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Influence of endogenous auxins and extracellular proteolytic enzymes in somatic embryogenesis of tamarillo (*Solanum betaceum*)

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor Jorge Manuel Canhoto (Faculdade de Ciências e Tecnologia da Universidade de Coimbra) e da Doutora Sandra Isabel Correia (Faculdade de Ciências da Universidade de Coimbra)

André Filipe Chagas Ferreira Caeiro

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2,4-D - 2,4-Dichlorophenoxyacetic acid

4-CI-IAA - 4-Chloroindole-3-acetic acid

ABA - Abscisic acid

AMC - Amino methyl cumarine

APS - Ammonium persulfate

ARF - Auxin responsive factor

AUX1 – Auxin transporter protein 1

DNA – Deoxyribonucleic acid

E-64 - Trans-epoxysuccinyl-l-leucylamido-(4- guanidino)butane

EC - Embryogenic callus

EDTA - Ethylenediaminetetraacetic acid

GA_3 - Gibberellic acid

GC - Gas chromatography

HPLC - High performance liquid chromatography

IAA - Indole-3-acetic acid

IBA - Indole-3-butyric acid

IPA - Indole-3-propionic acid

LOD - Limit of detection

LOQ - Limit of quantification

MS - Mass spectrometry

MU-G - Methylumbelliferyl-β-D-gucopryanoside

MU-NAG - 4-methylumbelliferyl-acettyl-β-D-glucosaminide

NAA - 1-Naphthaleneacetic acid

NEC - Non-embryogenic callus

PAA - 2-Phenylacetic acid

PAGE - Polyacrylamide gel electrophoresis

PGRs - Plant growth regulators

PIN – Pin-formed

RFU - Relative fluorescent units

SAUR - Small auxin-up RNAs

SD – Standard deviation

SDS - Sodium dodecyl sulphate

SE -Somatic embryogenesis

TCA - Trichloroacetic acid

TEMED - Tetramethylethylenediamine

TIBA - 2,3,5-Triiodobenzoic acid

TIR1 - Transport inhibitor responsor 1

TLC - Thin layer chromatography

TLCK - Tosyl-L-lysyl-chloromethane hydrochloride

TPCK - Tosyl phenylalanyl chloromethyl keton

Abstract

Solanum betaceum (= *Cyphomandra betacea* (Cav.) Sendtn.), usually known as tamarillo, is a small tree cultivated in several world regions for its fruits. This plant can be propagated by classical methods as well as *in vitro* techniques. One of these techniques is somatic embryogenesis, a process by which one or several somatic cells form a somatic embryo without fecundation. This technique is an important biotechnological tool, both for agronomic applications and fundamental scientific research. In spite of this, the molecular mechanisms underlying this phenomenon are still elusive.

A great number of studies in this field have been carried out in classical plant models, such as *Arabidopsis thaliana* and *Daucus carota*. However, many species, particularly woody plants, are still recalcitrant to somatic embryogenesis. Because of this, alternative models taxonomically closer to these plants are required. In this sense, the somatic embryogenesis of tamarillo has been extensively studied in our laboratory, particularly the induction protocols, which have been greatly optimized.

The objective of the present work was to further characterize two distinct factors that have been related with the regulation of somatic embryogenesis, namely the endogenous auxin content, particularly indole-3-acetic acid (IAA), and the extracellular proteolytic profile of embryogenic and non-embryogenic cells of tamarillo.

The results showed that the endogenous auxin content is considerably higher in embryogenic sample, with a tendency to increase as the dedifferentiation of the original explant evolves. Furthermore, differences in the degradation rate of endogenous IAA were found, with the non-embryogenic callus presenting higher levels in comparison to

the embryogenic ones. The importance of the endogenous level of auxin in the maturation phase of the somatic embryos has also been hinted, by the analysis of the effect of the presence of auxin transport inhibitors, such as TIBA, on the maturation medium.

The extracellular proteolytic profiles revealed differences, both qualitative and quantitative, between the embryogenic and non-embryogenic callus. In quantitative terms, the specific activity of proteases in the embryogenic callus is higher while the qualitative analysis suggested the presence of serine and metalloproteases. On the other hand, in the non-embryogenic callus, serine proteases seem to be dominant. Other types of hydrolytic enzymes, namely glycosidases, have also been found to express significantly different activities in the two types of calluses.

Key-words: embryogenic and non-embryogenic callus; embryogenic competence; HPLC; *in vitro* culture; indole-3-acetic acid; serine proteases

Resumo

Solanum betaceum (=*Cyphomandra betacea* (Cav.) Sendtn.), vulgarmente designado tamarilho, é uma árvore de pequeno porte cultivada em várias regiões do mundo devido ao interesse dos seus frutos. Esta planta pode ser propagada por vários tipos de métodos convencionais, bem como através de técnicas de cultura *in vitro*. Uma destas técnicas é a embriogénese somática, um processo no qual uma ou várias células somáticas originam um embrião sem que haja fecundação. Esta técnica é uma importante ferramenta biotecnológica, tanto para aplicações agronómicas como para investigação científica fundamental. No entanto, os mecanismos moleculares envolvidos neste tipo de morfogénese não são ainda profundamente conhecidos.

Um elevado número de estudos nesta área tem sido levado a cabo em sistemas-modelo claássicos, tais como *Arabidopsis thaliana* ou *Daucus carota.* No entanto, muitas espécies, particularmente plantas lenhosas, são recalcitrantes em relação à embriogénese somática. Por isso, são necessários novos sistemas-modelo alternativos, mais próximos filogeneticamente destas. Neste sentido, a embriogénese somática em tamarilho tem sido estudada e otimizada no nosso laboratório, particularmente no que diz respeito aos processos de indução.

O objetivo do presente trabalho foi a caracterização de dois fatores distintos ligados à regulação da embriogénese somática, nomeadamente os níveis de auxinas endógenas, particularmente o ácido indol-3-acético (AIA), e o perfil proteolítico extracelular de calos embriogénicos e não embriogénicos de tamarilho.

Os resultados mostram que o nível de auxinas endógenas é consideravelmente mais elevado em calo embriogénico, apresentando uma tendência para aumentar com a

desdiferenciação celular do explante original. Além disso, foram detetadas diferenças na taxa de degradação da auxina endógena, com o calo não embriogénico a apresentar este parâmetro mais elevado. Os resultados sugerem também a importância da auxina endógena no processo de desenvolvimento dos embriões somáticos através da análise do efeito da presença de inibidores do transporte polar de auxinas, como o TIBA, no meio de cultura.

Os perfis extracelulares de enzimas proteolíticas revelaram diferenças, tanto qualitativas como quantitativas. Em termos quantitativos, a atividade proteolítica específica no calo embriogénico apresentou-se mais elevada, enquanto a análise qualitativa sugeriu a presença de protéases serínicas e metaloproteases. Por outro lado, no calo não embriogénico, as proteínas serínicas parecem ser dominantes. Outros tipos de hidrólases, nomeadamente glicosidases, foram também detetadas com diferentes expressões nos dois tipos de tecidos.

Palavras-chave: Ácido indol-3-acético; calo embriogénico e não embriogénico; competência embriogénica; cultura *in vitro* HPLC; protéases serínicas

1. Introduction

1.1.General introduction

The first descriptions of *in vitro* somatic embryogenesis were made independently by Steward *et al*. (1958) and Reinert (1959), working in carrot (*Daucus carota* L.). Since these pioneer studies, the process of somatic embryogenesis has been described in a great number in both gymnosperms and angiosperms (Yang *et al.,* 2012), with carrot remaining the main model, and certainly the most studied plant, in terms of somatic embryogenic studies (Yang and Zang, 2010). *Arabidopsis thaliana*, the central model of plant genetic and molecular studies, was the first plant to have its genome completely sequenced (Arabidopsis Genome Initiative, 2000) and is the plant species for which more scientific information is available. Indeed, several somatic embryogenesis protocols have been also described for *A. thaliana* (Pillon *et al*., 1996; Gaj, 2001; Ikeda-Iwai *et al*., 2002), making this organism one of the first models to consider in molecular and biochemical embryogenic studies.

However, many plant species remain recalcitrant to both somatic embryogenesis induction and regeneration protocols and the specific molecular mechanisms underlying the formation of somatic embryos and its regulation remain largely unknown, making this complex topic a key issue in plant science (Vogel, 2005; Elhiti *et al.,* 2013). Advancements in this field require alternative model plants that allow the testing of the data acquired in the classical models. Furthermore, many of the recalcitrant plants have very different characteristics than those of the aforementioned models as, for example, woody plants.

Tamarillo is a solanaceous tree in which somatic embryogenesis was described for the first time at the Laboratory of Plant Biotechnology of the University of Coimbra (Guimarães *et al*., 1988) and further investigated in several studies (Guimarães *et al*.,

1996; Lopes *et al*., 2000; Canhoto *et al*., 2005; Correia *et al*., 2012b). From this point forward the protocol for somatic embryogenesis in tamarillo has been optimized and can be carried out from several explants in the presence of different plant growth regulators (PGRs) (Correia and Canhoto, 2012). Also, in specific conditions, the same explant can originate both embryogenic and non-embryogenic calluses, allowing a comparison of several biochemical and physiological parameters between the two types of cells. Thus, the studies that have been conducted in tamarillo indicate this species as a good model for the study of somatic embryogenesis in trees. In spite of this, further characterization of the embryogenic process in this plant is required.

Beyond this scientific interest, tamarillo is also a plant of economic importance due to the production of edible fruits much appreciated in several world regions. Thus, suitable protocols for *in vitro* cloning and improvement of this plant are necessary to respond to the demands of markets.

1.2. Tamarillo

Tamarillo is a small (2 to 4 meters high) solanaceous tree (Fig. 1A) indigenous to South America, specifically the Andean region of Peru, Chile, Ecuador and Bolivia, and currently cultivated around the globe, namely in California and New Zealand, the greatest exporters (Meadows, 2002). In its natural environment, tamarillo is a subtropical plant found between 700 and 2000 meters, preferring lower altitudes in colder climates (Lewis and Considine, 1999).

It is grown for its edible fruits, which can be consumed fresh, incorporated in recipes (Bilton, 1986) or used to prepare jams or other types of processed foods or drinks (Duke and duCellier, 1993). Moreover, the fruit presents low caloric and high vitamin content, as well as several natural anti-oxidants with potential therapeutic

activities, making it a possible source of interesting secondary metabolites (Kou *et al.,* 2008; Correia and Canhoto, 2012).

It is a perennial plant with large (20 to 40 cm long and 20 to 35 cm wide) simple deciduous leaves which release a strong musky odour (Prohens and Nuez, 2010). Flowers (Fig. 1B) are fragrant, rose-shaped with a white-pink corolla and developed in clusters of 10 to 50 in the top of the branches and blossom primarily in the transition from spring to summer, but can occasionally appear out of this period (Bohs, 1989; Richardson and Patterson, 1993; Lewis and Considine, 1999). Pollination requires insects or other pollination agents like wind, but the species is mainly autogamic which can, to a great extent, explain the low variability found in natural populations (Pringle and Murray, 1991). Fruits (Fig. 1C) are usually egg shaped, though round and elongated to elliptical forms are also common, with 4 to 8 cm length, 3 to 5 cm in width and long peduncles, appearing isolated or in small groups of 3 to 12 and can present different colours, from yellow to purple (Phrohens and Nuez, 2010; Correia, 2011). Pulp colour also varies from orange-red to yellow and each fruit encloses many small round flat seeds.

Figure 1. *Solanum betaceum*. (A) Tamarillo tree growing at the Botanical Garden of the University of Coimbra; (B) Flowers at different developmental stages; (C) fruits. From Correia (2011).

Tamarillo can be propagated by seeds, cuttings or grafting into wild *Solanum mauritiantum* trees (Prohens and Nuez, 2001). The seeds germinate easily but because they do not guarantee genetic uniformity, this technique cannot be used for propagation of selected genotypes. The asexual methods can be used, but the evidence of low compatibility within the species coupled with its phytosanitary problems make them improper for propagation and improvement of tamarillo cultivars (Mossop, 1977). Thus, the use of biotechnological tools, such as *in vitro* cloning and genetic manipulation are far more suitable to replace or complement the more conventional methods (Barghchi, 1998).

The biotechnological methods currently available allow for the manipulation and propagation of economical important species from virtually any type of cell. The set of *in vitro* techniques is very diverse with several methods applicable to the same species. (Das *et al.,* 2013). Tamarillo is one of such species in which several types of *in vitro* propagation have been reported (Correia and Canhoto, 2012). The first *in vitro* technique achieved was micropropagation through axillary shoot proliferation (Cohen and Elliot, 1979) followed by regeneration by organogenesis on leaf explants (Obando *et al.,* 1992), and later from protoplasts (Guimarães *et al.,* 1996). Somatic embryogenesis (SE) has also been extensively reported in tamarillo (Guimarães *et al.,* 1988; Canhoto *et al.,* 2005; Correia *et al.,* 2011).

1.3. Somatic embryogenesis in tamarillo

Somatic embryogenesis (SE) can be defined as a process by which a somatic cell or tissue originates a structure that resembles an embryo (somatic embryo) without fecundation (Rodrígez *et al.,* 1995). It has embryonic characteristics, such as its bipolar organisation, lack of vascular contact with the parental tissue and, through a series of embryogenic stages (globular, heart-shaped torpedo and cotyledonary), germinates into a plant (Williams and Maheswaran, 1986; Jiménez, 2001).

In vitro SE is a biotechnological process with a vast array of applications, being used as a tool in applied and commercial context as well as for scientific purposes. As a research tool, somatic embryogenesis is a model for plant development and biochemical and molecular studies, especially in early phases of embryo development since, in higher plants, the developing zygotic embryo is confined inside the maternal ovule and ovular tissues, being of difficult access (Yang e Zhang, 2010). In fact, many early

events in embryogenesis have been elucidated by somatic embryogenesis experiments in a few species of plants (Fehér *et al.,* 2015).

As already indicated, the first successful study of somatic embryogenesis in tamarillo was reported by Guimarães and co-workers (1988) in mature zygotic embryos and hypocotyls. In this protocol, embryogenesis was induced using the auxin 1-naphthaleneacetic acid (NAA) and, after the formation of a reduced callus, zygotic embryos differentiate in somatic embryos, in a "one-step" system. On the other hand, if the auxin used was either 2,4-diclopheenoxyacetic acid (2,4-D) or picloram, the zygotic embryos and young leaf explants produced an embryogenic callus that could be successfully maintained by successive subcultures in the same auxin-containing medium (Lopes *et al.,* 2000). Additionally, in both induction systems, the embryogenic yield was greatly increased by the addition of high levels of sucrose (9%) to the culture medium (Canhoto *et al.,* 2005).

In the last situation, the induced embryogenic masses, developed into somatic embryos when transferred to an auxin-free medium or a medium containing 0.1 mg/L of gibberellic acid (GA_3), in a "two-step" system. Particularly interesting is the observation that, in this system, non-embryogenic cells are induced simultaneously to the embryogenic ones. The induced non-embryogenic callus, when transferred to similar conditions does not originate somatic embryos. The different steps of plant regeneration through tamarillo somatic embryogenesis are shown in figure 3.

