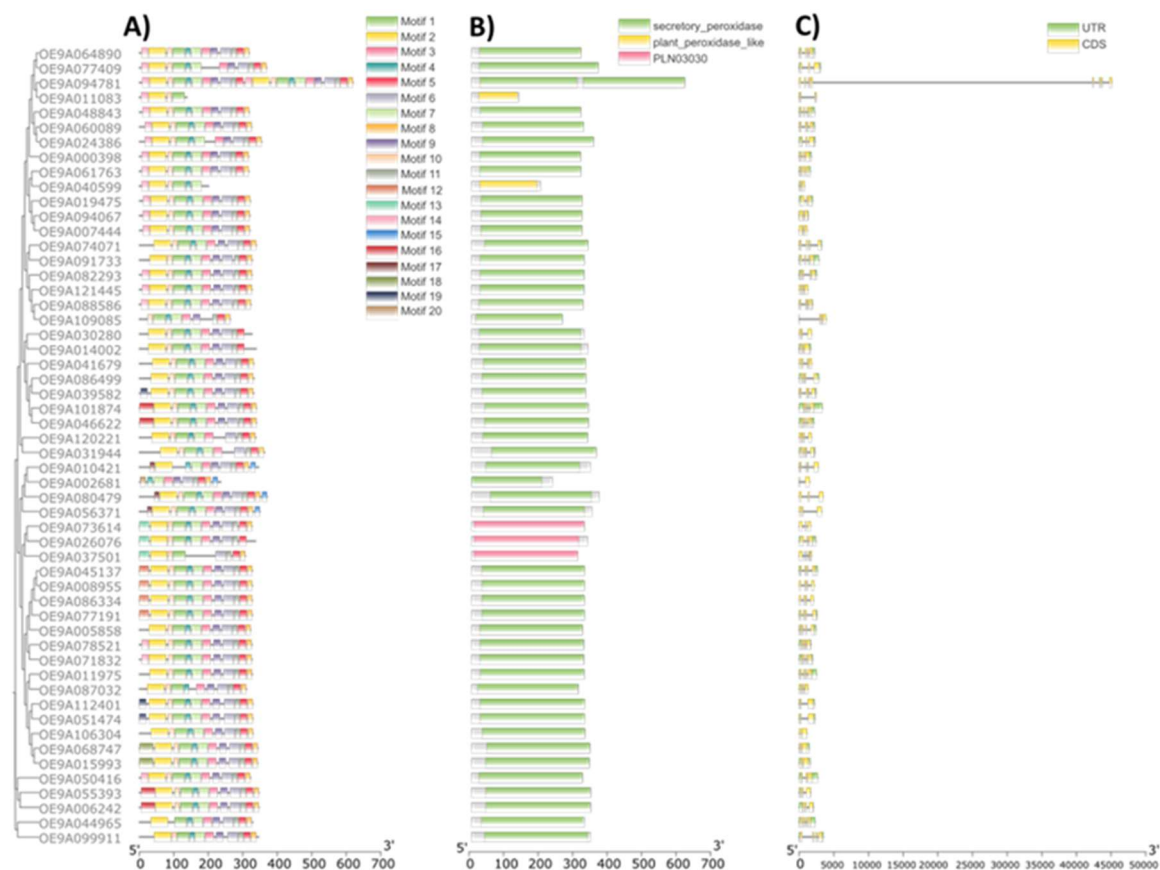


Figure VIII.5. Motif composition, conserved domains and gene structure of the 54 predicted class III POD genes of *Olea europaea* subsp. *europaea*. (A) Conserved motifs of *O. europaea* class III peroxidases (PODs) were identified by MEME, and different motifs are represented by specific colours (Motif 1 – 20). (B) Catalytic domains of *O. europaea* class III PODs, as identified by CDD database. (C) Gene structure of class III PODs in olive tree. Yellow boxes and black lines represent exons and introns, respectively.

VIII. 4.6. Expression Analysis of Class III *OePOD* genes

To validate the transcriptional patterns associated with SE competence, the relative expression levels of four selected *OePOD* genes were analysed by RT-qPCR in HE and LE embryogenic olive lines (Fig. 6). Overall, all analysed genes exhibited higher expression in HE lines compared with LE lines, supporting their possible involvement in embryogenic competence and somatic embryo development.

Among the analysed genes, OE9A064890 showed the highest differential expression, with transcript levels markedly higher in HE lines, particularly in A140, compared with consistently low expression in LE lines. Similarly, OE9A000398 was significantly upregulated in HE lines. The lines A140 and A148 showed the highest expression values, whereas all LE lines maintained reduced transcript abundance. This expression profile indicates that OE9A000398 may be involved in pathways

specifically activated during embryogenic competence acquisition. The gene OE9A030280 also displayed higher expression in HE lines compared with LE lines. Although transcript levels were lower than those observed for OE9A064890, a clear separation between embryogenic groups was evident, particularly in the A140 genotype, which exhibited significantly the highest transcript accumulation among all analysed lines. LE lines showed weak expression, especially lines A23 and A158, reinforcing the association between this gene and embryogenic responsiveness. A comparable trend was observed for OE9A073614, although the A148 line exhibited expression levels like those observed in LE lines. The A55 line showed the highest transcript accumulation; however, no significant differences were observed compared with the LE line A158.

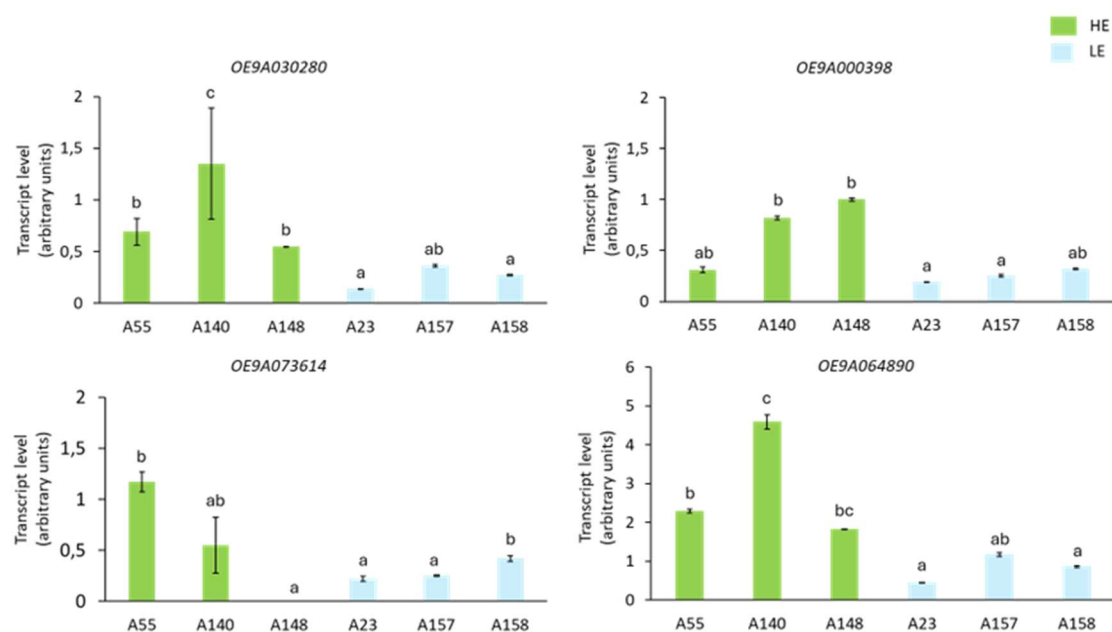


Figure VIII.6. Expression of all Class III *OePOD* genes, using *OeUBI* and *OeEF-1 α* as reference genes for data normalisation. The relative expression values are represented as the mean and standard error of biological replicates. Different letters correspond to statistically significant differences ($p \leq 0.05$).

VIII. 4.7. Peroxidase Expression by Western Blot

POD accumulation was analysed by Western blot in three HE and three LE lines, using an anti-horseradish peroxidase (HRP) antibody. Three technical replicates were analysed for each embryogenic line. Immunoblot analysis revealed several immunoreactive bands; four bands with distinct apparent molecular weights were selected for quantitative analysis of expression and are hereafter referred to as HRP1, HRP2, HRP3 and HRP4 (Figure VIII.7).

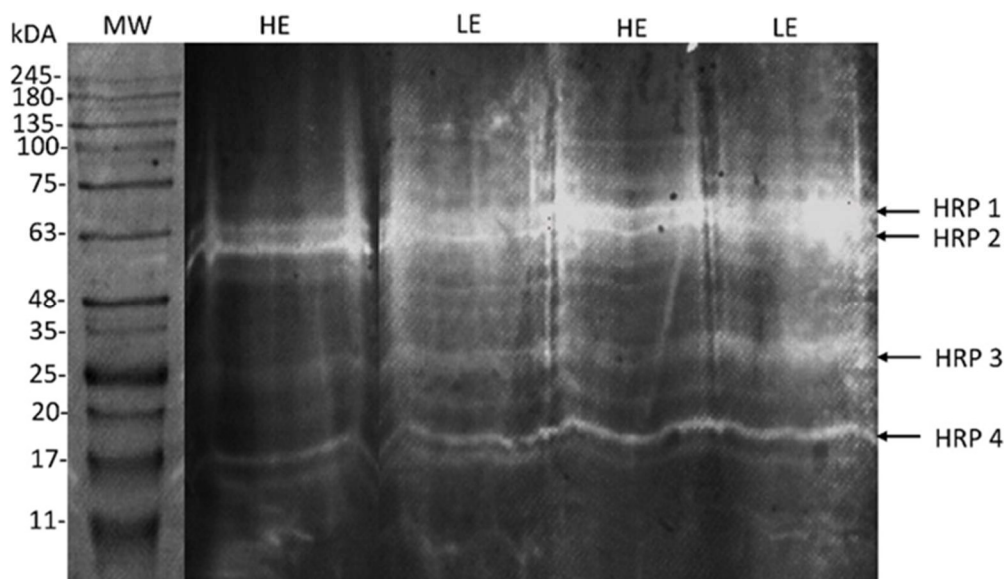


Figure VIII.7. Representative Western blot membrane showing HRP-immunoreactive peroxidase bands (HRP1–HRP4 in high-efficiency (HE) and low-efficiency (LE) embryonic olive lines.