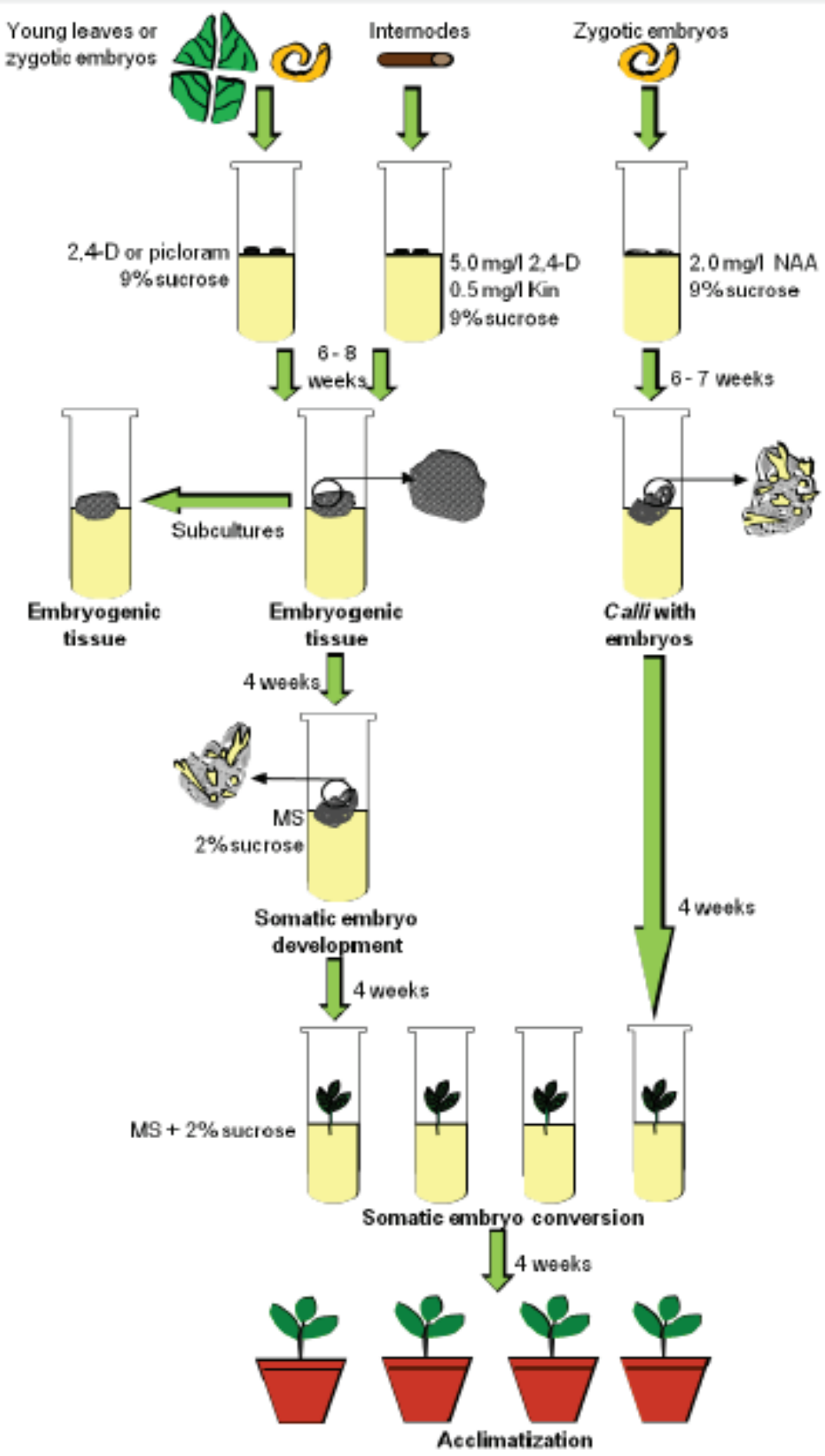

Figure 2. Schematic representation of the protocols for SE induction and plant regeneration in tamarillo. Adapted from Canhoto *et al.* (2005).

In the “two step” process the initial explants are either zygotic embryos or leaf segments (Figs. 3A and 3B). In the induction medium after a period of 8 to 10 weeks proembryogenic masses form on the explants (Fig. 3C) where both embryogenic and non-embryogenic calluses can be removed and subcultured in the same medium (Fig. 3D and 3E). After the transfer of masses of embryogenic callus to an auxin-free medium, the somatic embryos begin their development (Fig. 3F). Somatic embryos with fused or undifferentiated cotyledons and abnormal morphology (Fig. 3G) are relatively common. After the maturation phase, the somatic embryos can germinate, originating plantlets that can be rooted (Fig. 3H and 3J). Several studies have shown that different plant growth regulators as well as culture conditions such as the sucrose concentration or light/dark cycles can optimize the somatic embryo germination and decrease the abnormalities observed (Guimarães *et al*., 1996; Correia *et al*., 2012).

The mechanism of somatic embryogenesis is related to a transition from a somatic or gametophytic cell (or tissue) to an embryogenic one (Mordhorst *et al*., 1997). Despite the fact that *in vitro* ES has been described for many species, the underlying molecular mechanisms are still poorly understood and the protocols for each species are, in great extent, achieved by trial and error for particular explants (Jiménez, 2005; Fehér, 2015).In the next section, these factors are briefly outlined.

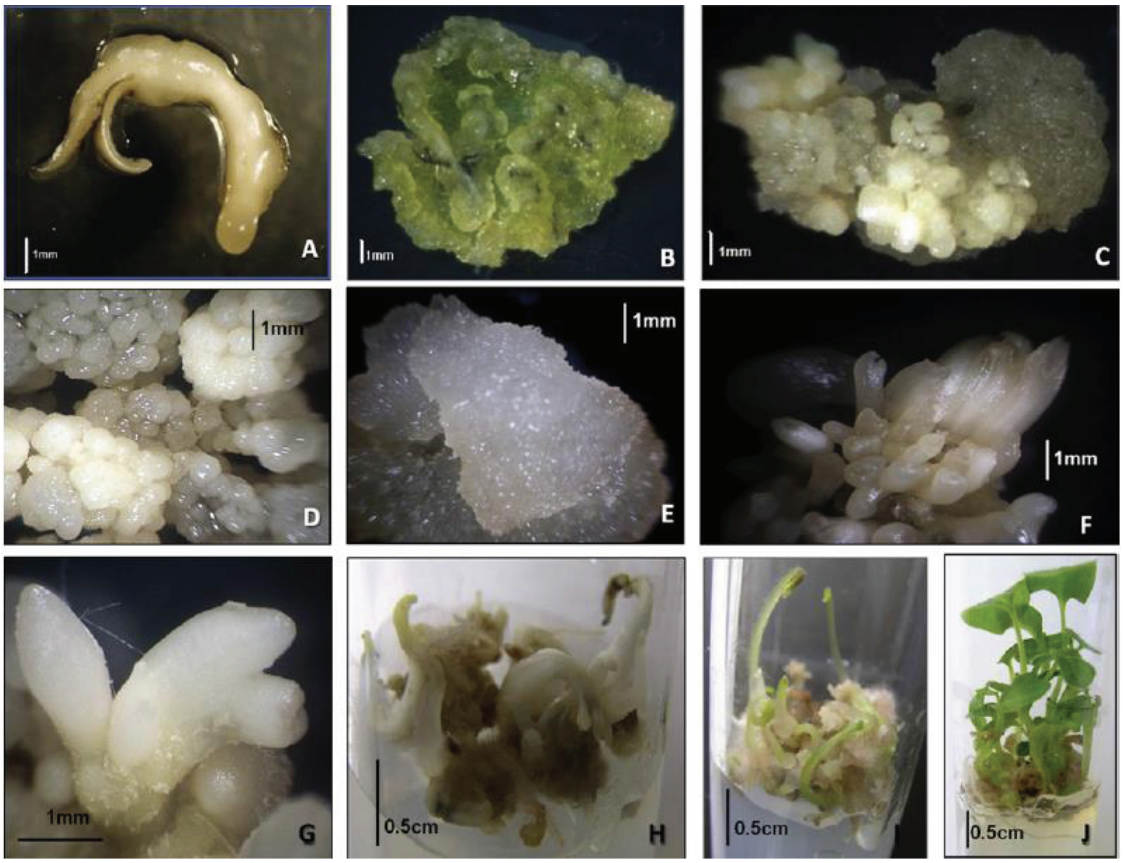

Figure 3. Somatic embryogenesis in tamarillo. (A) Zygotic embryo after 2 weeks on a 2,4-D containing medium; (B) Leaf explant after 1 month in the induction medium - MS supplemented with picloram; (C) Embryogenic and non-embryogenic (darker) zones formed on a leaf explant after 10–12 weeks of culture; (D) Embryogenic tissue; (E) Non-embryogenic callus; (F, G) Somatic embryos at different developmental stages after 3 weeks in a MS basal medium without auxins; Embryo with abnormal morphology (G); (H–J) Somatic embryo conversion and plantlet development, after 6 weeks on MS medium. From Correia and Canhoto (2012).

1.3.1. Factors controlling somatic embryogenis

The great intraspecific variability has been described in relation to the embryogenic response, which can be attributed to the genotype effect within a species. Thus, despite the fact that there is no universally applicable signal that results in SE (Mordhorst *et al*., 1997), there are certain factors that are common to practically all the

in vitro SE protocols described, namely the presence of stress factors (pH, thermic or oxidative) and plant growth regulators, typically auxins (Jiménez, 2001).

Stress factors seem to be decisive for SE induction and are apparently present in all *in vitro* protocols, with some researchers defining SE as an extreme response to stress (Jiménez and Thomas, 2005; Zavattieri *et al*., 2010). In fact, some data suggests the 2,4-D may act in a greater extent as stress substance rather than a phytohormone when used in SE protocols (Karami and Saidi, 2010). These stress factors have been extensively studied (reviewed in Zavattieri *et al*., 2010).

Additionally, the cell reprogramming required for the transition from a somatic to an embryogenic pathway requires a necessary change in the proteome and transcriptome of cells with embryogenic competence (Gruszczyńska and Rakoczy-Trojanowska, 2011). The changes are related with the genetic information of the plant, and, therefore, are considered to be internal factors of the SE process, with several studies trying to identify these genetic factors (Jiménez, 2001). In fact, several genes have already been identified as fundamental in the embryogenic process by studying mutants of *Arabidopsis thaliana* (Yang e Zhang, 2010). Furthermore, comparative proteomic studies have shown differences between embryogenic and nonembryogenic calluses induced from different explants and in the presence of distinct artificial auxins (Correia *et al.,* 2012a). Protein secretion studies, with emphasis on proteolytic enzymes, also differentiate between embryogenic and nonembryogenic tissues, with the type and activity of certain enzymes correlating with the embryogenic competence and morphology of the somatic embryos (Mo *et al.,* 1996).

Endogenous auxins have also been described as inducing the expression of certain genes related with SE (Jiménez, 2005) thus being hypothesized as having an

extremely important role in the induction and mainetance of the embryogenic tissue (Vondráková *et al*. 1992).

Therefore, both the influence of the endogenous plant growth regulators and the protein expression profiles of the tissue induced are complementary for a deeper understanding of the biochemical and molecular events underlying SE. In the next sections these factors are briefly reviewed, as well as the fundamental techniques that allow their analysis.

1.4. Auxins

Auxins (Fig. 4) are a family of plant growth regulators involved in several physiological mechanisms, namely tropistic growth, apical dominance, embryo and fruit development or acquisition of cell totipotency (Kepinski and Leyser, 2005). The existence of a "signal responsible for the unilateral movement of canary grass (*Phalaris canariensis*) in response to light" was first proposed by Darwin (Darwin, 1880). Several decades later, in 1934, this signal was identified as IAA, the major natural auxin and the first plant hormone to be described (Koegl and Kostermans, 1934). After this and other pioneer studies several other natural auxins have been reported, namely indole-3-butyric acid (IBA), 2-phenylacetic acid (PAA) and 4-chloroindole-3-acetic acid (4-CI-IAA) (Taiz and Zeiger, 2015). These auxins are less studied and their mechanisms of action are thought to be similar to those of IAA (Simon and Petrášek, 2011).

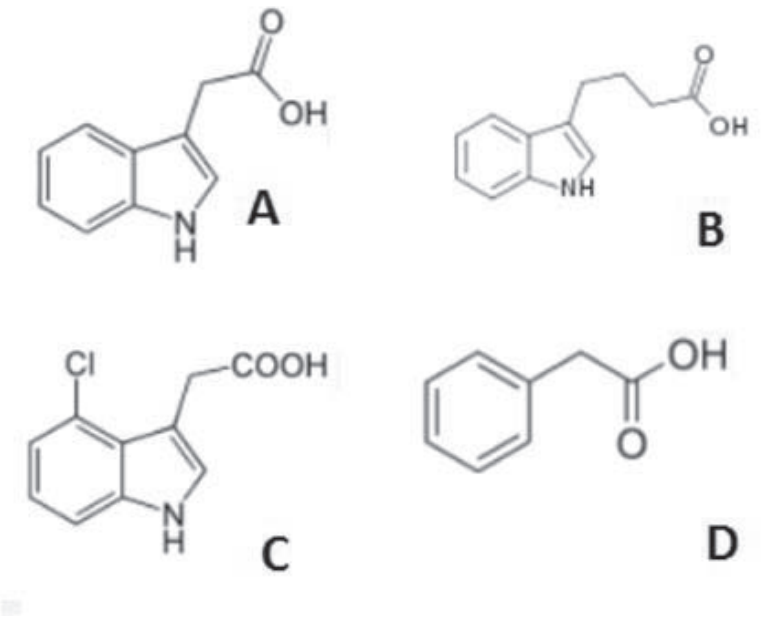

Figure 4. Natural Auxins. (A) Indol-3-acetic acid (IAA); (B) indole-3-butyric acid (IBA); (C) 4-Chloroindole-3-acetic acid (4-CI-IAA); (D) 2-phenylacetic acid (PAA).

Synthetic compounds with auxin-like effect, called synthetic auxins (Fig. 5), such as 2,4-diclophenoxacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (picloram) are also known and regularly used to mimic auxins or as herbicides (Grossmann, 2007).

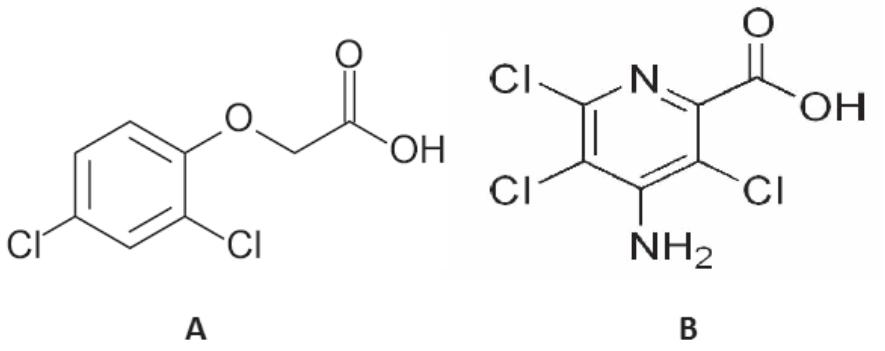

Figure 5. Synthetic Auxins. (A) 2,4-diclophenoxyacetic acid (2,4-D); (B) amino-3,5,6-trichloropicolinic acid (picloram).

The role of auxins in plant development is largely dependent upon differential concentrations, called auxin gradients, established in plant tissues (Paciorek and Friml, 2006). These appear to be largely established by a complex mechanism, involving several families of protein-transporters with a specified, sub-cellular location, namely the influx and efflux proteins AUX1 and PIN, respectively. The influx carriers are less characterized than the PIN proteins, as the auxin cellular influx is greatly influenced by the ionization state of IAA (a weak acid). However, heterologous expression studies of the *AUX1* genes on *Xenopus* oocytes have shown a pH-driven, saturable intake of IAA, confirming the importance of these carriers in the auxin transport (Yang *et al*., 2006). The PIN efflux carriers are substantively more studied, in both biochemical characteristics and physiologic importance, particularly in plant models such as *A. thaliana*, for which eight *PIN* genes are known (Křeček1 *et al*., 2009). In terms of function, PIN proteins seems to act as secondary gradient-driven transporters, acting cooperatively with ATP –binding primary transporters (Blakesle *et al*., 2007).

The interaction between these two types of transporters, as well as the intra and extracellular chemical environment, specifically the pH, leads to an asymmetric transport of auxins to specific cells, generally referred as auxin polar transport (Fig. 6), forming the auxin gradients needed for biological responses (Weijers *et al*., 2005).

Some compounds, called auxin polar transport inhibitors, such as trans-cinnamic and 2,3,4-triiodobenzoic acid (TIBA), inhibit the polar mechanism of auxin transport, without directly antagonizing the auxin effect (Katekar e Geissler, 1980). Studies with these compounds have demonstrated the importance of auxin in early embryogenic events, such as the acquiring of embryonic bilateral symmetry (Liu *et al*., 1993).