The HRP3 band, with an apparent molecular weight of approximately 25 kDa, displayed similar signal intensities across all analysed lines, with no statistically significant differences detected between HE and LE groups (Figure VIII.7). In contrast, the HRP1 band (≈ 63 kDa) showed, overall, higher signal intensity in HE lines compared with LE lines (Figure VIII.7). Nevertheless, one HE line did not differ significantly from the two LE lines, which showed the lowest signal intensities (A23 and A157), while another LE line (A158) exhibited signal intensities comparable to those of both HE and LE lines. Regarding the HRP2 band (≈ 60 kDa), HE lines A140 and A148 were characterised by markedly higher signal intensities (Figure VIII.7). This trend was not observed in HE line A55, which exhibited signal levels comparable to those detected in LE lines. Although LE lines A157 and A158 generally showed lower signal intensities, no statistically significant differences were observed between HE and LE lines as groups. Finally, the HRP4 band, with an apparent molecular weight of approximately 17 kDa, showed higher signal intensity in HE lines relative to LE lines across all genotypes (Figure VIII.8). However, statistically significant differences were only detected for HE line A148 when compared with all LE lines. The HE lines A55 and A140 exhibited signal intensities comparable to both A148 and the LE lines.

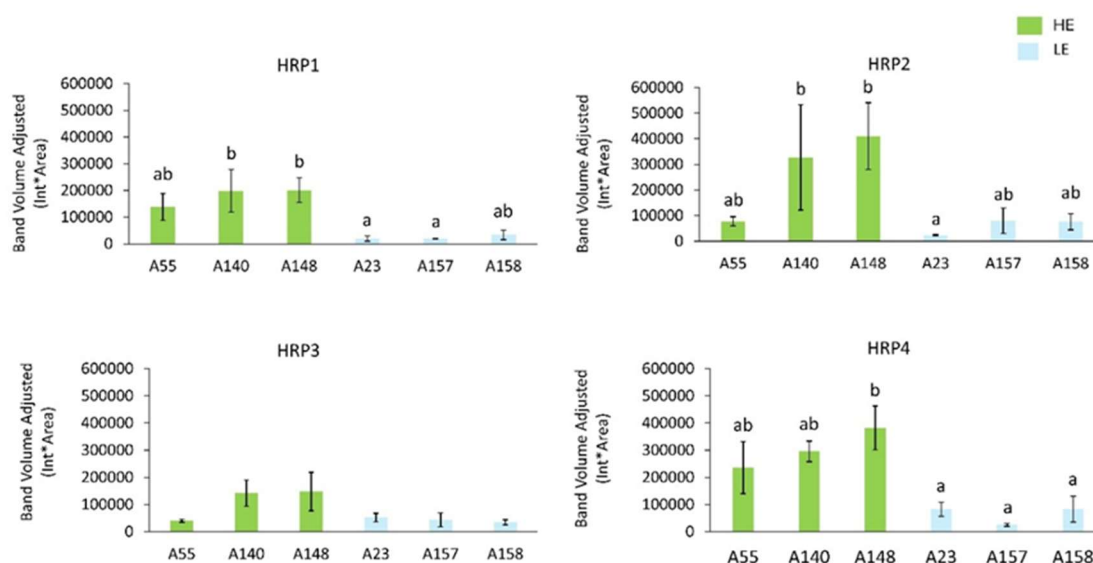


Figure VIII.8. Western blot analysis of peroxidases in three high-efficiency (HE) and three low-efficiency (LE) embryonic olive lines. Different lowercase letters indicate statistically significant differences ($p \leq 0.05$) within each group, whereas capital letters denote significant differences between groups (HE vs. LE).

VIII. 4.8. Determination of Hydrogen Peroxide

Based on the differential expression of Class III *OePODs* in HE lines identified by LC-MS/MS, H_2O_2 quantification was performed to assess potential variations in oxidative status among embryonic lines that could be related to enzyme activity. Quantification of H_2O_2 levels across the different embryonic lines revealed a distinct profile between the HE and LE groups (Figure VIII.9). H_2O_2 concentrations were consistently higher in the HE lines, with no significant differences detected among them. Within the HE group, A140 exhibited the highest H_2O_2 level ($1.43 \pm 0.3 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$), followed by A55 ($1.28 \pm 0.4 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$) and A148 ($0.99 \pm 0.06 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$). In contrast, significant differences were observed between the LE lines A23 and A158. Within the LE group, A23 ($0.38 \pm 0.06 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$) and A157 ($0.43 \pm 0.3 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$) had the lowest concentrations; however, A157 did not differ significantly from the other LE lines.

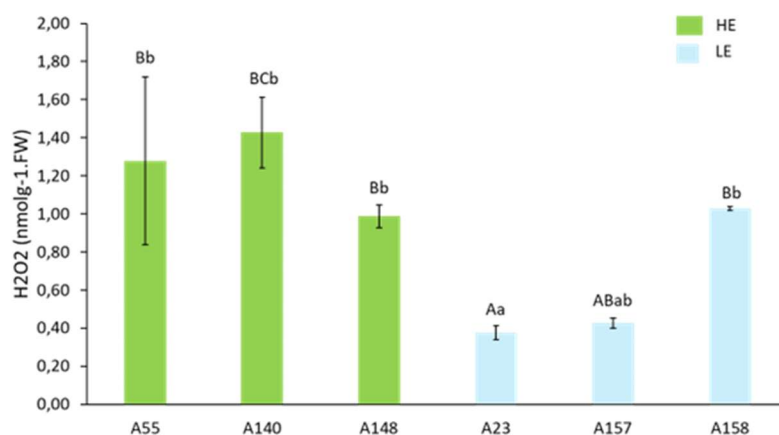


Figure VIII.9. Determination of hydrogen peroxide (H₂O₂) levels in three high-efficiency (HE) and three low-efficiency (LE) embryogenic olive lines. Different lowercase letters indicate statistically significant differences ($p \leq 0.05$) within each group, whereas capital letters denote significant differences between groups (HE vs. LE).

Notably, there were no significant differences between the HE lines and the LE line A158 (1.0 ± 0.01 nmol·g⁻¹ FW), suggesting variation within the LE group. These findings highlight a clear distinction in H₂O₂ accumulation between HE and LE lines, suggesting a potential link between embryogenic capacity and oxidative state.

VIII. 5. DISCUSSION

The acquisition of embryogenic competence is known to involve extensive cellular reprogramming accompanied by major changes in redox balance, stress responses and extracellular signalling pathways. In the present study, comparative proteomic and secretomic analyses clearly distinguished HE from LE embryogenic olive lines, supporting the existence of a specific molecular signature associated with embryogenic competence. Similar observations have been reported in other plant species, where SE is characterised by the activation of stress-related and redox-associated pathways that promote cellular dedifferentiation and developmental reprogramming (Zavattieri et al., 2010; Fehér, 2015; Elhiti & Stasolla, 2022). Proteins enriched in HE lines were mainly associated with redox regulation, signalling, and secondary metabolism, suggesting that high-embryogenic tissues maintain an active oxidative and signalling environment. In particular, the increased accumulation of POD P7-like, thioredoxin reductase and thiol-disulfide oxidoreductases reinforces the importance of cellular ROS homeostasis during SE. ROS are now recognised not only as toxic by-products of metabolism but also as important signalling molecules regulating cell division, differentiation and SE induction (Zhou et al., 2016; Rose, 2019). Controlled ROS accumulation has been associated with the

acquisition of embryogenic competence in several plant systems (Orłowska & Kępczyńska, 2020; Alam et al., 2025; Chen et al., 2026). In contrast, LE lines displayed enrichment of proteins mainly related to primary metabolism, translation and mitochondrial activity, including ribosomal proteins and components of the respiratory chain. This pattern may reflect a more differentiated physiological state with reduced developmental plasticity, whereas HE tissues appear to prioritise signalling and adaptive pathways required for embryogenic transition. Similar metabolic shifts between embryogenic and non-embryogenic tissues have previously been described in proteomic studies of SE, in which embryogenic tissues were characterised by enrichment of stress-related, redox-regulatory and signalling proteins, while non-embryogenic tissues showed higher accumulation of proteins associated with primary metabolism, translation and energy production machinery (Correia et al., 2012; Sharifi et al., 2012; Chen et al., 2023). In *Gossypium hirsutum* (Zhu et al., 2018) and *Zea mays* (Liu et al., 2018), embryogenic calli were particularly enriched in proteins linked to oxidative stress regulation, glycolysis and developmental reprogramming, whereas non-embryogenic tissues accumulated proteins associated with constitutive cellular metabolism and protein synthesis. Likewise, transcriptomic analyses in *Coffea arabica* demonstrated that embryogenic competence is accompanied by activation of regulatory, stress-responsive and hormone-related pathways, contrasting with the more basal metabolic profile observed in non-embryogenic tissues (Awada et al., 2023). In our study, one of the most relevant findings was the differential behaviour observed between intracellular and extracellular protein accumulation. Several proteins exhibited discordant abundance patterns between proteome and secretome fractions, indicating that selective secretion may represent an important regulatory mechanism during SE. Notably, secreted class III *OePODS* showed a pronounced enrichment in the secretome of HE lines, whereas intracellular non-secreted PODs displayed comparatively limited variation.