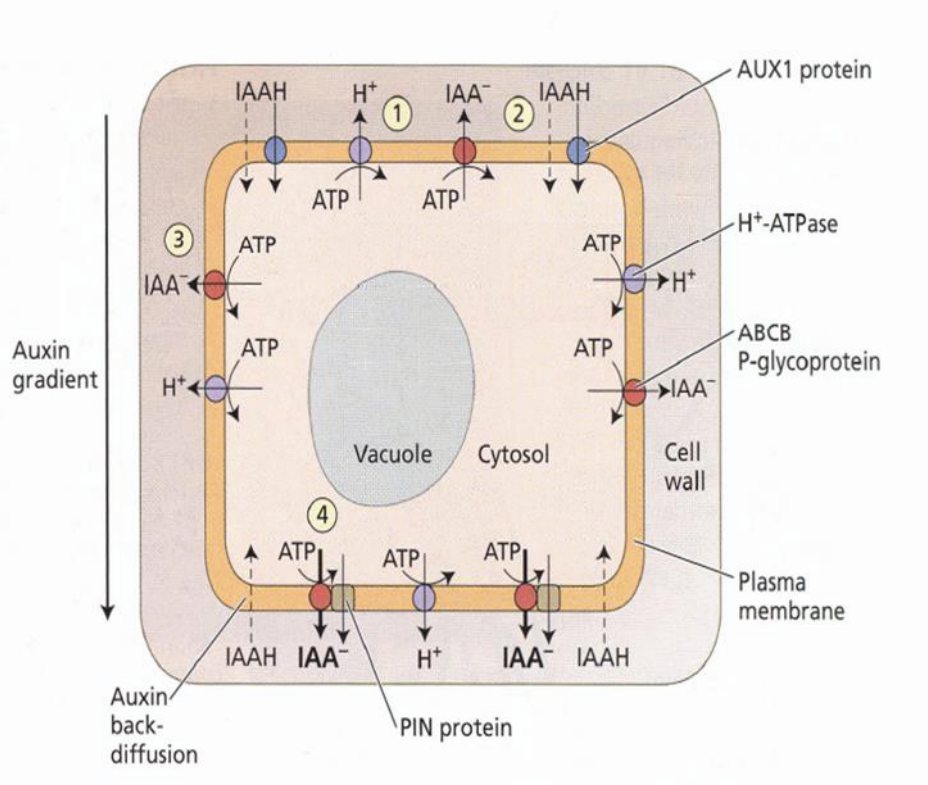

Figure 6. Mechanism proposed for auxin polar transport. The ionization state of IAA, a weak acid, is an important factor in the intracellular transport of auxins. From Taiz and Zeiger (2015).

The asymmetric distribution is also dependent on several other factors, such as the biosynthesis, degradation, conjugation and transport and accumulation of auxins (Ljung *et al*., 2005).

The biosynthetic pathways of auxins are still poorly understood, especially at the genetic level (Mano and Namoto, 2012). However, two main pathways are thought to be fundamental, one dependent from tryptophan (Trp), while the other is Trp-independent (Woodward and Bartel, 2005; Chandler, 2009; Normanly, 2010). Little is known about the Trp-independent pathway, but indole-3-glycerol or indole are the likely precursors (Zhang *et al.,* 2008). The Trp-dependent pathway is better known, with several pathways being postulated, namely the indole-3-acetamide acid, indole-3-pyruvic acid, tryptamine and indole-3-acetadoxime, and with several genes putatively

identified as involved in these biochemical pathways (Zhao, 2010). As Trp is synthetized in the chloroplast it is likely that this organelle is closely related with the Trp-dependent pathway (Zhong *et al*, 2010).

In terms of degradation, auxins and particularly IAA seem to be enzymatically degraded by one of two distinct pathways involving two different classes of enzymes (Sembdner *et al.,* 1981). The first pathway involves the oxidation of the side chain of IAA, catalysed by peroxidases (E.C. 1.11. 1.7) that require hydrogen peroxide (H_2O_2) as a cofactor, in a somewhat unspecific reaction (Pujari and Chandi, 2002). On the other hand, the second pathway is catalysed by indole-3-acetaldehyde oxidase (EC 1.2.3.7), commonly known as IAA oxidase, that acts on the indole ring, in the presence of molecular oxygen, oxidising IAA to oxindole-3-acetic acid allowing further conjugation with other molecules, namely glucose, resulting in the permanent inhibition of IAA (Taiz and Zaiger, 2015).

Finally, the mechanism of action is related with gene expression modulation. The principal auxin-responsive protein families identified (e.g. Aux/IAA repression family, Small auxin-up RNAs (SAUR) and Gretchen Hagen 3 (GH3) families) are codified by genes that contain a specific auxin-response element (AuxRE) with the sequence TGTCTC, that allowed the isolation of the transcription factor, ARF (Auxin Response Factor) that binds to this sequence (Ulmasov *et al*., 1997). Although the precise mechanisms of IAA genetic regulation are still not fully elucidated, the broad picture of auxin homeostasis (Fig. 7), from its biosynthesis to the mechanism of action is more or less clear, with an established connection between the physiological effects of auxin and its underlying molecular mechanisms (Paciorek and Friml, 2006).

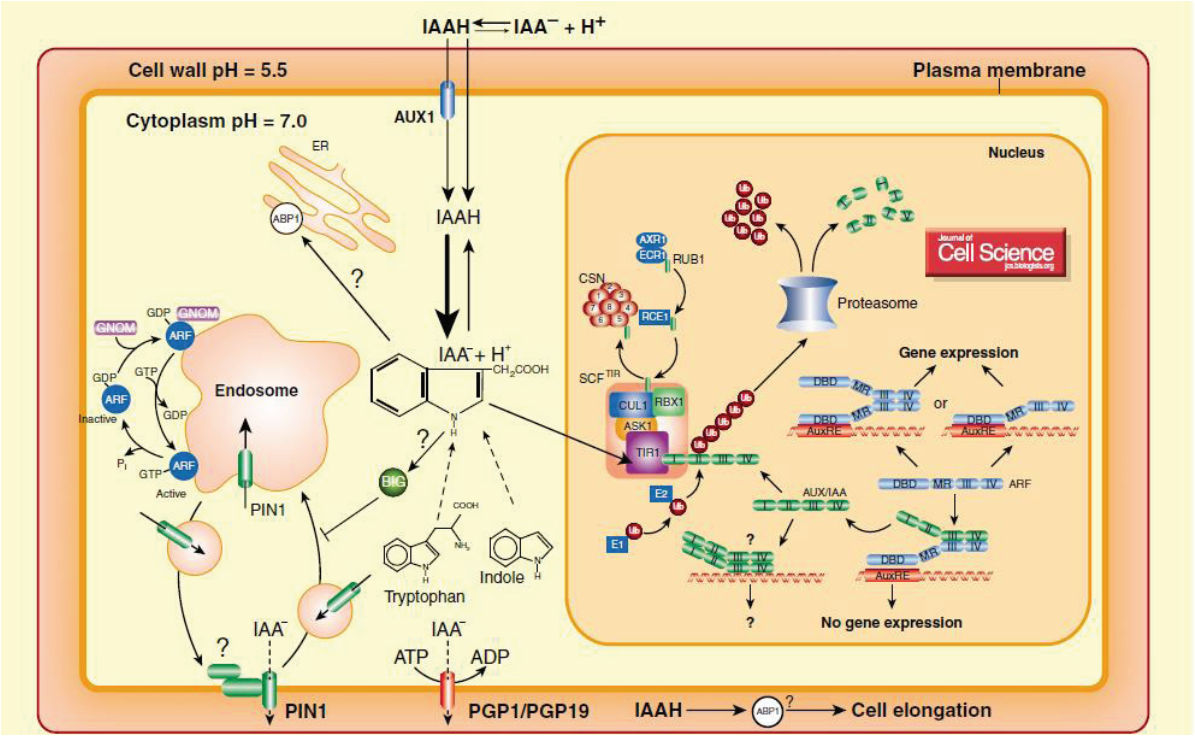

Figure 7. Auxin intracellular traffic. The principal mechanisms of transport, biosynthesis, degradation and nuclear action are depicted. From Paciorek and Friml (2006).

The importance of auxins in higher plants physiology is recognized and has been extensively studied and reviewed (Petrášek and Friml, 2006; Simon and Petrášek, 2011). These type of plant growth regulators are also fundamental in *in vitro* processes, such as SE, particularly in some species like tamarillo. The role of auxins in SE is addressed in the next section.

1.4.1. Role of auxins in somatic embryogenesis

Auxins, particularly 2,4-D, are the main plant growth regulators used in somatic embryogenesis experiments, with most protocols evolving an induction phase in an auxin supplemented medium followed by an embryo maturation phase on auxin-free or auxin-reduced medium (Raghavan, 2004). This typical induction scheme, varying the

types of synthetic auxins depending on the original explant, has been followed in tamarillo (Correia and Canhoto, 2012).

The level of endogenous plant growth regulators, namely auxins, is considered one of the most important embryogenic controlling factors (Gaj, 2004), with auxin gradients related with the establishment of bilateral symmetry being necessary for proper embryo development (Jiménez, 2005).

Studies with several plant models have demonstrated a variety of genes induced by auxins, such as the *H3-1* and *H3-11*, two histone-coding genes (Kawahara *et al*., 1992). This gene induction, compatible with the mechanism of modulation of gene expression proposed for auxin action, is a possible explanation to the transition normally required between and an auxin-rich and auxin-poor medium. In fact, it is postulated that the continued presence of auxins leads to a differential change in gene expression, probably associated with an increased level of DNA demethylation (Lo Schiavo *et al*., 1989) that allows the synthesis of the gene products required for completion of the first stages of embryogenesis (Litz and Gray, 1995). However, these conditions also favour the presence of mRNA and protein products that inhibit further embryogenic development, which upon removal of the auxin, are inactivated allowing the ontogenic program to continue (Jiménez, 2001). This idea seems to be sustained by some studies in carrot, in which embryogenic lines were able to develop up to the globular stage but no further in an auxin-rich medium, suggesting that additional genetic products are needed, and they occur only when the auxin level decreases (Yang *et al.,* 2012).

One of the major cellular mechanism regulating the auxin signal response in embryogenic development is the ubiquitin/proteasome pathway (Fig. 8) responsible for

the breakdown of specific auxin/indole-3-acetic acid (Aux/IAA) signal repressor proteins through the action of the ubiquitin protein ligase SCF^{TIR1} (Karami *et al.,* 2009). *TRANSPORT INHIBITOR RESPONSOR 1* (TIR1) mutants show reduced auxin response (Ronemus *et al.,* 1996).

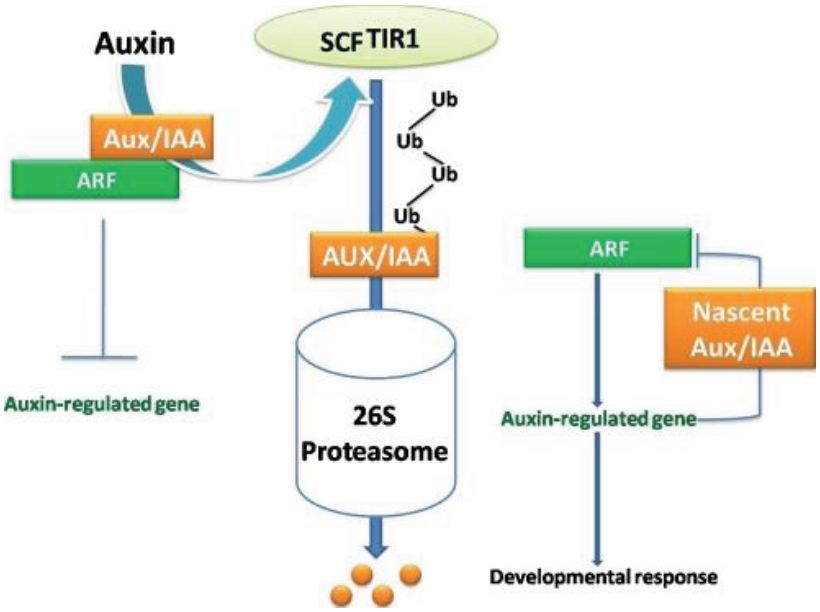

Figure 8. Auxin intracellular traffic. The principal mechanisms of transport, biosynthesis, degradation and nuclear action are depicted. From Paciorek and Friml (2006).

Another important aspect of *in vitro* auxin-mediated embryogenesis, particularly in tamarillo, as already described, is the development of two types of embryogenic cells with different embryogenic competences when "two-step" protocols are used. This difference can potentially be explained in terms of endogenous auxin concentration, since previous studies have demonstrated the need for high auxin levels in embryogenic tissues (Jiménez, 2005). Also, the relation between exogenous applied auxin and endogenous levels has been described in several studies in carrot (Michalczuk *et al.,* 1992). Thus, the endogenous level of IAA in response to the exogenously applied auxin,

mediated by yet unknown tissue or cellular factors, can decisively influence the embryogenic competence of the explant originating the embryogenic and non-embryogenic tissue observed in these two-step protocols.

Furthermore, the later stage of somatic embryo maturation can be influenced by this endogenous concentration. In fact, studies in cotton showed that after a sharp increase in the endogenous levels of IAA during the formation of the somatic embryos, a decrease was observed in the later stages of embryonic development, with the profile concentration showing a minimum during these stages (Yang *et al.*, 2012).

1.4.2. Methods of auxins analysis

Since the isolation of IAA and the identification of the auxin family several methods have been used to directly or indirectly quantify the endogenous level of auxins in plants. The first detection tests were bioassays, such as the *Avena* curvature or the split pea test (Went and Thimann, 1937). These tests measure a biological response dependent on the auxin level, but are susceptible to several sources of error, namely different auxin tissue sensibilities (Kim *et al.,* 2006).

Auxins can also be qualitatively and quantitatively measured by a colorimetric reaction based on the interaction of specific reagents, notably Salkowski (Salkowski, 1885) and Ehrlich (Anthony and Street, 1969) with the indole ring. These reactions were firstly used in conjunction with another techniques like thin layer chromatography (TLC) to identify auxin and its derivatives in mostly qualitative protocols of analyses (Ehmann, 1972). However, they can be adapted to quantitative protocols with an acceptable specificity (than can be improved by optimization of assay conditions) and accuracy, particularly the Ehrlich reagent based method (Anthony and Street, 1969).

Immunoassays based on IAA-specific antibodies have also been developed, with ELISA kits commercially available (Agrisera antibodies No. AS11 1749). Although precise and sensitive, these techniques present some weaknesses for extract quantification like cross reactions with other products, particularly methylated and conjugated IAA. Another setback of this method is the high cost of the required reagents and their relative short life span.

The most accurate and widely used techniques for auxin measurement today are chromatographic methods, with gas chromatography associated with mass spectrometry (GC-MS) being considered the most sensitive technique available (Mezzetti *et al*., 2004). However, the high-cost and operational difficulties of this technique, that requires stable isotope-labelled IAA as an internal standard, makes the use of GC impractical for routine use, particularly in non-specialized laboratories. To overcome this difficulty, high performance liquid chromatography (HPLC) protocols have been developed using a non-isotopic internal standard and relying on properties of the indole ring to detect a signal by either UV-visible spectrometry or fluorescence. A molecule used as internal standard is indole-3-propionic acid (IPA). IPA is very similar in structure to IAA, possessing an indole group and a side chain with an odd number of carbon atoms (3), in opposition to the even number that active auxins present, 2 in IAA and 4 in indole-3-butyric acid. Additionally, this compound is absent in plant tissues (Robson *et al.,* 1961) making it a good non-isotopic stable internal standard for GC (Mezzetti *et al*., 2004) and HPLC (Kim *et al.*, 2006).

1.1.Proteolytic profiles

As already described, somatic embryogenesis induction involves a deep change in the proteome of the cells resulting from the transition from a specific somatic state to

acquisition of embryogenic competence (Fehér, 2015). These changes have been studied in several classic ES models, namely carrot (Mitsuhashi *et al*., 2014) but also tamarillo (Correia *et al.,* 2012a)

Another important factor during the SE process are the secreted proteins, with nearly all *in vitro* culture plants presenting some degree of excretion, mainly thought to be involved in plant defence mechanisms (Quiroz-Figueroa *el al.,* 2006). Recent studies have shown a distinct extracellular proteolytic profile in embryogenic tissues on different stages of embryogenic development as well as different profiles between embryogenic and non-embryogenic tissues (Rakleova *et al.,* 2010). In fact, the importance of proteolytic signalling cascades has been known to be a biochemical process of extreme importance in many biological systems in several kingdoms, and in light of these findings, is considered relevant in SE as well (Mitsuhashi *et al.,* 2014). Although the referred studies are generally in an early stage, the results show both the presence of several types of proteases in the extracellular medium and the shifting in the expression profile along the developmental stages (Mitsuhashi *et al*., 2014).

These studies are generally conducted with cell suspensions of embryogenic and non-embryogenic tissues in auxin-rich media and somatic embryos in auxin-poor medium, with varying conditions dependent upon the species and explant used. The induction phase is normally carried out in a solid medium applying previously established SE protocols (Mitsuhashi *et a*l., 2014). The initial culture conditions, particularly the cell suspension density, has a demonstrated importance in the outcome of the experience, as shown by Maës and co-workers (1997) in embryogenic cell suspensions of grapevine rootstock, where initial populations higher than 5×10^3 cells/ml inhibited the later stages of embryo formation, namely the cotyledonary stage. This

inhibition has been linked to the presence of extracellular macromolecules (Maës *et al.,* 1997). Previous work in tamarillo cell suspensions has been carried out allowing optimized growth parameters and a comparison between the extracellular protein profiles in embryogenic and non-embryogenic calluses (Alves, 2012).