Class III PODs are plant-specific haem-containing enzymes belonging to large multigene families involved in ROS metabolism, cell wall modification, lignification, auxin catabolism and stress responses (Freitas et al., 2024). Their predominantly extracellular localisation is particularly relevant during SE, since apoplastic PODs participate in redox-mediated signalling processes that regulate cell fate transitions and developmental reprogramming (Hazubska-Przybył et al., 2020). Among ROS, H_2O_2 acts as an important oxidative signal during SE, contributing to polarity establishment and embryo patterning, as demonstrated in *P. dactylifera* (Din et al., 2021). Embryogenic tissues typically exhibit elevated H_2O_2 production due to their intense metabolic and mitotic activity (Din et al., 2022); however, controlled ROS accumulation is essential because moderate H_2O_2 concentrations function as signalling molecules regulating gene expression, protein synthesis and embryogenic induction (Kairong et al., 1999). In the present study, the strong enrichment of secreted class III PODs in HE lines suggests that these enzymes contribute to establishing an extracellular oxidative environment favourable for embryogenic development. Higher POD activity in HE tissues supports the idea that tightly regulated H_2O_2 homeostasis is required during early cellular reprogramming, allowing ROS to promote cell cycle

reactivation and embryogenic transition while preventing oxidative damage (Jomova et al., 2024). Moreover, apoplastic H_2O_2 may also function as a secondary messenger linking POD activity with hormonal regulation, since controlled ROS accumulation has been associated with activation of auxin biosynthesis and repression of cytokinin pathways involved in embryogenic competence (Lu et al., 2025). Similar associations between POD activity and embryogenic potential have been reported in several species, including *G. hirsutum* (Zhou et al., 2016), *Fraxinus mandshurica* (Yang et al., 2021), and *Larix* spp. (Zhu et al., 2024).

Western blot analysis further demonstrated stronger accumulation of HRP-immunoreactive POD isoforms in HE lines compared with LE lines, indicating that specific POD isoenzymes are associated with embryogenic competence in olive. Comparable tissue-specific POD isoform patterns have been observed in other species, including *G. glabra*, where distinct isoenzyme profiles were linked to different physiological states of calli tissues (Sarkar, 2024). In embryogenic cultures, enrichment of specific POD isoforms has been associated with the establishment and progression of SE (Galego et al., 2014; Oubli et al., 2021). Beyond ROS regulation, PODs also participate directly in cell wall remodelling through oxidative cross-linking of extensins and lignin precursors (Mishler-Elmore et al., 2021), thereby influencing cell elongation, division and acquisition of embryogenic identity. Consistent with these observations, HE lines exhibited higher POD activity and accumulation of proteins with molecular weights characteristic of extracellular class III PODs. Their predicted extracellular localisation reinforces their likely involvement in apoplastic redox regulation and cell wall dynamics during embryogenic development. Collectively, these findings strengthen the association between POD-mediated redox control, cell wall remodelling and embryogenic competence, supporting the potential use of PODs as biochemical markers of SE capacity in olive.

Given the prominent accumulation of class III PODs in HE secretomes, a comprehensive characterisation of the *OePOD* gene family was subsequently performed to better understand their structural diversity and potential functional roles during SE in olive. At present, the POD gene family has been reported in several plant species, including *Arabidopsis thaliana* (Tognolli et al., 2002), *Betula pendula* (Cai et al., 2021), *Malus domestica* (Lu et al., 2025), *Nicotiana tabacum* (Chen et al., 2022), *Psidium gualva* (Gull et al., 2024), and *Sesamum indicum* (Vishwakarma et al., 2024); however, comprehensive identification and functional analysis of the POD gene family in *O. europaea* remain unavailable. PODs are encoded by large multigene families in plants, although the number of POD members varies considerably among species (Shi et al., 2025). In the present study, based on the genomic information of *O. europaea*, a total of 54 POD family members were identified. Subsequently, phylogenetic relationships, subcellular localisation, conserved motifs and gene structures were analysed. The 54 *OePOD* proteins contained 20 conserved motifs, although slight differences in motif number and type were observed among subgroups, suggesting that these conserved motifs are associated with

essential structural and functional features of *OePOD* proteins (Mathé et al., 2010). Gene structure analysis further revealed substantial variation within the *OePOD* family. The *OePOD* genes displayed different gene architectures associated with a different number of exons and introns, which change among subgroups. Such structural diversity is considered an important factor in the evolution and functional diversification of gene families (Cai et al., 2021; Tao et al., 2023). Taken together, variations in conserved motifs and gene structure indicate the evolutionary complexity of the *OePOD* family in olive and may be associated with functional differentiation among its members. Plant PODs are known to display diverse subcellular localisations, including extracellular, membrane-associated and intracellular compartments, reflecting their functional diversity (Ren et al., 2014; Lüthje & Martinez-Cortes, 2018). In contrast, the *OePODs* identified in the present study were consistently predicted to localise to the extracellular space, indicating a predominant association with the cell wall and apoplast. This exclusive extracellular localisation supports the view that olive PODs primarily participate in apoplastic redox regulation and cell wall-related processes, functions that are particularly relevant during developmental reprogramming events such as SE.

These findings collectively indicate that embryogenic efficiency in olive is linked to significant intracellular metabolic reprogramming and synchronised extracellular remodelling, as evidenced by the secretome. The differential accumulation of proteins related to redox regulation, signalling, stress responses and cell wall-associated processes indicates that both intracellular and extracellular environments contribute to the establishment of embryogenic competence. Among these proteins, class III PODs emerged as particularly relevant components due to their consistent enrichment in HE lines and their putative roles in ROS homeostasis, extracellular signalling and cell wall dynamics. The coordinated modulation of proteome and secretome profiles, therefore, highlights the importance of integrated cellular and extracellular regulatory networks during SE and supports the potential use of PODs, together with other redox- and signalling-related proteins, as biomarkers of HE embryogenic olive lines.

VIII. 6. CONCLUSION

This study provides an integrated analysis of proteome, secretome and class III POD dynamics associated with SE in olive. Comparative analyses between HE and LE embryogenic lines revealed that embryogenic competence is linked to extensive metabolic and redox reprogramming, involving both intracellular and extracellular environments. The HE lines were characterised by enrichment of proteins related to signalling, stress responses and redox metabolism, while secretome profiling demonstrated a pronounced accumulation of extracellular class III PODs, highlighting the importance of apoplastic regulation during embryogenic development. Biochemical, immunological and transcriptional analyses consistently supported the association between enhanced POD activity and high embryogenic

competence. In parallel, genome-wide identification and characterisation revealed substantial structural diversity within the family, suggesting functional specialisation among members.

Overall, these findings strengthen the hypothesis that coordinated proteome–secretome regulation and POD-mediated redox homeostasis are central components of the molecular network controlling SE in olive. Future studies should focus on the functional validation of candidate *OePOD* genes, particularly those highly expressed in HE lines, through gene silencing or overexpression approaches. In addition, investigation of the interaction between POD activity, ROS signalling and hormonal pathways may provide further insight into the mechanisms governing embryogenic competence and contribute to the development of improved protocols for olive regeneration and propagation.

VIII. 7. REFERENCES

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CHAPTER IX

Concluding Remarks

IX. CONCLUDING REMARKS

The olive is one of the most emblematic and economically important perennial crops in the Mediterranean basin, with a long-standing cultural heritage and increasing global relevance driven by the recognised nutritional and health benefits of olive oil. As demand for high-quality olive-derived products continues to rise, the sector faces growing pressure to enhance productivity, sustainability, and resilience to biotic and abiotic stresses. However, the improvement and propagation of elite cultivars remain constrained by the limited efficiency of conventional vegetative propagation methods and the intrinsic recalcitrance of this species to *in vitro* regeneration, particularly when adult tissues are used. In this context, somatic embryogenesis (SE) emerges as a key biotechnological tool, offering unique opportunities for large-scale clonal propagation, germplasm conservation, and the implementation of advanced genetic improvement strategies. Nevertheless, its practical application in olive is still delayed by an incomplete understanding of the molecular and physiological determinants of embryogenic competence. Addressing this knowledge gap requires integrated approaches that combine robust experimental systems with multi-level molecular characterisation to decipher the regulatory networks governing SE and unlock its full potential in olive biotechnology.

Chapter III successfully established a robust SE system for *Olea europaea* using zygotic embryo-derived explants from the cvs ‘Galega Vulgar’ and ‘Arbequina’, while generating embryogenic lines with contrasting developmental efficiencies. The clear genotype-dependent response observed, both between and within cultivars, reinforces the intrinsic recalcitrance of olive and highlights the importance of explant origin, with radicle-derived tissues showing enhanced embryogenic competence. The maintenance of high (HE) and low-efficiency (LE) embryogenic lines through cyclic SE, without loss of their distinctive behaviour, represents a significant methodological advance, providing a stable and reproducible platform for comparative studies. Furthermore, the successful conversion of somatic embryos into plants, coupled with the overall chromosomal stability detected, supports the applicability of this system for clonal propagation, although the occurrence of occasional aneuploidy underscores the necessity of routine genetic screening. Importantly, the establishment of lines with divergent embryogenic capacities creates a valuable experimental framework for subsequent investigations into the determinants of SE responsiveness and recalcitrance in olive.

Building upon this experimental framework, Chapters IV and V focused on the molecular characterisation of embryogenic competence, with particular emphasis on the extracellular environment as a regulatory interface, including extracellular proteome and metabolome analysis (secretome and exometabolome, respectively). Proteomics has become a fundamental tool in plant research, substantially advancing our understanding of the molecular processes underlying SE. In this context, extracellular biomolecules play a pivotal role, as they can differentially induce, inhibit, or modulate SE through complex signalling and metabolic interactions. Nevertheless, most proteomic studies in SE have

traditionally focused on tissues, often leading to the over-representation of constitutively expressed and highly abundant proteins, thereby limiting the detection of low-abundance regulatory components. Proteomics approaches, particularly those targeting the extracellular space, offer a powerful alternative to overcome these limitations and provide deeper insights into cell-to-cell communication and signalling processes. The extracellular medium represents the first interface of interaction between cells and their environment, acting as a dynamic hub for signalling molecules involved in developmental reprogramming.