These extracellular proteolytic activity identification works are generally conducted by studying the protein profiles by using classical denaturing electrophoresis techniques, complemented with native separation and activity tests with broad substrates such as gelatine, as well as spectrometric or fluorometric activity assays. Furthermore, the use of specific protease class inhibitors allows the identification of the dominant classes of proteases.

1.5.1 Classes of Proteases

Proteases, or proteolytic enzymes, are a class of hydrolases that catalyse the cleavage of peptide bonds in protein or protein-based substrates. These enzymes have a key role in many fundamental biological processes: i) degradation of proteins in nutritive or catabolic cycles; ii) regulating the targeting and location of many proteins; iii) regulating the biological activity of proteins, namely enzymes by the proteolytic activation on proenzymes; iv) modulation of protein-protein interaction; v) intervening in some forms of cell signal transduction (Walsh, 2014).

This family of enzymes is characterized by a great structural and functional diversity, with a broad divergence of the polypeptides chains and their three-dimensional organization, which can range from single chains of 20 kDa to multi-subunit complexes of several kDa. Functionally, these proteins also present different characteristics, some present a broad substrate specificity while others hydrolyse a single peptide bound in a particular protein substrate (Walsh, 2014). The great diversity

of these family of enzymes gave origin to several classification criteria, such as the position of the peptide bound hydrolysed. Thus endopeptidases, are those that cleave internal peptide bounds whereas, exopeptidases, act near the carboxyl or amino terminus, with further classification criteria existing within these types (e.g. aminopeptidase, depetidyl peptitase, tripeptidly peptidase, carboxypeptidase and peptidyl dipeptidase) (Walsh, 2014). Another type of classification criterion is the mechanism of action, where a fundamental aspect of the catalysis mechanism, such as the essential presence of one or several amino acids in the active site is used as the identifying characteristic (Neurath, 1999). According to these scheme of classification there are six categories (Table I): metalloproteases, serine, cysteine, aspartic, glutamic and thereonine proteases.

Serine proteases are characterized by the presence of an essential serine residue in the active site and are the most common class of proteases, being present in all kingdoms as well as viruses. They can be divided into several subfamilies according with structural similarities, one of these families being the subtilisins, of great technological interest. Two classical inhibitors used in serine proteases studies are tosyl phenylalanyl chloromethyl ketone (TPCK) and tosyl-L-lysyl-chloromethane hydrochloride (TLCK) (Hedstrom, 2002). Aspartic proteases contain an essential aspartic acid residue and generally display maximum activity in an acidic pH and can be inhibited by pepstatin A (Harlow and Lane, 1999; Walsh, 2004). Cysteine proteases are characterized by the presence of a cysteine and histidine residue which form a catalytic dyad essential for activity and can be inhibited by, among others, trans-epoxysuccinyl-l-leucylamido-(4- guanidino)butane (E-64). Threonine proteases contain a threonine residue at the active centre, the main example of this kind of catalysis being the proteasomes found in eukaryotes, some bacteria and Archaea. Glutamate proteases,

initially thought to be a family of aspartic proteases, are characterized by a catalytic dyad of glutamic acid and glutamine residues and are predominantly found in filamentous fungi (Sims *et al.,* 2004). Finally, the metalloproteases exhibit activity only in the presence of divalent metal ions and can be inhibited by the presence of ethylenediamine tetraacetic acid (EDTA) and phenathroline (Walsh, 2004).

Table I. Classes of proteolytic enzymes.

Protease class	Essential residue/molecules	Known inhibitors	Example
Serine	Ser	TPCK; TLCK	Trypsin; subtilisins
Aspartic	Asp	Pepstain A	Pepsin; Chymosin
Cysteine	Cys; His	E-64; Bestatin	Papain; ficin
Threonine	Thr	Sodium orthovanadate	The proteasome
Glutamic	Glu; Gln	EPNP	Fungal proteases
Metalloprotease	Divalent ions	EDTA; 1,10-phenanthroline	Collagenase

1.5.2 Proteases related with somatic embryogenesis

Plant genomes typically encode hundreds of proteolytic enzymes of several different and unrelated families with the biological significance of these proteases still largely unknown. However, the great number of studies recently undertaken to address this issue seems to indicate that these great variety of proteases is closely related with the regulation of several biological important phenomena including embryogenesis (van der Hoorn, 2008).

One of the earliest studies of proteases involved in somatic embryogenesis was made in carrot and identified a trypsin inhibitor expressed in both embryogenic and non

embryogenic tissue, but only detected secretion to the extracellular medium on the embryogenic tissue (Carlberg, 1987). Although the results were insufficient to demonstrate or propose any biological function, this work was an indicator that the extracellular proteolytic enzymes are, to some degree, important in the establishment of embryogenic competence.

Later studies have demonstrated a wide array of extracellular proteases in carrot cell suspensions. Mitsuhashi and co-workers (2004) found four types of cysteine proteases, dynamically changing according to the developmental embryotic stage. Cysteine proteases have been subsequently found in suspensions of rice (*Oryza sativa*) and orchardgrass (*Dactylis glomerata*) (Rakleova *et al.,* 2010). Furthermore, treatment of grapevine somatic embryos with aprotinin, a cysteine protease inhibitor blocked the embryo development beyond the globular-shaped stage (Maës *et al.,* 1997), indicating the relevance of this particular class of protease.

Serine and subtilisin-like proteases have also been implied in the zygotic and somatic development of *A. thaliana* embryos, and, to a lesser extent, metalloproteases and aspartic proteases have been detected in carrot somatic embryos (Mitsuhashi *et al.,* 2004).

Previous studies on tamarillo extracellular profiles in our laboratory have identified by protein mass spectrometry several proteases, mainly subtilisin-like proteins distinctly expressed in embryogenic and non-embryogenic calluses, as well as other types of hydrolases including glucanases and xylanases (data not published). Most of these proteins have been identified using homology analyses.

Although these and other studies have demonstrated the importance of the extracellular proteins, particularly proteases and carboxypeptidases, their precise role in ES is still unclear, with some possible functions being the degradation of non-functional proteins, generation of active peptides or the modulation of the cell-wall in response to environmental stimuli (Hoorn, 2008).

1.2.Objectives

As already described, there are several protocols available for SE in tamarillo, namely a "two-step" indirect method that originates, in the same explant, embryogenic and non-embryogenic tissue. Both types of tissue can be maintained *in vitro* for relative long periods of time, making these cell lines a good material for the study of the underling factor that determines their embryogenic competence.

SE is a complex biological process influenced by many factors, such as stress agents, the presence of plant growth regulators and ultimately the genetic information of the plant and how this information is expressed in response to the environmental conditions, allowing the cellular reprogrammation responsible for embryogenic capacity.

The main aim of this work is to further characterize some of these factors in tamarillo: i) the role of endogenous auxin concentrations in the embryogenic tissue; ii) the differential extracellular proteolytic profiles in embryogenic and non-embryogenic calluses.

The endogenous auxin concentration has been found to be a determinant factor on other model species, and in this thesis it will be further studied on tamarillo. This study will be based on chemical quantifications of IAA, the main endogenous auxin, in

the embryogenic and non-embryogenic calluses in order to correlate this parameter with embryogenic competence. Furthermore, the quantitative studies will be carried out along the induction protocol, to establish an evolution of auxin concentration. Secondly, the importance of this phytohormone will be indirectly studied in the maturation phase of the somatic embryos by applying an auxin polar transport inhibitor (TIBA).

The extracellular proteolytic studies will continue previous work (Alves, 2012) and the core objective is to differentiate between the extracellular proteolytic profiles of embryogenic and non-embryogenic samples employing electrophoretic and fluorometric techniques to detect catalytic activity. To identify the main classes of proteases present, assays with classic inhibitors will be carried out.

The results of both these parameters can jointly contribute for a deeper biochemical and physiological characterization of the embryogenic tissue, thus allowing a deeper understanding of the process of somatic embryogenesis.

2. Material and Methods

2.1. Plant material and culture conditions

2.1.1. I*n vitro* propagation of tamarillo clones

Tamarillo plants used for SE induction, were propagated from previously established shoot cultures from *in vitro* germinated seeds, in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 3% sucrose, 0.2 mg/l of 6-benzylaminopurine (BAP) and 6 g/l of agar. The shoots (1 – 1.5 cm) were subcultured once a month using the same medium and kept in a growth chamber at 25 ºC, in a 16 h photoperiod, at 25-35 μmol $m^{-2}s^{-1}$ (white cool fluorescent lamps). The leaf segments used in the SE induction protocol were aseptically removed from these shoots, after one month in propagation medium.

2.1.2. Somatic embryogenesis induction

The induction medium used for SE induction on young leaf segments was based on MS medium with 9% (w/v) sucrose and a synthetic auxin, either 2 mg/l of 2,4-D for the embryos or 5 mg/l of picloram for the leafs (Lopes *el al.,* 2000). The pH was adjusted to 5.7 (using KOH or HCl solutions) and 2.5 g/l of Phyta gel (Sigma) was added before autoclaving at 121ºC for 20 min. The induction phase was carried out in dark conditions in a growth chamber at a temperature of 24ºC ± 1ºC during 8 weeks.

The *calluses* resulting from the induction stage were maintained *in vitro* in the same type of medium or transferred to a hormone-free maturation MS medium, with 4% (w/v) sucrose, to produce somatic embryos.

During the induction phase, samples of dedifferentiating explants were periodically (every 2 weeks) removed from the induction medium and frozen in liquid nitrogen for further analysis.

2.1.3. Cell suspensions

The calluses used in cell suspension cultures were previously induced and characterized in terms of its embryogenic competence (Correia, 2011). For the explants with reported embryogenic competence two lines were used, one induced from zygotic embryos in a 2,4-D induction medium, EC1, and one from young leafs induced in a picloram supplemented medium, named EC2. The non-embryogenic callus used was originated from young leafs (in a picloram supplemented medium) and was identified as NEC2. Additionally, a fourth line was used, with the same origin of EC2 and NEC2, which presented embryogenic-like morphology but a low embryogenic yield and was named EC3.

The calluses lines were grown on liquid basal MS medium supplemented with 9% (w/v) sucrose and either 5 mg/l picloram (EC2, EC3 and NEC2) or 2 mg/l 2,4-D (EC1), under dark conditions and agitation (120 rpm) for a period of 3 weeks. At the start of the growth period, the fresh mass of *calluses* was determined, using a standard reference ratio of 50 mg of calluses for 40 ml of liquid medium.

After three weeks of growth in these conditions, the medium was filtered under vacuum using a 0.45 μm Millipore® filter to obtain a cell free medium that was transferred to a sterile falcon tube. The *calluses* mass retained in the filter was weighed and immediately transferred to a solid culture medium with the same growth regulator as before (picloram or 2,4-D) and subsequently maintained in this medium.

2.2. Auxin Analysis

2.2.1. Polar auxin inhibitors assay

To test the influence of the auxin in the maturation phase of the *calluses*, the polar auxin inhibitor 2,3,5-triiodobenzoic acid (TIBA, from Sigma) was added to the maturation medium in a concentration range of 0.5 to 10 μM. 10 mM stock solutions of the inhibitor were prepared by dissolving the appropriate amount of TIBA in distilled water (using 1 ml of NaOH 1 M to allow solubilisation), sterilized by filtration with a 0.20 μm millipore® and stored at -20 ºC. The solutions were added to the maturation medium after autoclaving, at a temperature of about 60 ºC (to avoid thermal decomposition) and a control was prepared without any inhibitor. The medium was distributed in Corning® 100 mm × 20 mm Petri dishes. After the solidification of the culture medium, a mass of about 200 mg of the CE2 line was weighted was transferred to the medium and incubated in a culture chamber in dark conditions and a temperature of 21 ºC ± 1 ºC for 3 weeks. At the end of the growth period, the final mass of tissue was determined, in order to calculate the percentage of mass increment during the 4 week period (final mass – initial mass) / initial mass x 100 and the number of morphological normal and abnormal somatic embryos were counted.

All the concentration ranges, as well as the control, were made in t quadruplicate. The results are presented in number of somatic embryos per mg of initial mass.

2.2.2. Ehrlich reaction

The IAA content in the established calluses lines was first assayed using the colorimetric method described by Anthony and Street (1969). In this method, Ehrlich's

reagent reacts with the indol group of IAA in an acid medium, under optimized conditions for improved specificity. The reagent was prepared by dissolving 2 g of p–dimethylaminobenzaldehyde in 100 ml HCl 2.5 N. The plant material was grounded in a sterilized mortar with 2 ml of K-phosphate buffer 0.01 M (pH 6.0) and centrifuged (4800 g; 20 min). After centrifugation, the supernatant was used for the quantification. The reaction mixture was composed of 1 ml of diluted sample (10 x) in the K-phosphate buffer, 2 ml of trichloroacetic acid (TCA) 100% (w/v) and 2 ml of Ehrlich's reagent added in order. A blank solution of K-phosphate buffer was prepared simultaneously. After an incubation period of 30 min, the absorbance was measured at 530 nm in a Jenway 7305 spectrometer. A calibration curve was prepared using buffered solutions of IAA with concentrations between 2 and 50 μg/ml. The results are presented in μg of IAA per mg of fresh tissue.

2.2.3. Degradation of IAA

The oxidation of IAA by endogenous enzymes was assayed by a spectrophotometric procedure based on the oxidation of a known amount of IAA in a defined time period.

The tissue (approximately 500 mg) was grounded in a sterilized mortar with 2 ml of K-phosphate buffer 0.01 M (pH 6.0) and centrifuged (4800 g; 20 min) to remove cellular debris. The supernatant was collected and the reaction was initiated by addition of a solution of IAA (0.02 mM IAA; 0.02 mM $MnCl_2$) to a 1 mL of extract and incubated for 30 min. After the incubation period the IAA content was essayed using Ehrlich reaction and compared to a control without the extract. The results are expressed as mM of IAA oxidized per mg of fresh tissue per minute.

2.2.4. Quantification of IAA by HPLC

The method used allows the quantification of indole-3-acetic acid (IAA) by high performance liquid chromatography (HPLC), using an internal non-isotopic standard, indole-3-propionic acid (IPA) as descried by Kim *et al.* (2006) with modifications.

The plant material was extracted in 100% methanol (2.5 ml per gram of fresh weight tissue), IPA was added (10 µg/g.f.w.), and the resulting extract was cleared by centrifugation (16000 g, 10 min) at 4 ºC. Before the next steps, the polarity of the extract was increased by adding one volume of pure water. The sample was then extracted by two steps of serial partition against 100% ethyl acetate. In the first, the pH of the aqueous phase was adjusted to higher than 9 (1 M KOH) using pH indicator paper (Merck), in order to keep the IAA and IPA in their ionized form and, after separation of phases by centrifugation (16 000 g, 10 min.), the aqueous phase was transferred to a new tube and the pH was reduced to less than 3 (IAA and IPA protonated) and again partitioned against ethyl acetate. After separation of phases by centrifugation (16000 g, 10 min.), the organic phase was collected, completely dried in vacuum and dissolved in a minimal volume of 100% methanol, micro-filtered and injected in the HPLC apparatus.

The samples were then analysed in an HPLC system composed by a Gilson 234 injector, Gilson 305 pumps and 4.6 × 250 mm Waters Spherisorb® C_{18} column and a Gilson 170 diode array detector. An isocratic elution of 56% methanol and 44% water and orthophosphoric acid (pH =2.3) with a flow of 1 ml/min. The system used the control and analysis software Gilson Unipoint v 5.11. The detector wavelength was set at 282 nm, taking advantage of the indole group.

Calibration curves of standard concentrations of both IAA and IPA between 0.5 and 25 µg were prepared in triplicate. Also, the resolving power of the isocratic elution was tested with mixtures of both the components in different concentrations.

2.3. Extracellular protein analysis

2.3.1. Protein concentration

To follow protein secretion during cell suspension growth, the absorbance of the medium at 280 nm was weekly measured in a Jenway 7305 spectrophotometer using 5 mL silica spectrophotometer cells (b=1 cm) against a distilled water blank.

In order to precipitate the proteins secreted into the liquid culture medium, ammonium sulfate was added to a final concentration of 80% (w/v) and after its complete dissolution, the solution was centrifuged at 4 ºC (20 min; 10000 g) in a Beckman Avanti j-20xp refrigerated centrifuge. The supernatant was decanted and the pellet was dissolved in 2 ml of 30 mM sodium phosphate buffer (pH 6.0), dialyzed (MWCO: 12-14000 Daltons, medicell membranes) overnight at 4ºC against 1l of the same buffer to remove excess ammonium sulfate and then frozen (-20ºC) until further analysis.