Within this framework, Chapter IV established a robust and reproducible methodology for the simultaneous extraction and separation of proteins and metabolites from the conditioned medium (CM), ensuring minimal intracellular contamination and enabling their downstream analysis by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) and Nuclear magnetic resonance spectroscopy (NMR). Extending this methodological framework, Chapter V applied the workflow to compare the secretome and exometabolome of CM from embryogenic lines with contrasting embryogenic capacities. The results revealed distinct extracellular profiles associated with embryogenic competence, highlighting coordinated regulation of redox homeostasis, amino acid metabolism, and central carbon metabolism. In particular, the HE lines exhibited metabolic features consistent with effective carbon utilisation and controlled oxidative balance, whereas LE lines showed signatures of metabolic constraint and stress-related responses. Collectively, these findings underscore the extracellular space as a key regulatory compartment in SE and lay the foundation for functional validation of these molecular determinants. Bridging molecular insights and functional outcomes, Chapter VI advanced from molecular characterisation to experimental validation by assessing the biological activity of CM in modulating morphogenic responses. Applying a nurse culture-inspired methodology, CM was obtained from an HE embryogenic line of cv. ‘Arbequina’ considerably enhanced the morphogenic response of the LE line, facilitating calli proliferation and somatic embryo development in a concentration-dependent manner during consecutive subcultures. These findings provide clear experimental evidence supporting the regulatory role of extracellular factors in coordinating cellular reprogramming during SE. Furthermore, the application of CM to adult explants of the recalcitrant cv. ‘Galega Vulgar’ demonstrated its capacity to induce the formation of meristematic nodules, particularly under liquid culture conditions, highlighting the importance of the physical culture system in modulating the bioavailability and activity of signalling molecules. Collectively, these results validate the biological relevance of the extracellular components previously characterised and establish a direct link between extracellular molecular composition and morphogenic outcomes.

Complementing the extracellular-focused analyses, Chapters VII and VIII shifted the focus to the intracellular environment, aiming to refine the molecular framework underlying embryogenic competence through tissue-level proteomic characterisation and targeted validation. Proteomics has

emerged as a powerful high-throughput approach to investigate global protein dynamics in SE, yet methodological factors, particularly sample preparation strategies, can significantly influence proteome coverage and subsequent biological interpretation. In this context, Chapter VII provided a comparative assessment of two distinct proteomic workflows, proving that both approaches achieved extensive and consistent proteome coverage, while differing in their capacity to capture specific functional protein subsets. Importantly, the choice of sample preparation strategy plays a critical role in proteomic analyses, influencing both proteome coverage and the ability to capture biologically relevant proteins associated with embryogenesis. Across both approaches, embryogenic competence was consistently associated with coordinated stress-adaptive and redox-regulated networks, reinforcing patterns previously identified at the extracellular level. Building upon these findings, Chapter VIII further validated selected molecular targets through complementary gene- and protein-level approaches, confirming the differential abundance of key proteins, including members of the class III peroxidases (POD) family, in HE embryogenic lines. This integrative strategy supports the reliability of the identified molecular signatures and establishes a coherent link between intracellular processes and extracellular signalling dynamics.

Taken together, the work presented in this thesis establishes a comprehensive and integrative framework for advancing SE in olive. By combining the development of robust embryogenic systems with multi-compartment molecular characterisation and functional validation, this study provides new insights into the coordinated regulatory mechanisms underlying embryogenic competence, particularly those associated with redox homeostasis, vesicle-mediated transport, cellular signalling, cell wall remodelling, and the regulation of carbon and amino acid metabolism. Importantly, this work represents one of the first integrative secretome–exometabolome analyses linked to functional SE assays, providing a multidimensional perspective on the extracellular regulation of embryogenic competence in olive. The identification of consistent molecular signatures across extracellular and intracellular environments highlights the central role of metabolic, redox, and signalling networks in cellular reprogramming, while demonstrating the culture medium as an active regulatory component rather than a passive substrate. Importantly, the ability to modulate morphogenic responses through CM reinforces the translational potential of these findings. Collectively, these results contribute to a deeper understanding of the molecular basis of embryogenic competence and provide a foundation for the development of improved regeneration and propagation strategies for olive and other recalcitrant woody species.

CHAPTER X

Future Perspectives

X. FUTURE PERSPECTIVES

Future research should focus on the functional investigation of the molecular networks associated with somatic embryogenesis (SE) competence, particularly those related to redox regulation, stress adaptation, and extracellular signalling. The extracellular space plays a key role during the transition between non-embryogenic and embryogenic states, although the precise functional roles of its components remain to be clarified. Future research should prioritise extracellular signalling processes, with particular emphasis on extracellular vesicle-mediated communication, to elucidate how vesicle-associated cargo is selectively released, perceived, and integrated during cellular reprogramming in SE. The biological activity observed in conditioned medium (CM) derived from high-efficiency (HE) embryogenic lines further supports the relevance of this compartment for the regulation of morphogenic responses, highlighting the contribution of extracellular factors. In this context, identifying vesicle-associated molecules and other key extracellular components linked to embryogenic competence will be essential to clarify their functional role and evaluate their potential application in improving SE efficiency, particularly in recalcitrant adult-derived tissues of elite olive cultivars.

Although this thesis identified several candidate proteins and metabolites potentially involved in the regulation of SE, their specific biological functions and interactions remain largely unresolved. The integration of transcriptomics, targeted metabolomics, and functional genomics approaches will therefore be essential to clarify the regulatory hierarchies controlling cellular reprogramming and totipotency acquisition in olive.

Equally important the identification of reliable molecular markers associated with embryogenic competence during the early stages of SE. The consistent differential abundance of specific extracellular and intracellular proteins and metabolites identified between HE and LE embryogenic lines highlights their potential as predictive biomarkers of embryogenic responsiveness. Future validation across different genotypes and developmental stages may support the development of early screening strategies for responsive cultures, contributing to improved efficiency and reproducibility of SE systems in olive.

In parallel, future efforts should aim to integrate SE systems with emerging functional genomics, genome editing, and precision breeding technologies. Efficient regeneration platforms constitute a fundamental basis for the implementation of advanced genetic improvement strategies in olive, including the modulation of traits related to stress tolerance, productivity, and adaptation to climate change. In this context, the candidate genes and molecular pathways identified in this thesis may constitute promising targets for functional validation through gene silencing or overexpression approaches aimed at clarifying their function in embryogenic competence. Furthermore, the optimisation of SE protocols for adult-derived tissues may have particularly high translational value,

facilitating the direct improvement of agronomically important cultivars while preserving their elite genetic background.

Collectively, these future directions reinforce the potential of SE not only as a propagation tool but also as a central platform for the development of next-generation olive biotechnology applications. The results presented in this work demonstrate that integrating complementary experimental approaches, from extracellular and intracellular molecular profiling to functional assays, provides a more accurate representation of the biological processes underlying embryogenic competence, increasingly aligning experimental outputs with the *in vivo* physiological context. This integrative perspective is becoming essential to move beyond descriptive analyses towards a mechanistic understanding of SE. In this framework, future research should also explore the epigenetic regulation of cellular reprogramming, as epigenetic modifications are likely to play a key role in controlling developmental plasticity in olive. In addition, further work should aim to elucidate the molecular basis of the observed effects of CM, identifying the specific bioactive molecules responsible for modulating morphogenic responses and characterising the downstream molecular changes they trigger during embryogenic induction and progression.

APPENDIX A

Supplementary Material – Chapter IV

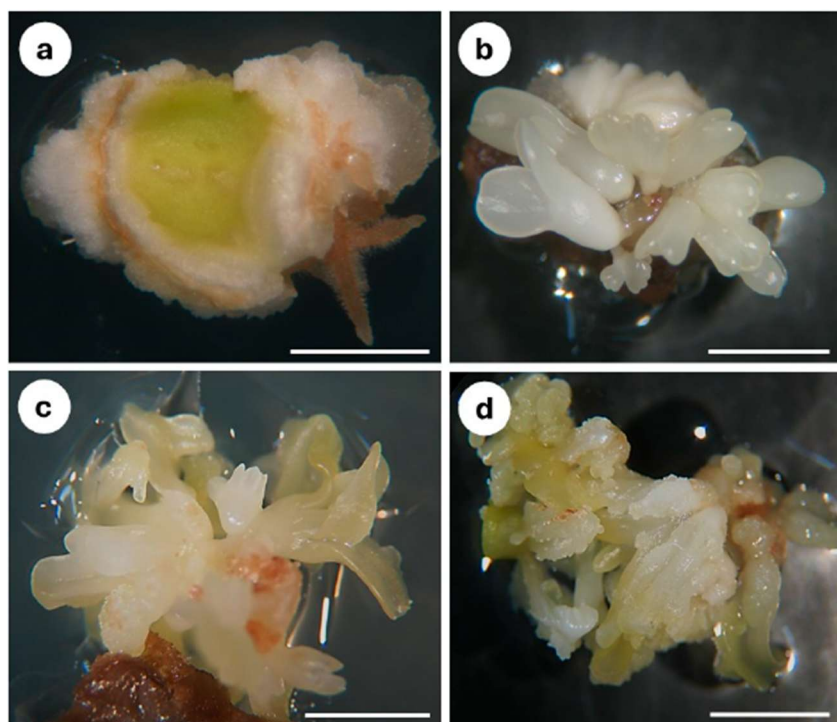


Figure A.1. Explants from cv. ‘Galega Vulgar’ at the end of the induction phase (a) and expression phase (b). High-efficiency (HE) (c) and low-efficiency (LE) embryogenic line (d) in cyclic embryogenesis in liquid ECO culture medium. Bars: 0.5 cm.

Table A.1. Expression level of protein bands (numbered 1 to 6) considered for the comparative analysis of precipitation methods. Significant differences in expression band levels among the three protein precipitation methods (Acetone, TCA/Acetone, and Methanol/Chloroform) are indicated by different letters, which denote statistically significant differences ($p \leq 0.05$).