2.3.2. Electrophoresis

After the concentration step, both pellets and supernatant were subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) following the method described by Laemmli (1970). The samples were incubated in a denaturing buffer (125 mM Tris-HCl, pH 6.8, 100 mM glycine; 40% (v/v) glycerol; 4% SDS (w/v); 200 mM DDT; 0.001% (w/v) bromophenol blue) for 15 minutes at 98 ºC and loaded into a 12.5% polyacrylamide gel (1.5 M Tris-HCl pH 8.8; 40% acrylamide : bis-acrylamide (29:1); 20% SDS (v/v); 10% ammonium persulfate (APS), 3 µL

tetramethylethylenediamine (TEMED) Milli Q water) vertically assembled in a Mini-Proteon Tetra Handcast (Biorad) and subjected to a 120 V potential for an hour and half at room temperature. After the electrophoresis, the gels were stained using 0.25% (w/v) Coomassie Brilliant blue R250 in 45% (v/v) methanol and 5% (v/v) acetic acid, for 15 minutes and distained by successive washing in a distaining solution (25% (v/v) methanol, 5% (v/v) acetic acid). When this method was insufficient to observe protein bands due to low protein concentration (below 100 ng), the gels were stained with silver nitrate. The gels were successively washed with 50% (v/v) ethanol and 30% (v/v) ethanol for 10 min, Milli Q water for 20 min twice, incubated in a silver nitrate solution (0.2 g/l) for 20 min and revealed (0.7 ml/l formaldehyde; 30 g/l anhydrous calcium carbonate; 10 mg/l sodium thiosulfate) until the bands were visible, and the revelation was stopped with a proper solution (50 g/l Tris and 2.5% (v/v) acetic acid). After staining the gels were kept in water.

2.3.3. Zymography

The dialyzed samples were subjected to a zymography to detect proteolytic enzymes in solution. The 12.5% acrylamide was prepared and assembled as before with addition of gelatin to a final concentration of 0.1% (substrate of the proteolytic enzymes). The samples were loaded into the gel using a buffer without reducing agents (125 mM Tris-HCl pH 8.8; 15% sucrose (w/v); 2.5% SDS (w/v), 0.001% (w/v) bromophenol blue) and subjected to a 120 V potential for an hour and half at 4ºC. After electrophoresis, the gel was washed in a renaturation buffer (30 mM phosphate buffer (pH 6.0), 1% triton X-100) for 1 hour and incubated at 37 ºC in sample buffer overnight. After the incubation period the gels were stained with Coomassie blue, appearing distained bands were there was proteolytic activity. Additionally, an SDS-

PAGE using the same loading buffer was made to verify the apparent molecular weight of the bands with activity. This gel was stained using silver nitrate.

2.3.4. Protein quantification

The total protein quantity was essayed using Bio-Rad Protein Assay based on Bradford's reaction (Bradford, 1976) in a 96-well microplate. A calibration curve was constructed using concentrations of BSA between 5 and 40 µg/ml. All measurements were made simultaneously and in triplicate at 595 nm in a SPECTRAmax PLUS 384 spectrophotometer.

2.3.5 Enzyme activity assays

The proteolytic specificity was essayed using AMC (Amino Methyl Cumarine) fluorogenic substrates coupled with the amino acids Arginine (L-Arginine- 4-methylcoumaryl- 7-amide) (Arg-AMC) and Phenylalanine (L-Phenylalanine-4-methylcoumaryl-7-amide) (Phe-AMC) (PeptaNova). The reactions were conducted in a SpectraMAX-GemiNI microwell fluorescent reader at 37º C with and excitation and emission wavelength of 380 and 460 nm, respectively, for 30 min, and were initiated with the addition of 2 µl of substrate to 200 µL of sample (final substrate concentration of 0.1 mM). The results were obtained as the slope of the reaction curve in relative fluorescent units (RFU) per second and converted to pmol AMC/min/µg of protein. To identify the main class of protease present in the extracts, another set of assays with 7 inhibitors was carried out: EDTA, bestatin, E-64, phenathroline, TLCK, TPCK and pepstatin A. Each inhibitor (final concentration of 1 mM) was incubated with 200 µl of extract for 10 min at room temperature. The reaction was initiated with the addition of 2 µl of Phe-AMC and carried out the conditions described previously. The results are

presented as % of inhibition in relation to a control prepared and tested at the same time as the samples.

Additionally, the specificity to other hydrolytic substrates was tested, using different types of fluorogenic substrates, namely 4-Methylumbelliferyl-β-D-gucopryanoside (MU-G) and 4-Methylumbelliferyl-acettyl-β-D-glucosaminide (MU-NAG) (Sigma-Aldrich), in a final concentration of 0.5 mM. The conditions were the same as before with exception of excitation and emission wavelength, in this case of 365 and 460 nm, respectively. The results were obtained as before and converted to pmol MU/min/μg of protein.

2.4. Statistical analyses

The homogeneity of variances was tested with the Brown–Forsythe test ($p<0.05$). In the case of homogeneity of variances, the data was analyzed with one-way analysis of variance (ANOVA) and, where necessary, the means were compared by Tukey test ($p<0.05$).

In the case of non-homogenous variances, the non-parametric Kruskal-Wallis one-way analysis of variance was used and, in this case, the means were compared by Dunn's multiple comparison test ($p<0.05$).

3. Results

3.1. Auxin analysis

3.1.1. Auxin polar transport inhibitors

The purpose of this assay was to establish the role of endogenous auxin concentration on tamarillo somatic embryo maturation from embryogenic callus by using TIBA, a known auxin polar transport inhibitor.

The embryogenic callus (EC2) was induced from leaf explants and proliferated in a picloram containing medium. The callus tissue was removed from the proliferation medium, weighted and the number of somatic embryos recorded. Somatic embryos in different developmental stages (globular, heart-shaped, torpedo and cotyledonar) were observed (Fig. 9A) as well as abnormal somatic embryos (Fig. 9B). Embryo development was not synchronized since different developmental stages could be present in the same sample, mainly globular but, in less number, also heart-shape, torpedo and cotyledonary embryos (Figs. 9C and 9D), Furthermore, proembryogenic masses proliferation without the differentiation of somatic embryos (Fig. 9E) was also observed.

The growth of embryogenic callus and the number of normal and abnormal embryos was counted and registered considering the final mass of the samples (Table II).

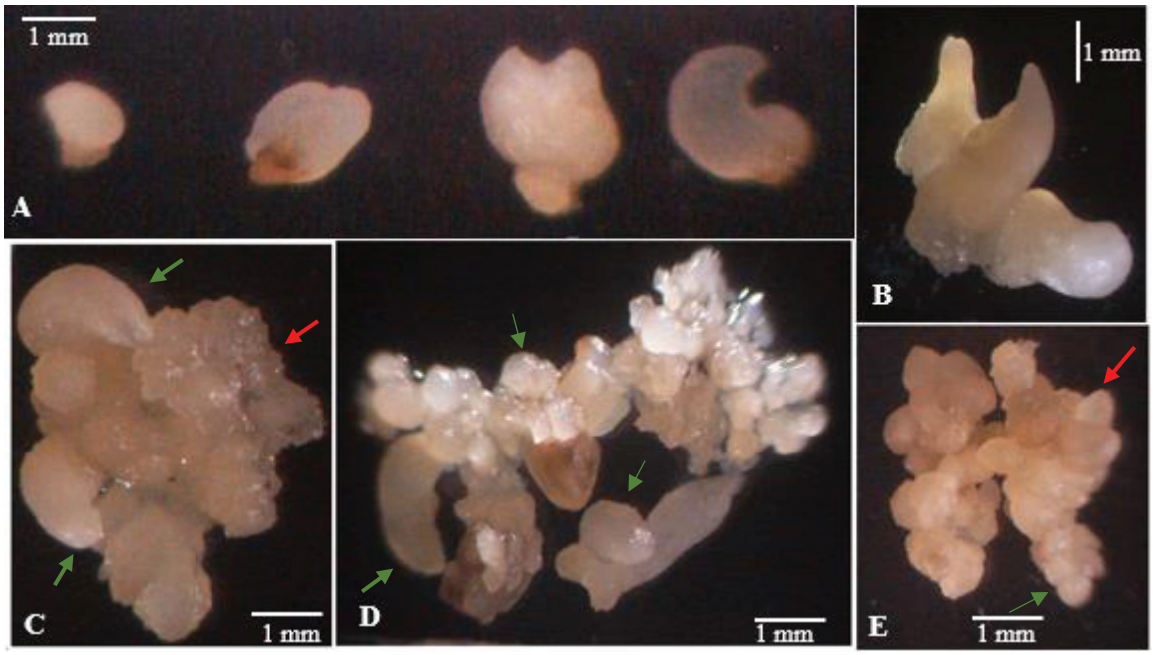

Figure 9. Somatic embryo development. (A) Normal somatic embryos in different developmental stages, observed in the control samples (without TIBA); (B) somatic embryos with abnormal morphology; (C) somatic embryos (green arrows) surrounded and proembryogenic masses (red arrows); (D) asynchronous embryo development, with several stages present and surrounded by proembryogenic masses; (E) proliferated proembryogenic masses (red arrows) with only some globular somatic embryos (orange arrows).

The mass increment used to evaluate the proliferation of the proembryogenic masses showed no statistical differences between the control group (without TIBA) and the treatments, while there was still an overall tendency for the increment to diminish with the increasing of TIBA concentration, as can be confirmed by the statistical difference between the lower (0.5 and 1 µM) and higher concentrations tested (5 and 10 µM).

Table II. Mass increment and number of somatic embryos registered in the auxin polar transport inhibitors assay. Values are presented as mean ± SD (n=4); values in the same column with different letters are statistically different by Tukey test (p<0.05) in the case of the mass increment and abnormal embryo formation. Normal embryo formation was analysed by Dunn's multiple comparison test (p<0.05). Results were taken after 3 weeks of growth on solid medium with varying concentrations of TIBA.

TIBA (µM)	Mass Increment (%)	Embryo formation (number/g of tissue)	
		Normal	Abnormal
0.0	81.52 ± 3.16 [a, b]	22.16 ± 9.58 [a]	9.08 ± 8.02 [b]
0.5	84.87 ± 2.10 [a]	17.42 ± 1.99 [a]	3.86 ± 1.18 [b, c]
1.0	84.94 ± 1.33 [a]	14.4 ± 3.45 [a]	1.71 ± 0.20 [b]
5.0	84.18 ± 0.22 [a]	7.77 ± 2.90 [b]	10.13 ± 2.62 [a, b]
10.0	76.63 ± 0.19 [b]	2.08 ± 1.50 [b]	1.51 ± 0.74 [b]

Normal embryo formation was not statistically different between the control and the lower TIBA concentrations used (0.5 and 1 µM). However, by comparing the samples with the higher concentrations it was found that an increasing in TIBA greatly reduced the formation of normal somatic embryos. Abnormal embryo formation only increased over the control when 5 µM TIBA were added to the culture medium. In all the other TIBA concentrations the results did not show significantly differences relatively to the control.

3.1.2. Ehrlich reaction

The first auxin quantification assay was carried with the embryogenic line EC 1 and the non-embryogenic line NEC1 induced from a zygotic embryo and maintained in a 2,4-D supplemented medium. Embryogenic callus (EC2) and non-embryogenic callus (NEC2) samples originated from leaf explants in a picloram-rich induction medium,

were also used, as well as a third type of embryogenic callus (EC3), showing a lower potential for somatic embryo formation.

As described in the methodology section, the samples were diluted tenfold before the addiction of TCA since it was detected that in undiluted samples the proteins present were precipitated, forming macroscopic suspensions that influenced the absorbance values of the Ehrlich samples. Thus, several dilutions were tried, and the tenfold was selected because it enabled the absorbance value to fall within the values of the calibration curve without causing observable precipitation. The results were normalized taking into account the initial mass of tissue used, being presented in µg of IAA per mg of initial tissue (Fig. 10).

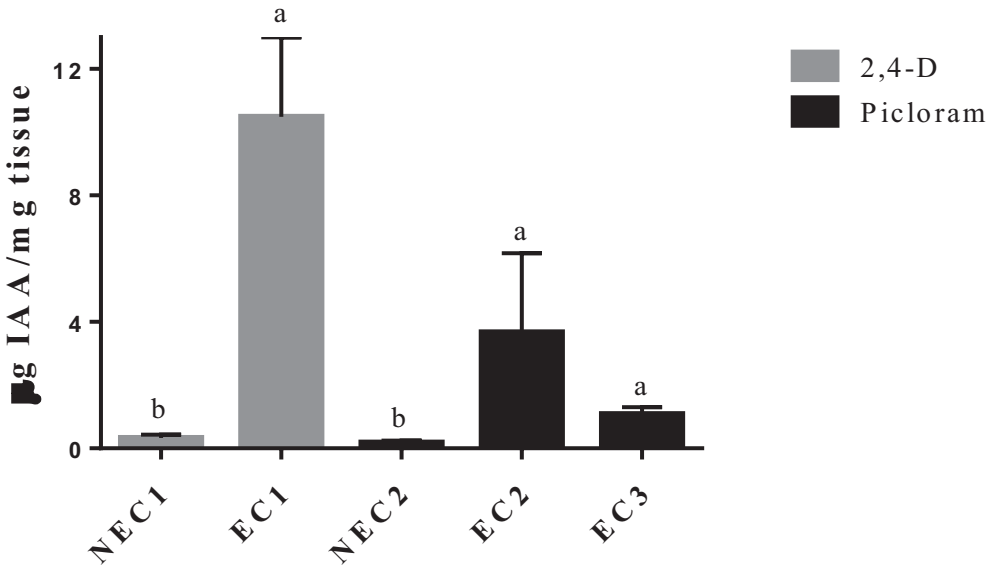

Figure 10. IAA quantification by Ehrlich reaction in different calluses samples. Results are presented as mean ± SD (n =3). Different letters in the same treatment - calluses induced with 2,4-D (grey bars) or calluses induced with picloram (black bars) - are statistically significant by Tukey test ($p<0.05$). Quantification was recorded after 4 weeks of culture.

Although some differences were observable between treatments, the statistical analyses were made on grouped samples. Because the callus samples used were induced and proliferated in the presence of different exogenous auxins (EC1 and NEC1 induced in 2,4-D; EC2, EC3 and NEC2 induced in picloram), the statistical comparisons were made taking into account this difference, as shown in figure 10.

The difference between the endogenous auxin levels in NEC1 and EC1 is statistically significant, with EC1 presenting the highest value of IAA registered (10.49 ± 2.51 μgIAA/mg fresh tissue). In the case of picloram induced calluses, the difference between embryogenic (EC2 and EC3) and non embryogenic callus (NEC2) is statistical relevant, with EC2 showing higher IAA values than EC3, although not statistically different.

This comparison indicated a significant statistical difference between the embryogenic tissues induced with different synthetic auxins, with picloram apparently leading to a marked increase in the endogenous level of IAA. On the other hand, no differences were found between non-embryogenic tissues.

3.1.3. Auxin degradation assay

IAA degradation analysis was carried out in the callus samples, through the quantification of the amount of auxin degraded over a known and fixed period of time (1 hour). The results are presented as the rate of destruction of IAA per min. by measuring the total amount of protein present in the extract. The total protein (Table III) was quantified using Bio-Rad Protein Assay kit as described in the methodology section, by taking a 40 μL sample of protein suspension after the centrifugation.

Table III. Total protein content in the different calluses**.** Results are presented as mean ± SD (n=3).

Tissue	Protein concentration (mg/ml) ± SD
NEC1	0.112 ± 0.029
NEC2	0.290 ± 0.067
EC1	0.693 ± 0.075
EC2	0.625 ± 0.071
EC3	0.388 ± 0.290

After the quantification of the protein, the reaction was initiated and incubated for 1 hour at room temperature. After this period, the residual IAA was measured using Ehrlich reaction and the amount of IAA consumed per minute calculated. The results (Fig. 11) were normalized taking into account the protein quantities presented in Table III.

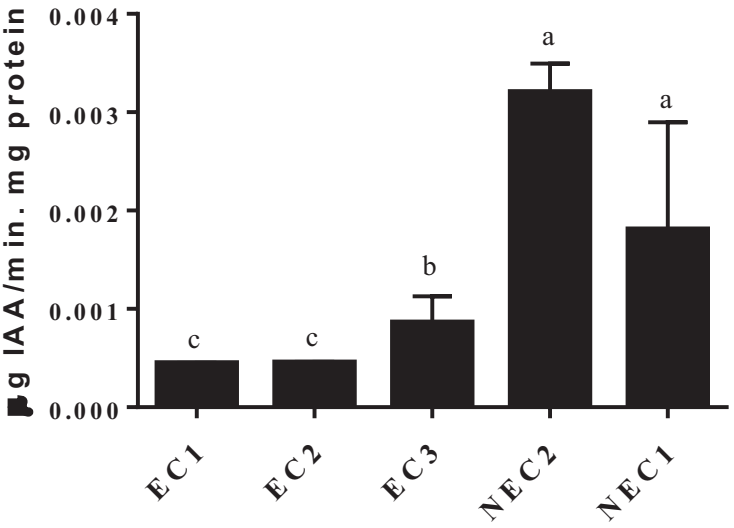

Figure 11. Auxin degradation. Results are present as mean ± SD (n=3). . Results are presented as mean ± SD (n=3). Different letters are statistically

significant by Tukey test ($p<0.05$). Quantification was recorded after 4 weeks of culture.

The results clearly show that high auxin degradation was particularly notorious in non-embryogenic callus especially in NEC1. Both embryogenic calluses were characterized by reduced levels of IAA degradation, whereas the low-yield embryogenic tissue (EC3) showed intermediate values between embryogenic and non-embryogenic calluses. Embryogenic calluses showed no relevant statistical differences between each other.

3.2. HPLC analysis

3.2.1. Method validation

Auxin content in embryogenic and non-embryogenic tissues was further investigated through HPLC. Preliminary assays showed that an isocratic mobile system composed of methanol : water pH=2.3 (orthophosphoric acid) (58:42 v:v) was enough to resolve both IAA and IPA in control samples prepared mobile phase and (Fig. 12), later on, in samples by measuring absorbance at 280 nm.

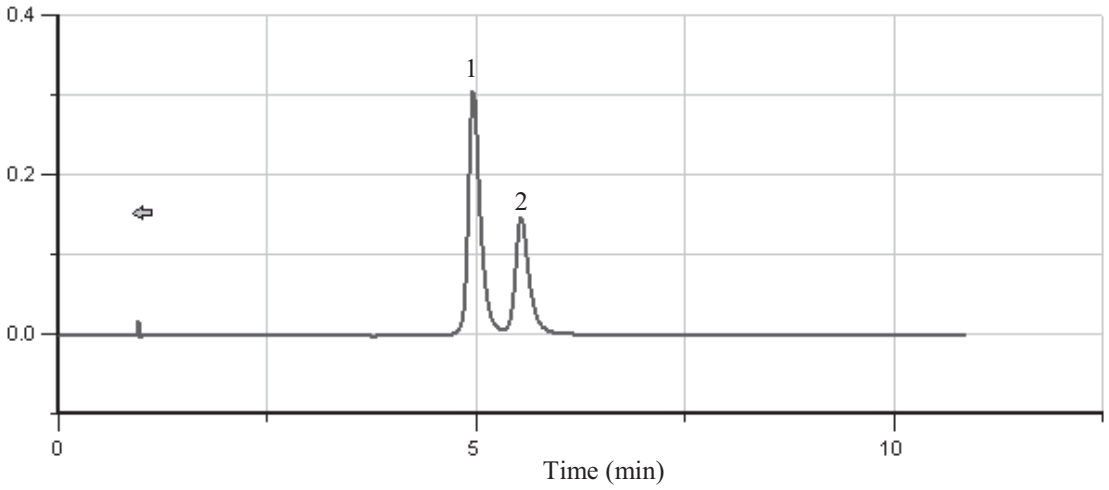

Figure 12. Chromatogram of the mixture of IAA and IPA. 1-IAA peak; 2-IPA peak, Abs – Absorbance measured at 280 nm.

To determine the linearity limits of the technique before sample quantification different concentrations (100 µg/ml to 0.1 µg/ml) of both compounds dissolved in the mobile phase were injected in triplicate. The average peak (n=3) was plotted against concentration, and calibration curves were obtained. From these, the limit of detection (LOD) and limit of quantification (LOQ) for each compound was determined. The recovery percentage of both compounds, as well as the average retention times of the standards were also recorded. The parameters of these tests are presented in table IV and were used to quantify IAA in the samples.

Table IV. Validation parameters of the HPLC quantification technique**.** The retention times are presented as the mean ± SD (n=3).

Parameter	IAA	IPA
Equation	$y = 3 \times 10^7$	$y = 2 \times 10^7$

Correlation coefficient (R^2)	0.9984	0.9997
LOD (µg/ml)	0.25	0.25
LOQ (µg/ml)	25.0	25.0
Recovery (%) (n=3)	88.31 ± 0.82	92.18 ± 1.99
Retention time (min.)	5.34 ± 0.25	6.01 ± 0.21

IAA in the samples was also identified by the characteristic UV absorption spectrum of IAA, identified in the peaks of standard solutions (Fig. 13) and samples.

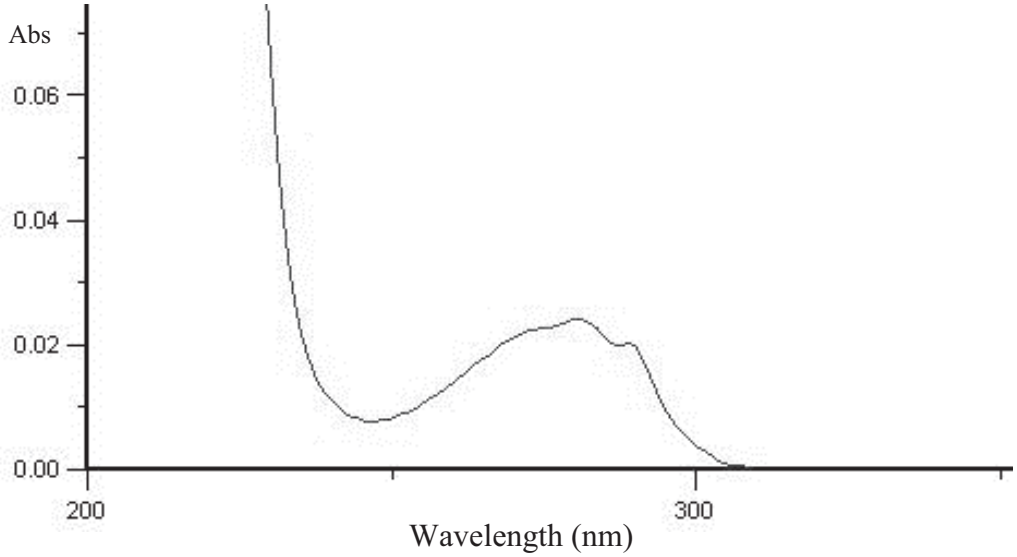

Figure 13. UV-absorption spectrum of IAA.

The spectrum reveals a shoulder at 273 nm, 282 nm and 290, characteristic of the indole group. The spectra of the peaks of IAA were analysed in combination with the retention times (Table III) during quantification to confirm that the peak was in fact IAA.

3.2.2Quantification of IAA by HPLC

Similarly to the Ehrlich reaction test, the first plant materials to be assayed were the embryogenic (EC1, EC2 and EC3) and non-embryogenic calluses. The samples were treated as described in the methodology section and after the chromatography the resulting chromatograms were analysed for the presence of IAA (a known concentration of IPA was added to each sample as an internal standard) and quantified using the validation values presented in table III. A chromatogram representative of these samples is presented in figure 14.

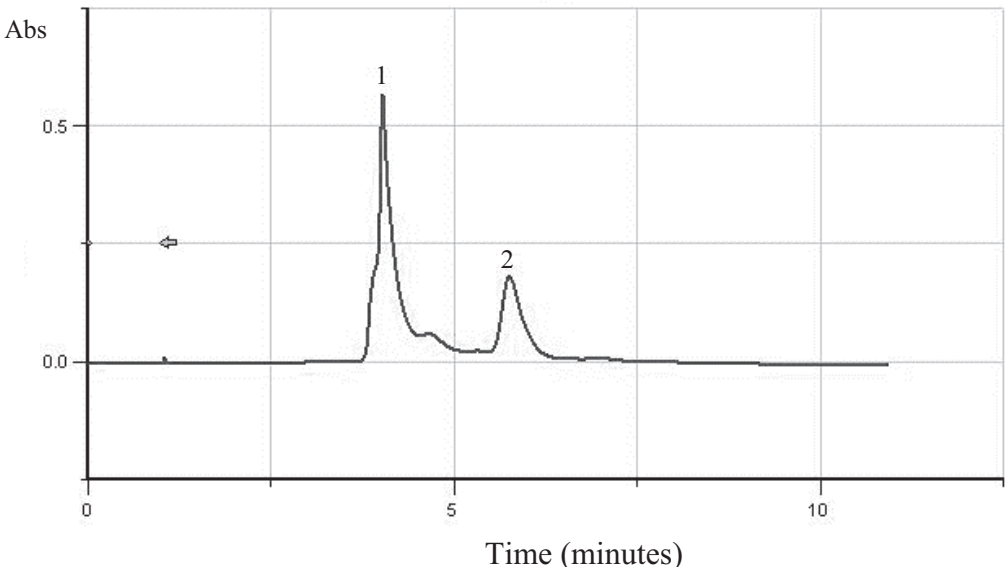

Figure 14. Chromatogram a callus sample (EC1). 1- IAA; 2- IPA , Abs – absorbance at 280 nm.

All samples were made in triplicate and the overall results are presented in figure 15.

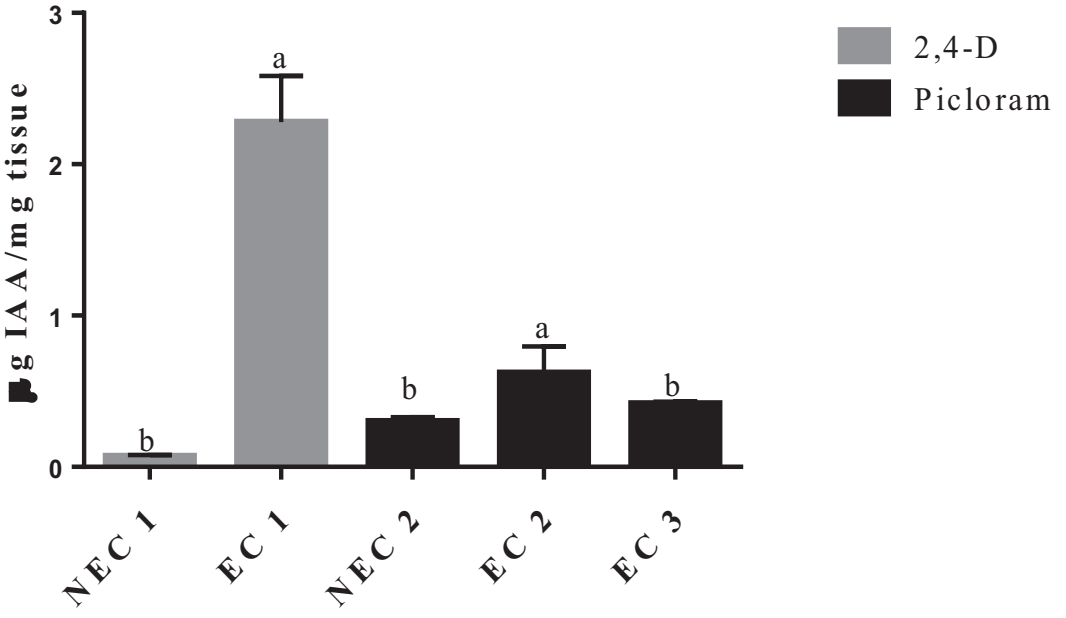

Figure 15. IAA quantification by HPLC in different callus samples. Results are presented as mean ± SD (n=3). Different letters in the same treatment - calluses induced in 2,4-D (grey bars) or calluses induced in picloram (black bars) - are statistically significant by Tukey test ($p<0.05$). Quantification was recorded after 4 weeks of culture.

Like in the Ehlrich assay the statistical analysis of the results was carried out considering the auxin used in the induction phase and the results were treated separately.

The data shows a significant difference between the IAA levels in NEC1 and EC1, both induced with 2,4-D from zygotic embryos and also between the calluses that were induced in the leaf segments in presence of picloram (EC2, EC3 and NEC2). The difference between the EC2 and EC3 is also statistically significant. Furthermore, a separate comparison between the embryogenic calluses (EC1, EC2 and EC3) also revealed statistically significant differences while in non-embryogenic tissues (NEC1 and NEC2) the amounts of auxin were not significantly different.

A comparison between the two techniques (HPLC and Ehrlich reaction) was made, by plotting the output given by the Ehrlich method against the value given by the HPLC analysis for each of the callus tissue analysed (NEC1, NEC2, EC1, EC2 and EC3). The results show the quantitative relation between the two quantification methods (Fig. 16).

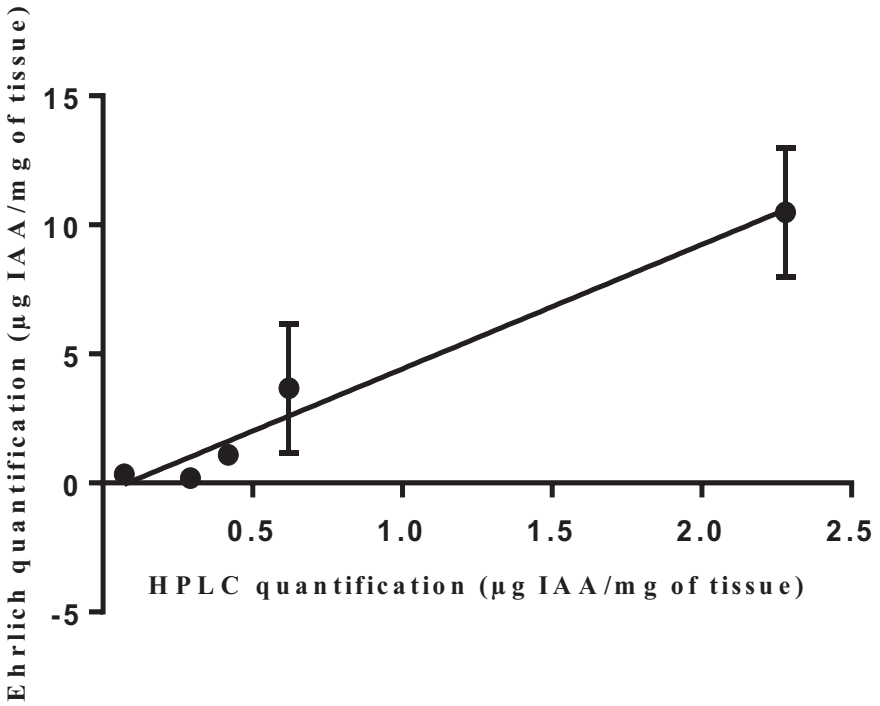

Figure 16. Comparison between the Ehrlich and HPLC methods. The linear regression curve has the equation y= 4.817x – 0.3959 and a coefficient of determination (R^2) of 0.8719.

The differences in embryogenic calluses induced in 2,4-D and picloram are also significant, with the one induced in 2,4-D (EC1) presenting the highest concentration of IAA, while the calluses induced in picloram presented intermediate values, statistically relevant when measured by HPLC but irrelevant when measured by the Ehrlich colorimetric method. The differences between the non-embryogenic calluses that

occurred in different induction media were also not statistically significant in any of the assays performed.

In a further experiment a time course evaluation of the endogenous levels of IAA during the induction phase was carried out. At time 0, the initial explants (young leaves) were used to quantify the initial levels of IAA present in tissues through HPLC (Fig. 17).

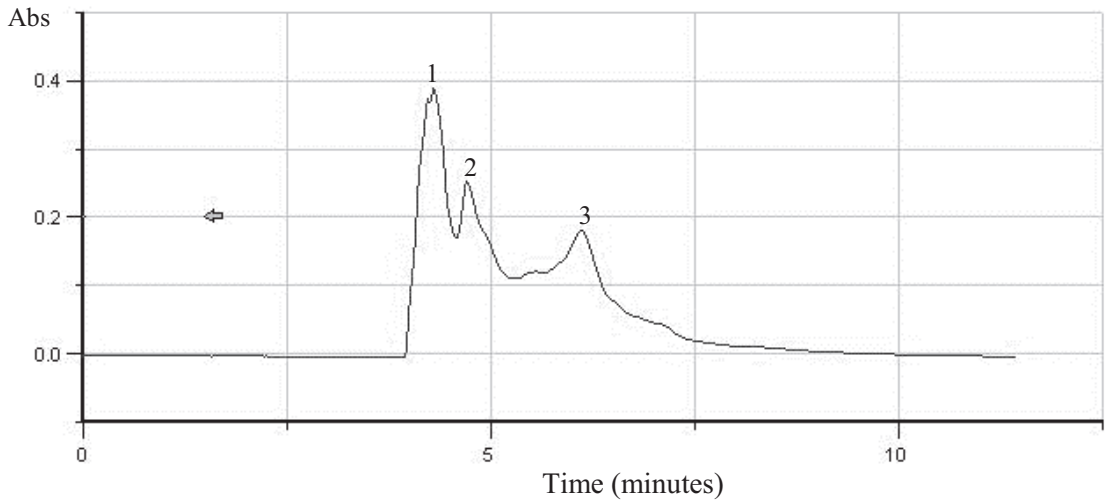

Figure 17. Chromatogram of tamarillo leafs. Peaks 1, 2 and 3 have retention times of 4.12, 4.83 and 6.29, respectively.