Precipitation Methods				
Lyophilized				
Bands	Acetone	TCA/Acetone	Methanol/ Chloroform	<i>p-value</i>
1	291.3 ± 291.3	1849.7 ± 464.5	381,7 ± 381.7	0.052
2	324.7 ± 324.7	1807.7 ± 158.4	378.3 ± 378.3	0.052
3	326.3 ± 326.3	1762.7 ± 33.7	319.3 ± 319.3	0.052
4	409.3 ± 409.3	851.7 ± 429.7	262.0 ± 262.0	0.5
5	456.4 ± 456.3 a	1598.0 ± 94.92 b	nt	0.035

6	381.0 ± 381.0	607.3 ± 607.33	525.3 ± 262.7	0.948
Not lyophilized				
1	146.0 ± 146.0	877.3 ± 114.9	547.7 ± 279.2	0.120
2	399.7 ± 201.6	506.0 ± 253.8	291.3 ± 291.3	0.794
3	nt	612.3 v 313.5	337.0 ± 337.0	0.281
4	184.3 ± 184.3	366.7 ± 366.7	nt	0.558
5	491.0 ± 249.5	522.7 ± 271.3	294.0 ± 294.0	0.854
6	nt	443.0 ± 443.0	nt	0.368

nt- not detected

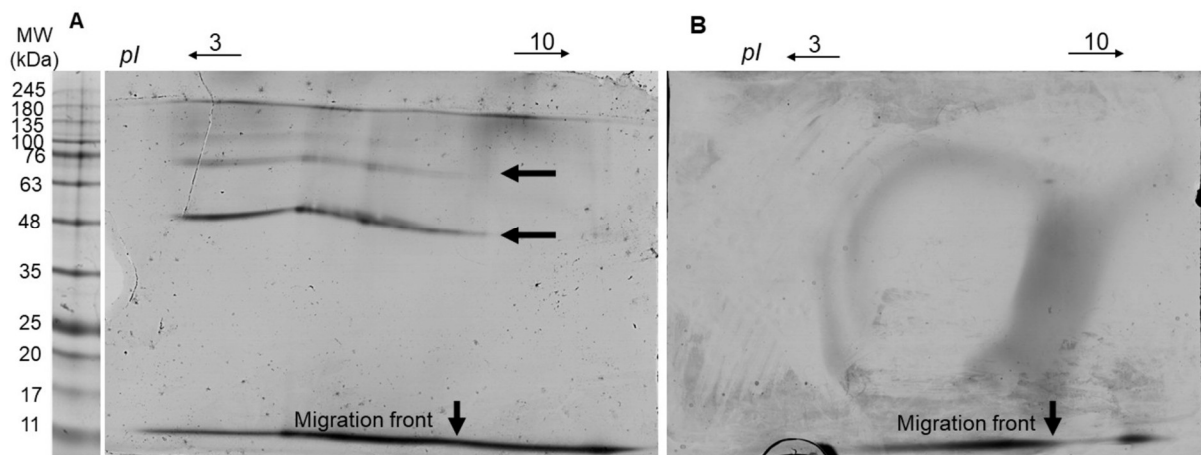


Figure A.2. Identification of MDH isoenzyme activity following isoelectric focusing on polyacrylamide gels stained with malate, NAD⁺, and nitroblue tetrazolium. The appearance of protein spots in the sample obtained from plant material indicates the presence of the MDH enzyme (black arrows) (A); the absence of those spots in conditioned medium (CM) reveals no presence of MDH enzyme and consequently no contamination by proteins in the precipitated secretome from the CM (B). MW – molecular weight marker (NZYA Colour Protein Marker II, Nztech) with the apparent size of each band indicated in kDa; pI – isoelectric point.

Table A.2. List of secreted proteins identified in conditioned medium from a high-efficiency embryogenic line of olive somatic embryogenesis by LC–MS/MS analysis. Protein identification was performed using Mascot, MsAmanda, MSFragger, and Sequest HT search engines against the *Olea europaea* UniProtKB database supplemented with the cRAP contaminant database. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD058411.

Due to its large size, this table is provided as Supplementary Material and is available at:

<https://www.mdpi.com/article/10.3390/horticulturae11030331/s1>

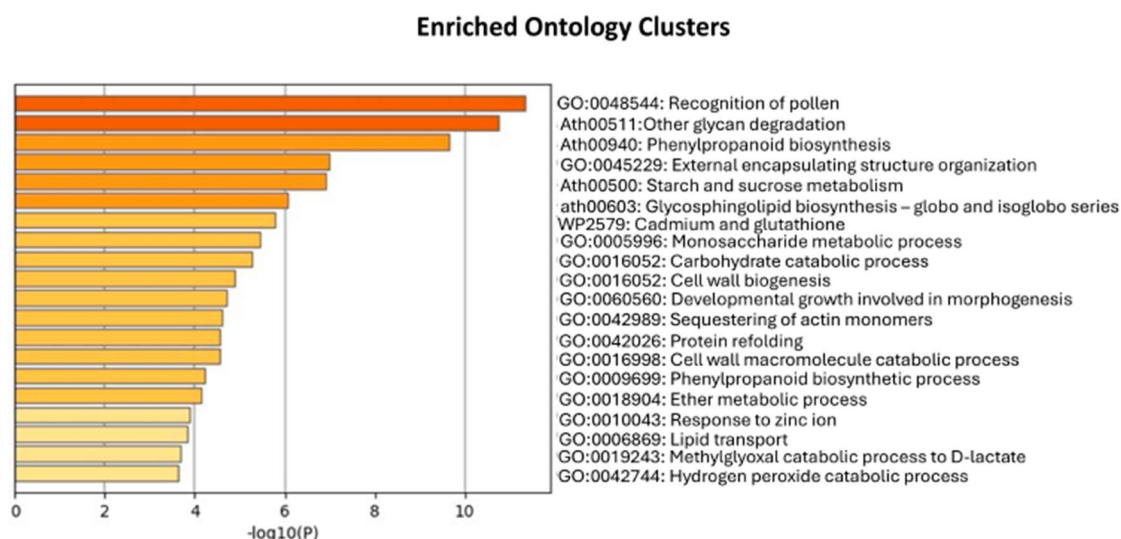


Figure A.3. Enriched ontology clusters corresponding to biological processes. Statistical significance of the enrichment is indicated by $-\log_{10}(p\text{-value})$.

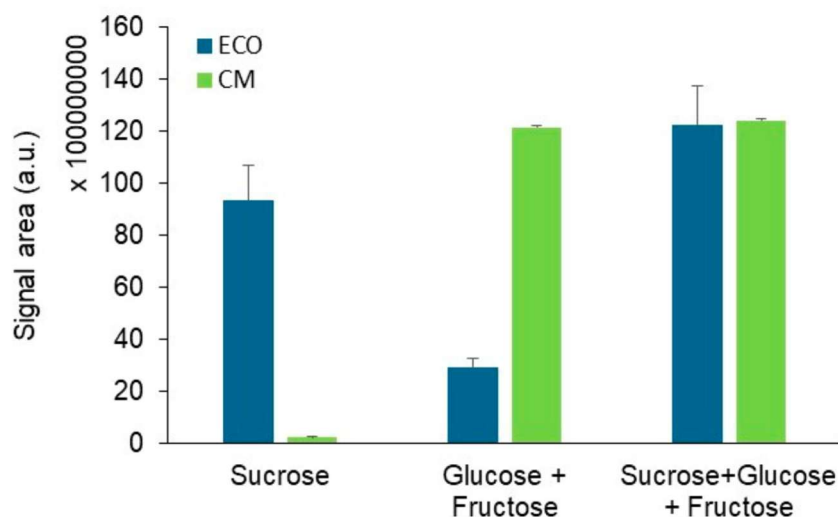


Figure A.4. Quantification of sucrose, glucose, and fructose levels in ECO (control sample established without plant material) and CM samples using NMR spectroscopy. The signal area (a.u.) for each sugar component, as well as their combinations, is displayed, highlighting differences between ECO (dark blue) and CM (light green) samples. Error bars represent the standard deviation of measurements.

APPENDIX B

Supplementary Material – Chapter V

Table B.1. List of secreted proteins identified in conditioned medium from high (HE) and low-efficiency (LE) embryogenic lines of *Olea europaea* cultivar ‘Arbequina’ by LC–MS/MS analysis. Protein identification was performed using Mascot, MsAmanda, MSFragger, and Sequest HT search engines against the *Olea europaea* UniProtKB database supplemented with the cRAP contaminant database.

Due to its large size, this table is provided as Supplementary Material and is available at:

<https://drive.google.com/drive/folders/1Q1FcF0BMBdJ-KcuiECf59rhBVTfdWnvJ?usp=sharing>

Table B.2. Differentially abundant proteins between high (HE) and low-efficiency (LE) lines in the secretome samples.

Due to its large size, this table is provided as Supplementary Material and is available at:

<https://drive.google.com/drive/folders/1Q1FcF0BMBdJ-KcuiECf59rhBVTfdWnvJ?usp=sharing>

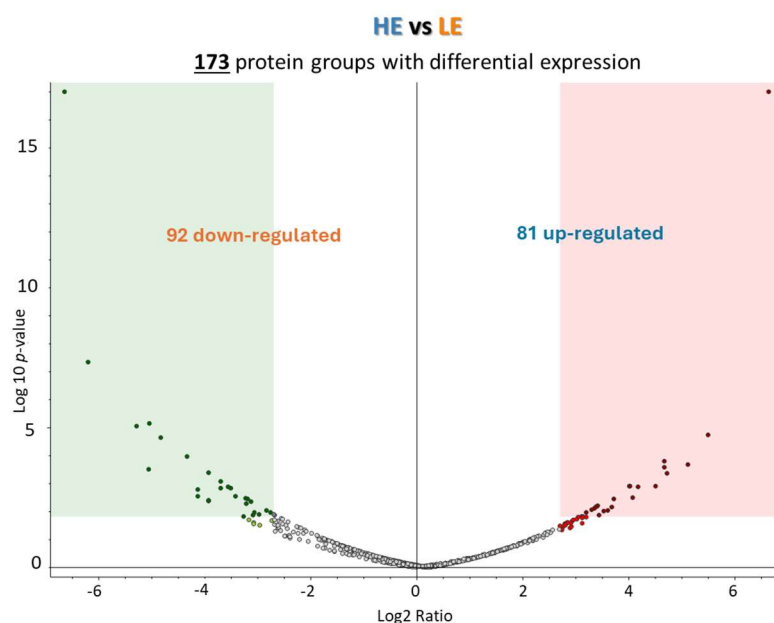


Figure B1. Volcano plot of differentially abundant proteins that distinguish high-efficiency (HE) from low-efficiency (LE) embryogenic lines of *Olea europaea* L. cv. ‘Arbequina’.