The chromatogram displayed in figure 18 shows that peaks 1 and 2 represent the average retention time of IAA while peak 3 is within the range of the retention time expected to IPA. However, spectral analysis indicated the presence of compounds unrelated either to IAA or IPA (Fig. 18). Therefore, the quantification of IAA and IPA in the initial explant was unsuccessful using this approach.

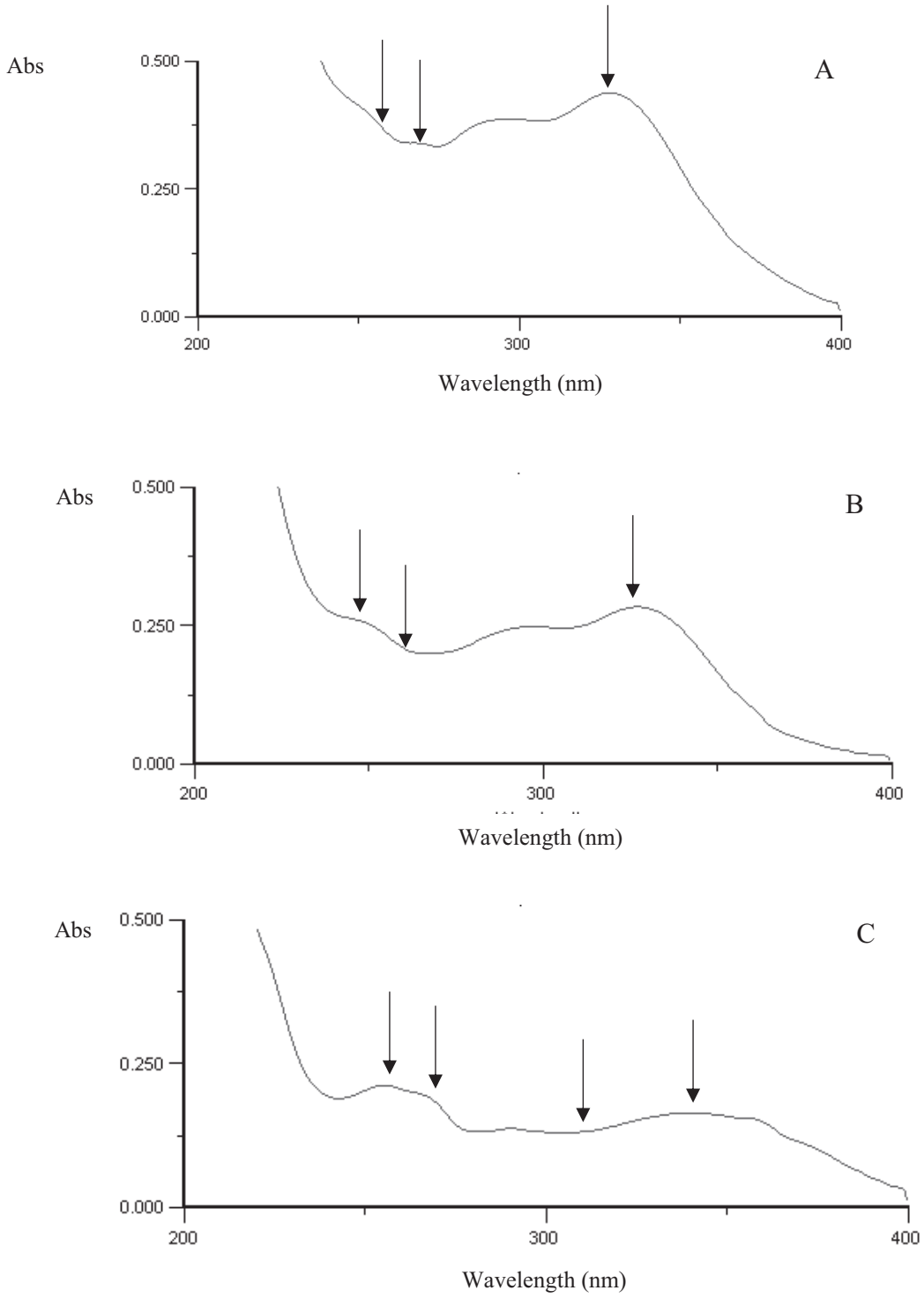

Figure 18. UV-absorption spectrum the compounds found in tamarillo leafs. **(**A) peak 1 on the chromatogram (263.7 – 275. 1 nm shoulder, 388.1 nm point of inflection, 328 maximum absorption band); (B) peak 2 on the chromatogram (252.2 – 265.8 nm shoulder, 308.1 nm point of inflection; 326.7 local maximum); (C) peak 3 on the chromatogram (254.7-265.5 shoulder, 279.1 nm point of inflection and 324.4 nm absorption band).

The spectral analyses of the peaks, revealed that the compounds are likely derivates of the phenol caffeic acid with a peak 1 being putatively identified as ferulic acid or a close compound, probably conjugated with a monosaccharide. Peaks 3 was identified as a quercetin glycoside whereas peak 2 is probably a quercetin derivative or a conjugate of this compound. The analysis presented is based on the qualitative analysis of the points of reference of the UV-spectrums presented and, therefore, is insufficient to securely identify the compounds.

IAA content was further detected in embryonic induced leaf segments at different stages of induction (Fig. 19) that ultimately originated EC2 (embryogenic callus from leafs induced in a picloram rich medium). Thus, after a period of 8 weeks, when callus tissue was already developed, samples were removed every 2 weeks and the IAA was measured by HPLC.

Figure 19. Different stages of the induction phase. (A) Leaf segment after 4 weeks on induction medium; (B) leaf segment after 8 weeks; (C) embryogenic callus maintained in a picloram containing medium (EC2).

Before IAA detection the samples were individually observed to determine the percentage of explants, forming embryogenic callus. These results are summarized in table IV.

Table V. Results of the somatic embryogenesis induction protocol on tamarillo leaf segments.

Parameter	Number
Total explants	25
Non responsive	2
Non-embryogenic tissue	12
Contaminations	1
Embryogenic tissue	11
Embryogenic yield (%)	44

The quantification of IAA was possible at 8 weeks and onwards, as the nelwly formed callus tissue did not present the compounds previously found on leafs (Fig. 20).

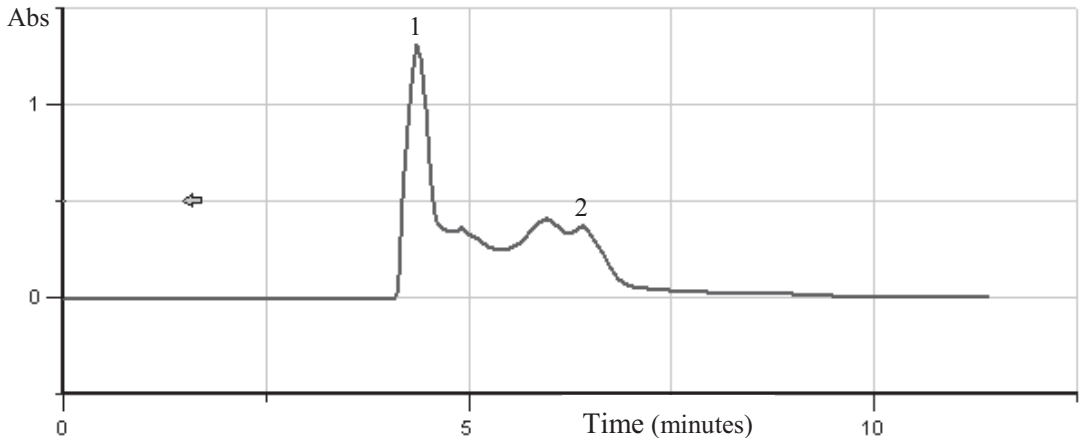

Figure 20. Chromatogram of induction phase leaf segments with 8 weeks. 1- IAA peak; 2 – IPA peak; Abs – absorbance at 280 nm.

Beyond IAA and IPA the samples showed some other compounds with approximately the same retention times. However the quantification was possible since spectrum analysis of peak 1 showed to be similar to the IAA UV-absorption spectrum. Thus, a profile of endogenous auxin was created, with 8, 10 and 12 week-old samples (Fig. 21).

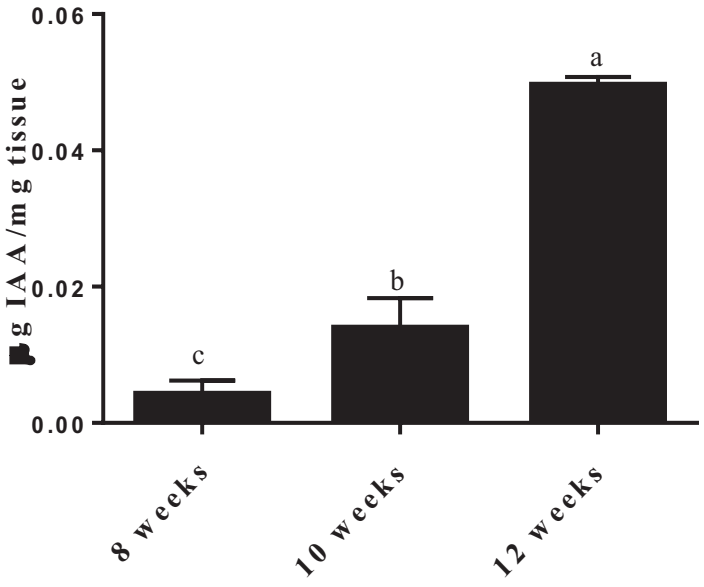

Figure 21. Comparison between the endogenous IAA levels in the induction time course periods. Different letters represent a significant statistical difference by Tukey test ($p<0.05$).

The results showed marked differences between the three time courses tested, with a constant increase in the endogenous levels of IAA. Thus, the data suggests that the concentration of endogenous auxin during the induction phase of SE increases steadily. However, by the 12th week of culture, the average amount of auxin (0.05 µg/mg tissue) is still about 12 times less than the corresponding embryogenic callus EC2 (0.62 µg/mg tissue).

3.3. Extracellular protein analysis of suspension cultures

3.3.1. Protein secretion and concentration

The calluses used for this study were previously induced and subcultured in solid culture medium and were the same lines previously characterized for endogenous auxin concentration (EC1, EC2, EC3 and NEC2).

During growth of the calluses it was observed that embryogenic lines grew as macroscopic aggregates of cells whereas non-embryogenic suspensions where characterized by the presence of long vacuolated isolated cells. (Fig. 22).

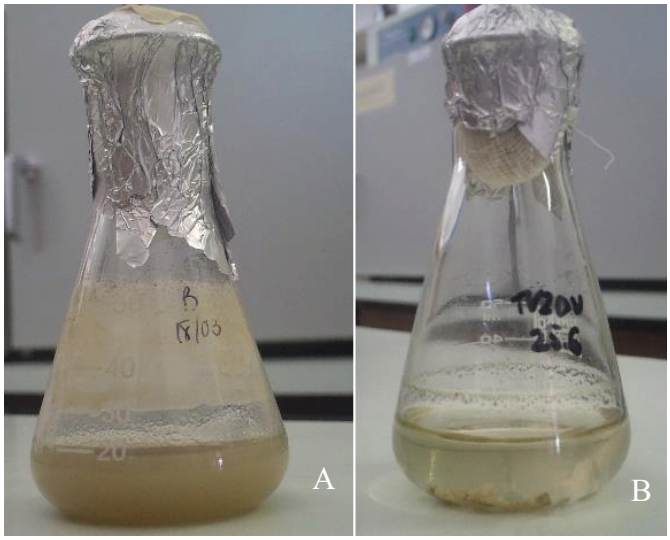

Figure 22. Callus tissue on liquid medium. (A) Non-embryogenic callus (NEC2); (B) embryogenic callus (EC2).

During growth, the absorbance of cell-free culture medium was weekly measured against a water blank to follow the protein secretion patterns of each of the samples. Additionally, the mass at the end of the growth period was also measured and plotted against the initial mass (Fig. 23).

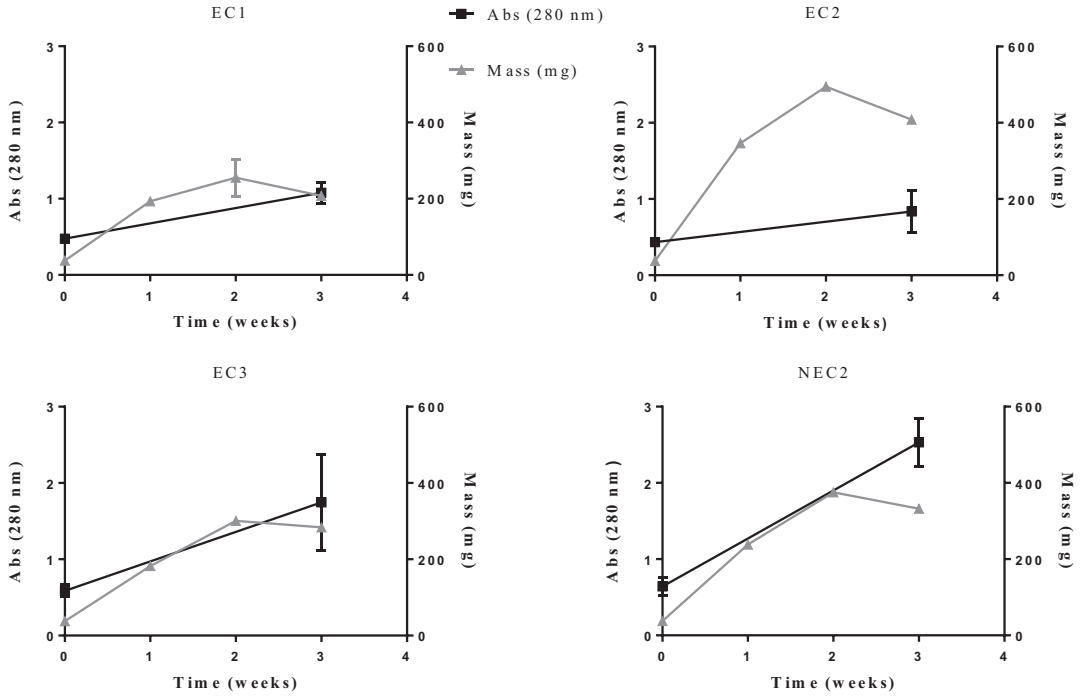

Figure 23. Liquid medium growth parameters**.** Absorbance and mass (mg) of embryogenic and non-embryogenic cultures during the first three weeks of culture on a medium containing 2,4-D (EC1) and picloram (EC2, EC3, NEC2). Results are presented as mean ± SD (n=3)**.**

As observed in figure 23, the average mass increment (fresh weight) of the non-embryogenic callus (NEC2) was the highest (302.19 ± 83.78 mg), while the embryogenic calluses EC1 and EC2 presented the lowest increments (120.50 ± 18.49 mg and 70.97 ± 50.70 mg, respectively). The EC3 presented an intermediate growth between the two extremes (235.1 ± 115.33 mg).

In terms of absorbance (A_{280}), all the samples presented a similar profile, with a steep increase in the first two weeks leading to a maximum followed by a small decrease in the 3rd week. EC2 and NEC2 presented the higher values after 2 weeks of culture (1.876 and 1.142, respectively). The initial value was taken from liquid medium

without the synthetic auxin since it was observed that the auxin greatly increased the absorption of the blank.

After the growth period, the protein in the extracellular medium was precipitated using ammonium sulfate and subsequently dialyzed to remove excess of salt. After dialysis the extracellular protein profile was evaluated by SDS-PAGE in denaturing conditions (Fig. 24).