APPENDIX C

Supplementary Material – Chapter VII

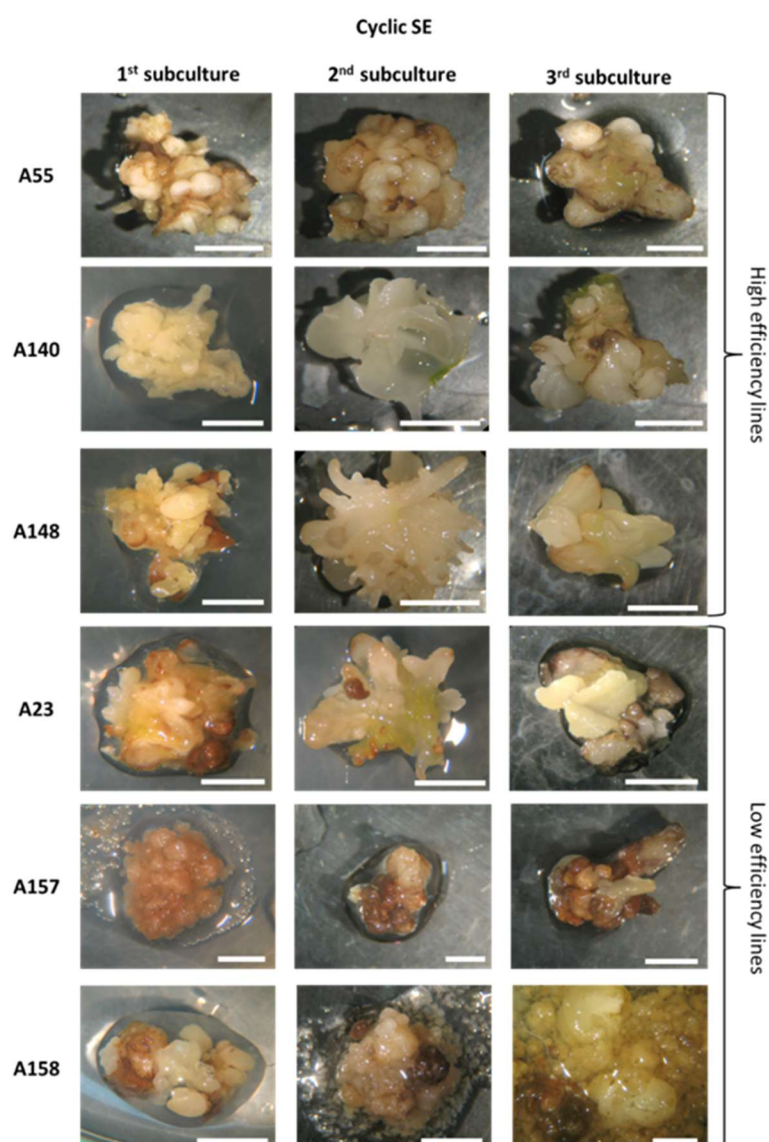


Figure C.1. High-efficiency (HE) and low-efficiency (LE) embryogenic lines derived from olive cv. ‘Arbequina’ during three successive subcultures in liquid ECO medium. Scale bars: 0.5 cm.

Table C.1. Proteome profile of embryogenic calli from high-efficiency (HE) and three low-efficiency (LE) embryogenic lines of *Olea europaea* cultivar ‘Arbequina’ analysed by LC–MS/MS. Protein identification was performed using Mascot, MsAmanda, MSFragger, and Sequest HT search engines against the *O. europaea* UniProtKB database supplemented with the cRAP contaminant database.

Due to its large size, this table is provided as Supplementary Material and is available at:

<https://drive.google.com/drive/folders/1Q1FcF0BMBdJ-KcuiECf59rhBVTfdWnvJ?usp=sharing>

Table C.2. Differential abundance analysis of embryogenic calli high-efficiency (HE) and low-efficiency (LE) lines of *Olea europaea* cultivar ‘Arbequina’ with proteins significantly associated with either Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) or Suspension-Trapping (S-Trap) methods. Analyses were performed using a two-way linear model (efficiency $\times$ extraction method) on log₂-transformed data using the limma package in R, with empirical Bayes moderation. Differentially abundant proteins were defined using $p < 0.05$ and $|\log_2FC| > 1$. Presence/absence patterns were also evaluated across conditions.

Due to its large size, this table is provided as Supplementary Material and is available at:

<https://drive.google.com/drive/folders/1Q1FcF0BMBdJ-KcuiECf59rhBVTfdWnvJ?usp=sharing>

Table C.3. Protein distribution associated with high-efficiency (HE) and low-efficiency (LE) embryogenic calli of *Olea europaea* cultivar ‘Arbequina’ identified using Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) and Suspension-Trapping (S-Trap) extraction methods.

Due to its large size, this table is provided as Supplementary Material and is available at:

<https://drive.google.com/drive/folders/1Q1FcF0BMBdJ-KcuiECf59rhBVTfdWnvJ?usp=sharing>

Table C.4. Specific proteins related to the high-efficiency (HE) and low-efficiency (LE) lines in the Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) and Suspension-Trapping (S-Trap) methods.

Due to its large size, this table is provided as Supplementary Material and is available at:

<https://drive.google.com/drive/folders/1Q1FcF0BMBdJ-KcuiECf59rhBVTfdWnvJ?usp=sharing>

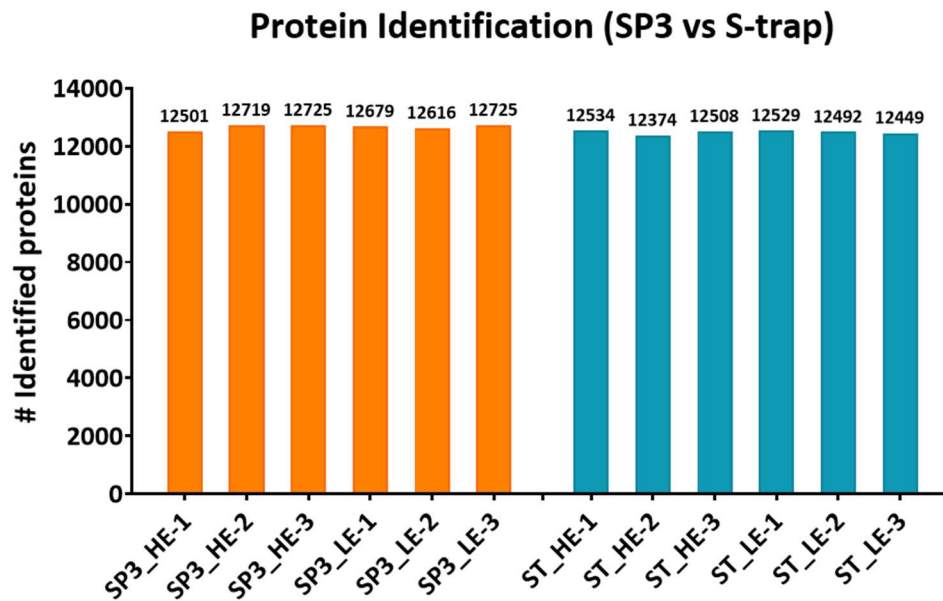


Figure C.2. Average number of proteins identified per biological replicate using the SP3 and S-Trap methods.

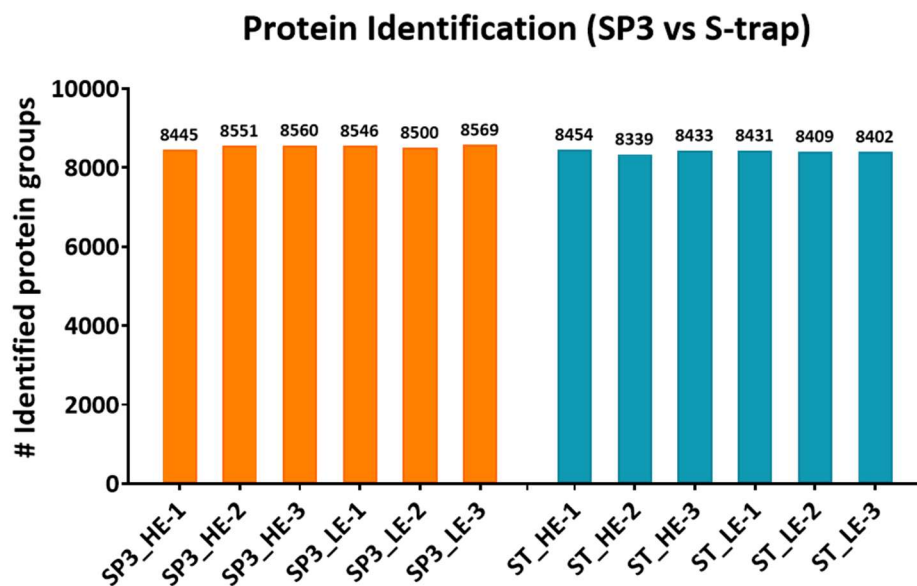


Figure C.3. Average number of protein groups identified per biological replicate using the SP3 and S-Trap methods.

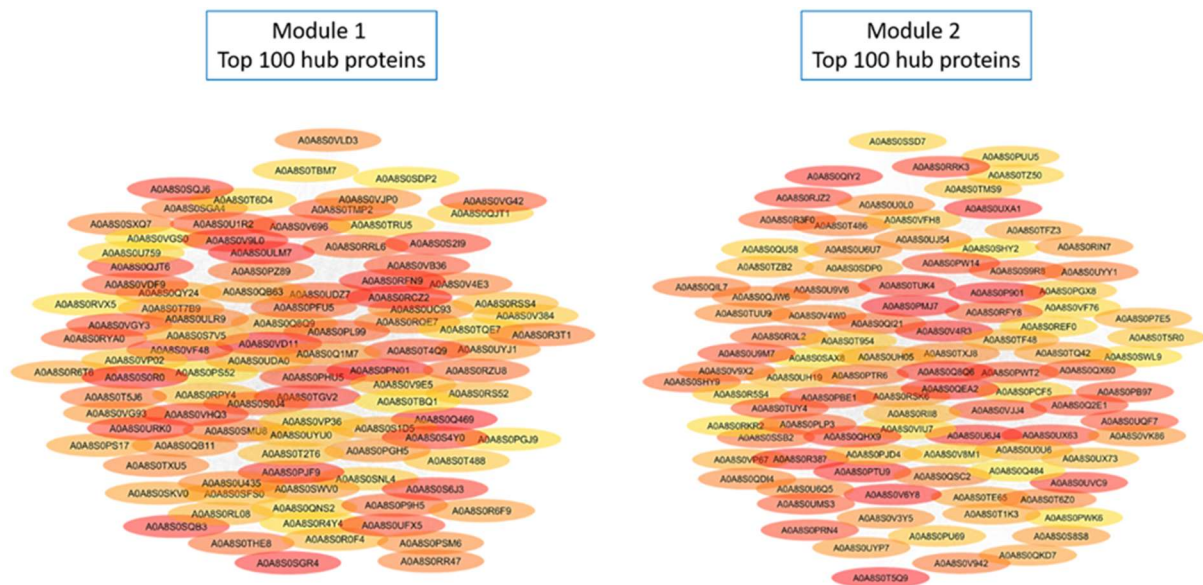


Figure C.4. Top 100 hub proteins for Module 1, correlated to SP3 extraction method, and Module 2 correlated to S-Trap extraction method, as ranked by cytoHubba plugin in the Cytoscape software.

APPENDIX D

D. Supplementary Material – Chapter VIII

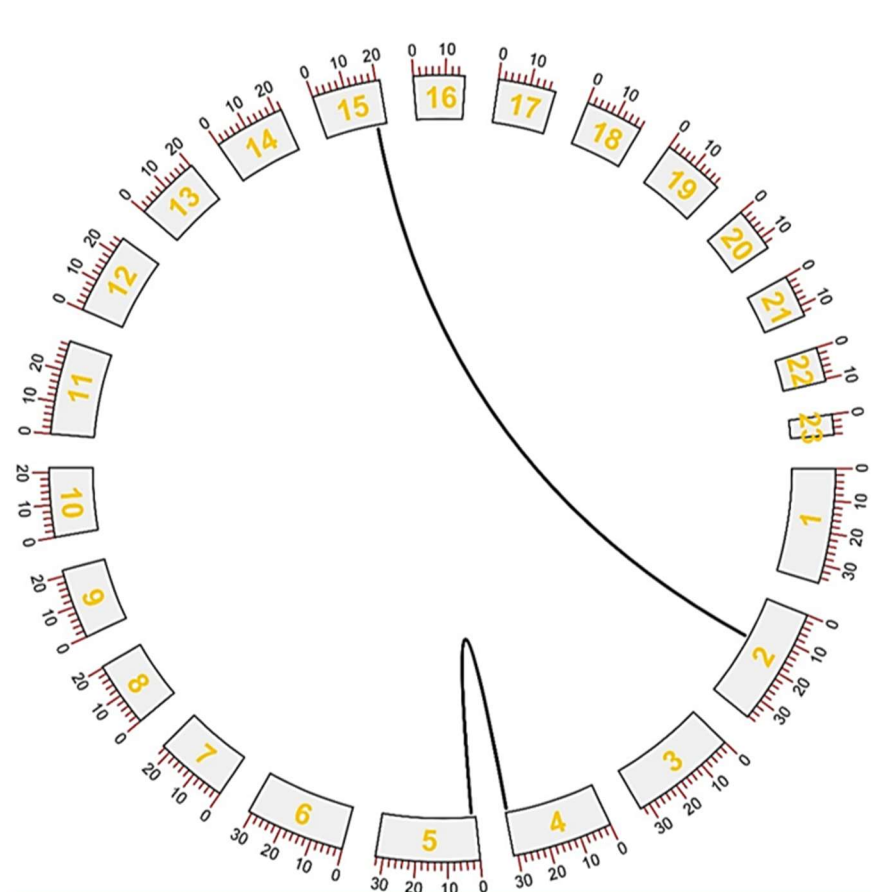


Figure D.1. Collinearity analysis of class III peroxidase genes in *Olea europaea*, showing the distribution of segmental duplication across chromosomes. Chromosomes are represented by yellow boxes, with the corresponding chromosome number indicated within each box. Segmental duplicated genes are connected with black lines.

Table D.1. Proteins identified in the integrated proteome–secretome dataset, with $\log_2$ fold-change values for the proteome ($\logFC_Prot$) and secretome ($\logFC_Sec$) in high-efficiency (HE) versus low-efficiency (LE) embryogenic lines and their assigned regulation pattern.

Protein	$\logFC_Prot$	$\logFC_Sec$	Pattern
A0A068C9M6	-0,264	-9,319	Intermediate
A0A8S0P7F5	-0,020	3,546	Intermediate
A0A8S0P7K1	-0,128	-5,589	Intermediate
A0A8S0PC55	0,091	7,620	Intermediate
A0A8S0PDL3	-0,027	0,971	Intermediate
A0A8S0PE42	-0,032	6,475	Intermediate
A0A8S0PF87	0,281	-6,807	Intermediate
A0A8S0PFV4	0,059	-2,045	Intermediate
A0A8S0PML6	-0,336	4,714	Intermediate

A0A8S0PTY5	0,405	4,960	Intermediate
A0A8S0PVD3	-0,032	-5,032	Intermediate
A0A8S0Q5Q3	-0,137	5,753	Intermediate
A0A8S0Q8R7	-0,174	-2,047	Intermediate
A0A8S0QBD8	-0,194	-4,614	Intermediate
A0A8S0QBM2	-0,021	1,236	Intermediate
A0A8S0QDS6	0,470	4,065	Intermediate
A0A8S0QHT3	0,246	-10,151	Intermediate
A0A8S0QJ05	0,214	7,285	Intermediate
A0A8S0QJI1	-0,294	-2,122	Intermediate
A0A8S0QM65	0,008	-3,937	Intermediate
A0A8S0QME8	-0,017	-3,446	Intermediate
A0A8S0QRI8	-0,106	-6,390	Intermediate
A0A8S0QWY5	-0,009	-0,590	Intermediate
A0A8S0R7I0	-1,136	0,478	Intermediate
A0A8S0RDM7	-0,065	1,384	Intermediate
A0A8S0REL7	0,061	-3,637	Intermediate
A0A8S0RF48	-0,151	7,332	Intermediate
A0A8S0RHI3	0,125	-2,860	Intermediate
A0A8S0RMU3	0,071	7,764	Intermediate
A0A8S0RX29	-0,241	-11,774	Intermediate
A0A8S0S017	0,250	8,106	Intermediate
A0A8S0S074	0,217	-1,691	Intermediate
A0A8S0S5P0	-0,369	3,368	Intermediate
A0A8S0S7D5	-0,106	-5,782	Intermediate
A0A8S0SBD2	-0,224	3,927	Intermediate
A0A8S0SEQ6	0,194	2,607	Intermediate
A0A8S0SH41	0,342	7,921	Intermediate
A0A8S0SJK9	0,134	-1,409	Intermediate
A0A8S0SRQ4	-0,119	1,772	Intermediate
A0A8S0SV34	0,264	0,561	Intermediate
A0A8S0SWJ4	0,135	4,730	Intermediate
A0A8S0SWK0	-2,444	0,099	Intermediate
A0A8S0SYI5	0,006	-5,352	Intermediate
A0A8S0T2P5	-0,338	4,237	Intermediate
A0A8S0T3Y9	0,183	1,505	Intermediate
A0A8S0T541	0,426	6,400	Intermediate
A0A8S0T6R9	0,031	-10,341	Intermediate
A0A8S0T7J8	0,221	-6,660	Intermediate
A0A8S0T7T6	0,102	-8,519	Intermediate
A0A8S0TBJ6	-0,261	-6,106	Intermediate

A0A8S0TD35	-0,308	-0,707	Intermediate
A0A8S0TNQ6	-0,281	-1,685	Intermediate
A0A8S0TPZ5	-0,087	-7,509	Intermediate
A0A8S0TQS7	0,039	-5,429	Intermediate
A0A8S0TTH7	0,298	5,993	Intermediate
A0A8S0TU97	-0,082	-3,579	Intermediate
A0A8S0TXT1	0,325	-6,304	Intermediate
A0A8S0TZ76	0,078	-6,032	Intermediate
A0A8S0U1P0	-0,084	4,903	Intermediate
A0A8S0U461	-0,116	-5,672	Intermediate
A0A8S0U5H5	0,269	-6,587	Intermediate
A0A8S0U7E8	0,161	-8,297	Intermediate
A0A8S0UCI0	0,069	1,736	Intermediate
A0A8S0UPR3	-0,134	-5,221	Intermediate
A0A8S0UVS7	0,153	-5,415	Intermediate
A0A8S0UZL4	-0,152	-4,191	Intermediate
A0A8S0V297	-0,143	7,126	Intermediate
A0A8S0V2I3	-0,129	7,002	Intermediate
A0A8S0V5I1	0,094	-7,311	Intermediate
A0A8S0VA89	0,121	2,477	Intermediate
A0A8S0VE21	0,101	3,725	Intermediate
A0A8S0VJV7	0,200	4,710	Intermediate
I6ZPI0	0,203	0,000	Intermediate
A0A8S0S5M5	-0,497	4,507	Intermediate
A0A8S0SHQ0	0,113	11,174	Intermediate
A0A8S0SI37	0,126	-7,488	Intermediate

Table D.2. Group of proteins showing concordant upregulation in both the proteome and secretome in high-efficiency (HE) embryogenic lines, with log₂ fold-change values for the proteome (logFC_Prot) and secretome (logFC_Sec).

Protein	logFC_Prot	logFC_Sec	Pattern
A0A8S0QNM8	1,926	11,729	High_High
A0A8S0T083	1,536	6,853	High_High
A0A8S0T582	2,657	6,185	High_High
A0A8S0T8H2	1,263	6,909	High_High
A0A8S0TNK9	0,836	5,788	High_High
A0A8S0UWN1	0,672	7,643	High_High
A0A8S0PU95	2,723	1,542	High_High

Table D.3. Group of proteins showing concordant upregulation in both the proteome and secretome in low-efficiency (LE) embryogenic lines, with log₂ fold-change values for the proteome (logFC_Prot) and secretome (logFC_Sec).

Protein	logFC_Prot	logFC_Sec	Pattern
A0A8S0PH43	-0,563	-2,082	Low_Low
A0A8S0Q765	-1,980	-3,762	Low_Low
A0A8S0QCZ2	-1,590	-8,768	Low_Low
A0A8S0QGJ8	-0,726	-2,702	Low_Low
A0A8S0QUI3	-3,166	-2,522	Low_Low
A0A8S0RKJ8	-0,927	-5,835	Low_Low
A0A8S0SMN4	-0,762	-4,653	Low_Low
A0A8S0TH18	-0,543	-6,247	Low_Low
A0A8S0TWQ2	-1,778	-5,930	Low_Low
A0A8S0UAD3	-0,712	-8,308	Low_Low
A0A8S0USZ0	-2,370	-3,766	Low_Low
A0A8S0UV31	-0,563	-7,128	Low_Low
A0A8S0V255	-0,661	-4,044	Low_Low
A0A8S0VKK0	-1,761	-4,519	Low_Low
A0A8S0Q4H6	-2,631	-4,565	Low_Low

Table D.4. Proteins classified as HighProt_LowSec, with log₂ fold-change values for the proteome (logFC_Prot) and secretome (logFC_Sec) in HE versus LE embryogenic lines.

Protein	logFC_Prot	logFC_Sec	Pattern
A0A8S0PU36	0,606	-1,739	HighProt_LowSec
A0A8S0QXN9	1,920	-6,372	HighProt_LowSec
A0A8S0RAU3	3,995	-3,842	HighProt_LowSec
A0A8S0RI49	1,000	-4,469	HighProt_LowSec
A0A8S0SCY5	1,599	-3,724	HighProt_LowSec
A0A8S0TA51	2,553	-4,576	HighProt_LowSec
A0A8S0TPE9	1,402	-3,548	HighProt_LowSec

Table D.5. Proteins classified as LowProt_HighSec, with log₂ fold-change values for the proteome (logFC_Prot) and secretome (logFC_Sec) in HE versus LE embryogenic lines.

Protein	logFC_Prot	logFC_Sec	Pattern
A0A8S0PEL9	-0,720	3,636	LowProt_HighSec
A0A8S0QAK5	-0,869	4,385	LowProt_HighSec
A0A8S0QB53	-3,714	5,128	LowProt_HighSec

A0A8S0QKB0	-1,162	1,928	LowProt_HighSec
A0A8S0T2H2	-1,087	10,013	LowProt_HighSec
A0A8S0TVB6	-1,453	8,666	LowProt_HighSec
A0A8S0UAZ1	-1,735	5,554	LowProt_HighSec
A0A8S0TM03	-2,116	9,527	LowProt_HighSec

Table D.6. Identification of *POD* gene family members from *Olea europaea* subsp. *europaea* var. *sylvestris*. Sequences were retrieved at the olive whole-genome project website (<https://www.ncbi.nlm.nih.gov/genome/?term=Olea+europaea+var.+sylvestris+genome> (accessed on 28 December 2025)).

Gene id	Chr.	Genomic location	Isoelectric Point	Orientation	Gene (bp)	Exons	Transcript	ORF (bp)	AA	Mass (kDa)	Uniprot
OE9A044965	1	34542397-34544690	4,6	Reverse	2328	4	1830	990	329	37	A0A8S0SLM7
OE9A051474	1	28,474,210-28,476,492	5,83	Forward	2320	2	1250	990	329	36	A0A8S0RV43
OE9A109085	1	1121523-1125380	8,87	Forward	3919	4	795	795	264	28	A0A8S0QKP6
OE9A000398	2	388849-390579	9,27	Reverse	1638	4	1286	954	317	34	A0A8S0QYF1
OE9A007444	2	22695858-22697030	8,76	Forward	1189	3	966	966	321	35	A0A8S0TT76
OE9A019475	2	22673102-22675060	9,39	Forward	1989	3	1546	969	322	35	A0A8S0TTQ9
OE9A031944	2	14699093-14701391	6,03	Reverse	2334	4	1609	1092	363	40	A0A8S0RE35
OE9A094067	2	22711245-22712540	6,51	Forward	1314	3	1087	966	321	35	A0A8S0TWP7
OE9A101874	2	20550139-20553480	8,95	Reverse	3394	4	2831	1023	340	37	A0A8S0UKQ0
OE9A074071	3	19121487-19124752	8,61	Forward	3316	4	1232	1020	339	37	A0A8S0TVE5
OE9A068747	4	553945-555422	9,41	Forward	1502	2	1400	1035	344	37	A0A8S0QMD4
OE9A078521	5	17764699-17766380	9,14	Reverse	1706	4	1321	984	327	36	A0A8S0VB57
OE9A087032	5	1555210-1556496	9,46	Reverse	1305	4	936	936	311	34	A0A8S0QIN3
OE9A077191	6	9889853-9892432	6,88	Forward	2620	4	1243	987	328	36	A0A8S0UQ95
OE9A094781	7	18825882-18871010	8,77	Reverse	45872	8	1863	1863	620	67	A0A8S0PGI0
OE9A011975	8	17747687-17750198	8,58	Reverse	2550	5	1789	987	328	35	A0A8S0QNM8
OE9A008955	9	20033743-20035920	8,37	Reverse	2210	4	987	987	328	36	A0A8S0UXP1
OE9A086334	9	20120156-20122221	5,77	Reverse	2097	4	987	987	328	36	A0A8S0UQR6
OE9A014002	10	19331506-19333183	7,55	Forward	1704	3	1491	1020	339	38	A0A8S0T8L6
OE9A006242	12	17368685-17370731	9,12	Forward	2079	3	1558	1044	347	38	A0A8S0Q9U4
OE9A050416	13	14374539-14377210	6,98	Forward	2076	3	3538	1620	323	35	A0A8S0SSW9
OE9A030280	14	3602040-3603845	8,1	Reverse	1834	3	984	984	327	36	A0A8S0U9I5
OE9A086499	15	10576079-10578930	9,24	Forward	2896	4	1541	1002	333	37	A0A8S0TMG7
OE9A120221	15	18125656-18127416	8,68	Forward	1787	4	1017	1017	338	38	A0A8S0QLF1
OE9A046622	17	14179611-14181752	7,55	Forward	2175	4	1665	1023	340	37	A0A8S0RHZ9
OE9A026076	22	7298370-7300760	8,36	Reverse	2428	3	1740	1014	337	37	A0A8S0PG50

OE9A002681	CACTIH010000054.1	358449-359962	8,5	Reverse	1514	2	711	1353	236	26	A0A8S0PKK2
OE9A056371	CACTIH010000054.1	404256-407463	9,18	Reverse	3208	3	1053	1070	350	38	A0A8S0PG25
OE9A080479	CACTIH010000054.1	390331-393792	8,57	Reverse	3518	3	1116	1116	371	41	A0A8S0PG50
OE9A064890	CACTIH010000223.1	31521-33782	6,88	Forward	2296	4	1450	960	319	34	A0A8S0PTA3
OE9A088586	CACTIH010000241.1	222793-224721	5,19	Forward	1958	4	1116	975	324	35	A0A8S0PU95
OE9A106304	CACTIH010000566.1	47372-48500	7,06	Forward	1146	2	993	993	330	36	A0A8S0Q9R8
OE9A112401	CACTIH010000835.1	8541-10734	7,58	Forward	2229	2	1247	990	329	36	A0A8S0Q7T4
OE9A091733	CACTIH010002064.1	80623-83467	4,56	Reverse	1937	4	1969	1003	328	35	A0A8S0R258
OE9A073614	CACTIH010004316.1	2720-4392	8,71	Forward	1698	3	984	984	327	35	A0A8S0SE44
OE9A039582	CACTIH010002076.1	229071-231535	8,55	Reverse	2502	4	1336	999	332	37	A0A8S0R1J2
OE9A060089	CACTIH010002438.1	18776-21004	8,12	Forward	2263	4	1335	981	326	34	A0A8S0RBM1
OE9A037501	CACTIH010003620.1	37765-39568	8,16	Forward	927	5	942	942	308	34	A0A8S0RIC9
OE9A121445	CACTIH010003736.1	382895-384154	6,45	Reverse	1278	4	984	984	327	35	A0A8S0RXD3
OE9A099911	CACTIH010004008.1	121617-125110	8,92	Forward	1432	4	1455	1058	346	39	A0A8S0SAY0
OE9A040599	CACTIH010004313.1	30606-31394	8,74	Forward	800	2	687	606	201	22	A0A8S0SFS5
OE9A005858	CACTIH010005446.1	649763-652186	9,5	Reverse	2461	4	1385	972	323	35	A0A8S0SKG8
OE9A061763	CACTIH010005464.1	358036-359655	6,87	Forward	1644	3	1387	957	318	34	A0A8S0SR19
OE9A082293	CACTIH010005479.1	125938-128459	6,08	Reverse	2561	4	1448	984	327	35	A0A8S0SQZ2
OE9A011083	CACTIH010006392.1	6503-8961	4,89	Forward	495	2	503	733	138	14	A0A8S0TJ63
OE9A024386	CACTIH010007397.1	314705-317023	8,38	Reverse	2356	3	1564	1068	355	38	A0A8S0U4X4
OE9A015993	CACTIH010007411.1	291038-292611	8,06	Forward	1598	2	1493	1032	343	38	A0A8S0U3V4
OE9A055393	CACTIH010007421.1	78019-79656	8,81	Reverse	1663	3	1149	1044	347	38	A0A8S0U923
OE9A041679	CACTIH010007632.1	52838-54651	7,55	Forward	1840	4	999	999	332	36	A0A8S0UGT7
OE9A048843	CACTIH010007734.1	41638-43846	6,25	Forward	2242	4	1459	960	319	34	A0A8S0UNM5
OE9A010421	CACTIH010008025.1	17650-20418	8,59	Forward	2769	6	1058	1058	475	38	A0A8S0UMR0
OE9A071832	CACTIH010009041.1	749873-751839	8,94	Forward	1526	4	1551	1000	327	35	A0A8S0UPS3
OE9A045137	CACTIH010009043.1	29420-32058	8,48	Forward	2680	4	1438	987	328	36	A0A8S0USA0
OE9A077409	CACTIH010009512.1	66832-69917	8,84	Reverse	3146	3	1507	1110	369	40	A0A8S0VKP1