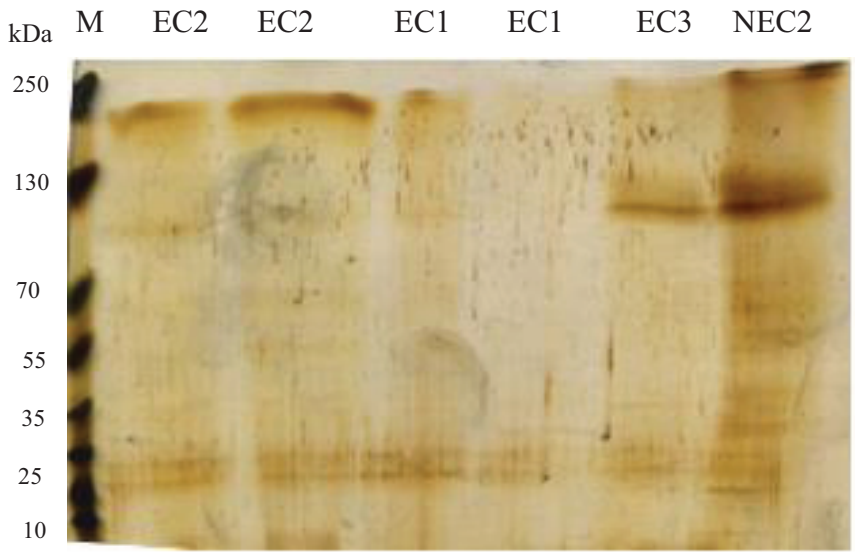

Figure 24. SDS-PAGE of the extracellular medium of embryogenic and non-embryogenic cultures. The gel was stained first with Coomassie blue and then with silver nitrate.

The protein profiles were similar in all samples. However, protein amounts seem to be higher in the non-embryogenic callus medium as some bands were visible when staining the gel with Coomassie blue. None of the bands on embryogenic callus medium were visible with Coomassie blue staining (about fifty times less sensitive than the silver nitrate protocol), so the protein appears to be more concentrated in the non-embryogenic callus (NEC2).

The molecular weight of the proteins in the gel was estimated by plotting the log of the molecular weight of the markers against the distance of the migration. The high molecular band found in NEC2 and EC3 and only faintly visible in the embryogenic callus is approximately 127 kDa. The lowest bands encountered in all tissues ranged from about 15 to 19 kDa.

Moreover, the intracellular protein content of the callus tissue was also evaluated. The samples were grounded and centrifuged to sediment the insoluble cellular constituents. After the centrifugation, it was observed that in the EC1 and EC2 samples there was an apparent viscous liquid phase distinguishable from the bulk of the cleared solution. Therefore, this "top" solution was also evaluated in the SDS-PAGE. The NEC2 and EC3 did not present this separation of phases. In all the samples, the insoluble fraction was also analysed by suspension of the solid particles in buffer, centrifuging the suspension again and analysing the resulting supernatant (Fig. 25).

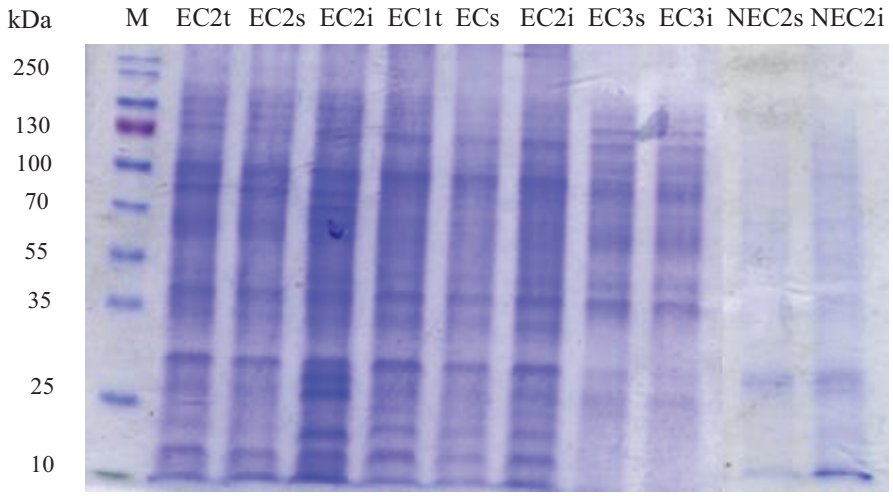

Figure 25. SDS-PAGE of the intracellular proteins. The letters after the name of the samples identify the phase in that lane: "t" represents the top liquid phase previously described in EC1 and EC2; "s" refers to the soluble aqueous phase; "i" designates the insoluble part.

The two liquid phases found in EC1 and EC2 do not vary significantly from each other. The insoluble phase also showed qualitatively similar profiles, although the bands appear to be more intense, indicating a greater concentration of protein in the insoluble phase. EC3 presented a slightly different profile than the previous tissue, with both soluble and insoluble fractions equally concentrated and bands of apparent molecular mass greater than 35 kDa with a similar distribution. However, when molecular masses decreased the intensity of the bands was weaker and the profile changed significantly.

The non-embryogenic callus (NEC2) showed a relatively lower protein concentration and an insoluble fraction with more stained bands. The more visible bands are of low molecular weight (approximately 10 and 30 kDa). These results confirm the lower concentration of intracellular protein previously assayed by the Bradford method (see table III).

After the qualitative evaluation of the extracellular and intracellular protein profiles, the proteolytic activity of the extracellular proteins was further studied.

3.3.2. Enzymes activity assays

The first activity assay made for the detection of proteolytic activity was made by zymography (Fig. 26A), in a polyacrylamide gel copolymerized with gelatine, a general substrate for proteolytic enzymes. An additional SDS-PAGE was made in the same conditions as the zymography (same loading buffer) without any boiling steps (Fig. 26B). The gel was stained using the silver nitrate technique.

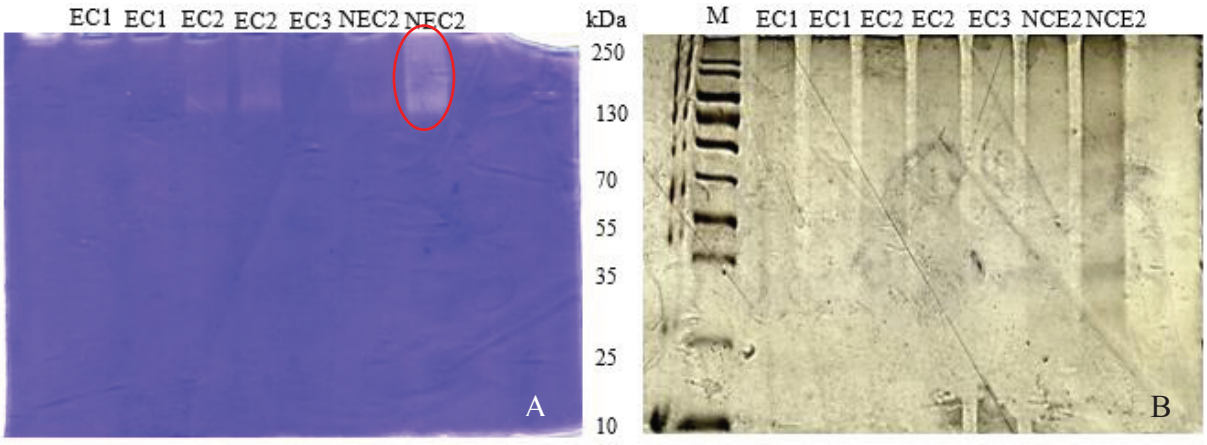

Figure 26. Analysis of proteolytic activity by electrophoretic techniques. (A) Zymogram; (B) SDS-PAGE in non-denaturing run in parallel with the zymography.

The proteolytic activity (marked area, Fig. 26A) was observed on a molecular weight zone of 130 to 250 kDa and it was more pronounced in the NEC2 and EC2 media, although it was also present on media where the other cultures were grown (Fig. 26B). Analysis of the SDS-PAGE gel revealed that some protein did not enter the gel properly. The apparent molecular masses here relate to the electrophoretic mobility of the native protein structures and, therefore, are distinct from the apparent molecular weights of unfolded structures.

After this study, the activity was measured by fluorescence, using the substrates described in the methods section. In order to obtain specific activities, the total protein of the extracellular extracts was measured by the Bio-Rad Protein Assay based on Bradford's method.

Table VI. Total extracellular protein. Results are presented as mean ± SD (n=3).

Tissue	Protein concentration (µg/ml) ± SD
NEC2	11.135 ± 2.364
EC1	5.767 ± 1.136
EC2	8.401 ± 0.697
EC3	6.653 ± 0.387

In the fluorescence assays two substrates were used, Arg-AMC and Phe-AMC, in the conditions previously described. With the Arg-AMC, activity on the dialyzed extract was not detected. The Phe-AMC substrate presented activity on all extracts (Fig. 27).

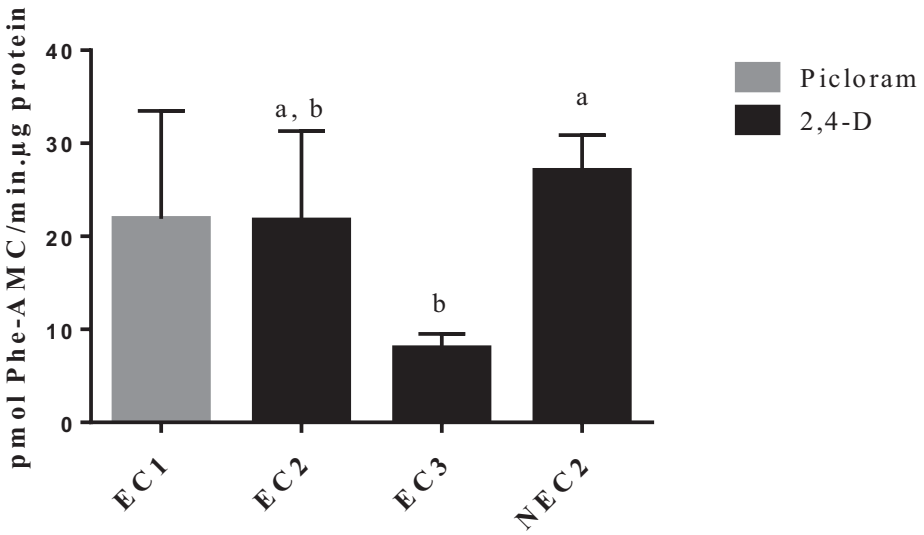

Figure 27. Specific Phe-AMC activity of the extracts. Results are presented as mean ± SD (n =3); EC 1 – medium from tissue induced in 2,4-D (grey bar); NEC 2, EC2 and EC3 – media from tissues induced in picloram (black bars). Different letters in the same treatment are statistically significant using

the Tukey Test ($p<0.05$). The EC1 medium was not analysed with the other media, as it was induced in a different auxin.

The results indicate values of activity very similar between all the samples, except for EC3 which displayed values considerably lower. However, as the culture medium of EC1 had a different type of synthetic auxin which was different from the other samples it was not evaluated in the statistical analysis. The data showed no differences between the embryogenic and non-embryogenic media (EC2 and NEC2), with the EC3 showing differences relatively to the NEC2 medium but not to the EC2. The outcome of this statistical test is likely due the smaller variance of the NEC2, as the mean values of EC2 and NEC2 are similar.

The affinity to glycoside hydrolases was also tested with fluorescent substrates, MU-G (Fig. 28) and MU-NAG (Fig. 29).

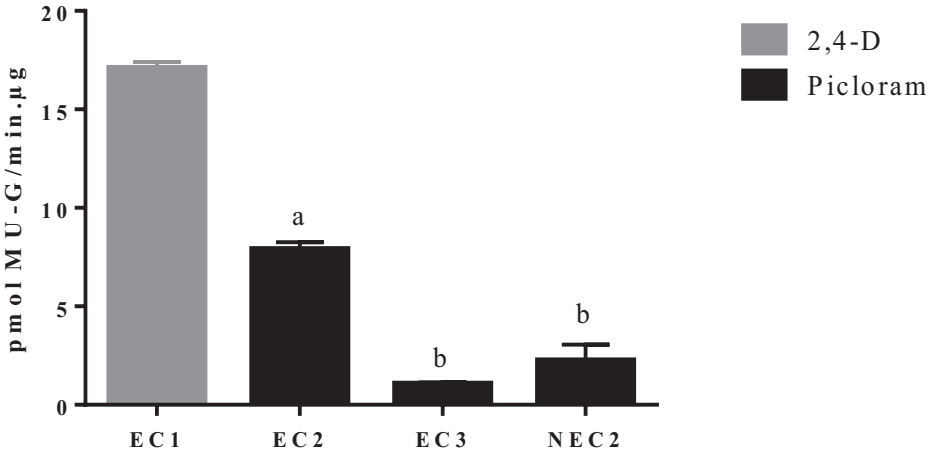

Figure 28. Glycoside hydrolases (MU-G) activity. Results are presented as mean ± SD (n =3); EC 1 – medium from tissue induced in 2,4-D (grey bar); NEC 2, EC2 and EC3 – media from tissues induced in picloram (black bars). Different letters for the same type of calluses indicate statistically significant differences using the Tukey Test ($p<0.05$). The EC1 medium was not analysed with the other media, as it was induced in a different auxin.

The data showed that the specific activity was significantly higher in EC2, with both EC3 and NEC2 presenting statistically insignificant differences.

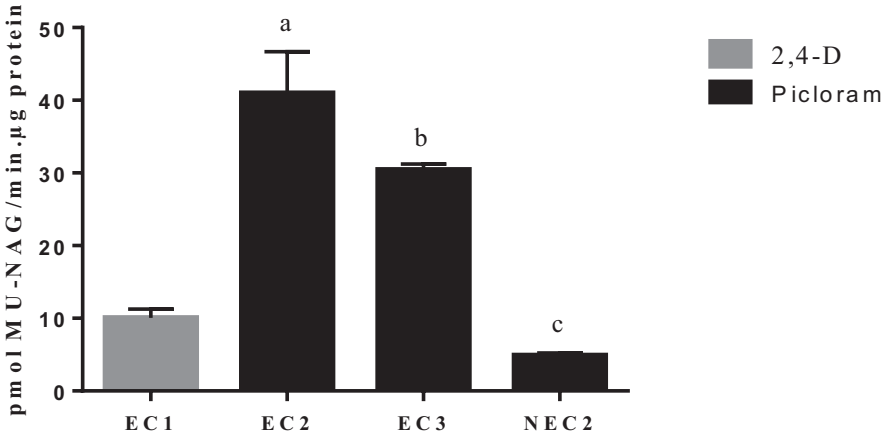

Figure 29. Glycoside hydrolases (MU-NAG) activity. Results are presented as mean ± SD (n =3); EC 1 – medium from tissue induced in 2,4-D (grey bar); NEC 2, EC2 and EC3 – media from tissues induced in picloram (black bars). Different letters in the same treatment are statistically significant by Tukey Test ($p<0.05$) The EC1 medium as not analysed with the other media, as it was induced in a different auxin.

In the case of MU-NAG specific hydrolases, the variations observed were statistically significant when calluses of each group were compared, with the highest specific activity observed on the EC2 and lowest on the NEC2. Again, EC3 gave intermediate values which in this case, were significantly different from the other *calluses* used.

3.3.3. Proteolytic enzymes inhibitors

The proteolytic enzyme inhibitor studies aimed either to confirm the activity observed in the protease assays or to identify the principal classes of these enzymes present in the medium.

The inhibition assays were carried out in EC2 (Fig. 30) and NEC2 (Fig. 31) samples. The substrate used was Phe-AMC and the conditions were the same as used in the activity assays previously described.

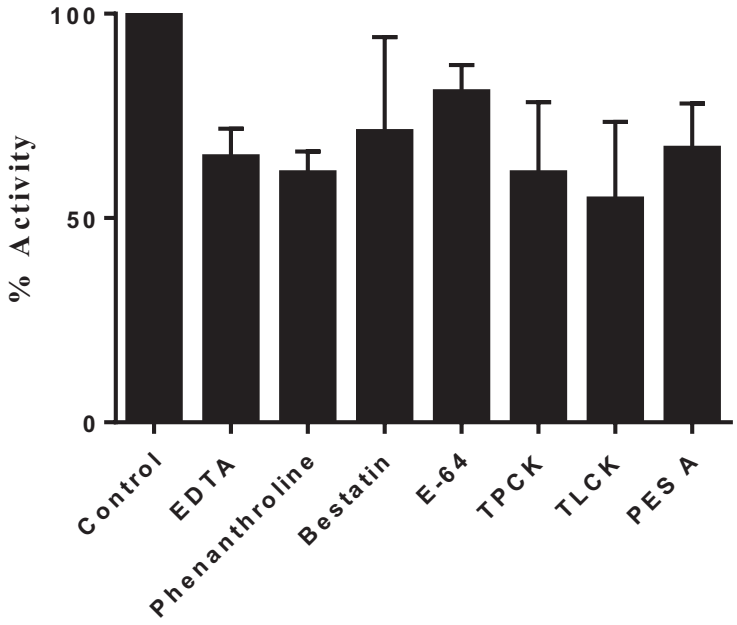

Figure 30. Protease inhibitor assay for EC2 samples. Results are presented as mean ± SD (n=3) relatively to the control (100%).

EC2 medium showed some inhibition in all the samples tested. In spite of this it was hard to define the class of protease present due to high standard deviations observed in some samples and to the high percentage of activity encountered for most of the conditions tested.

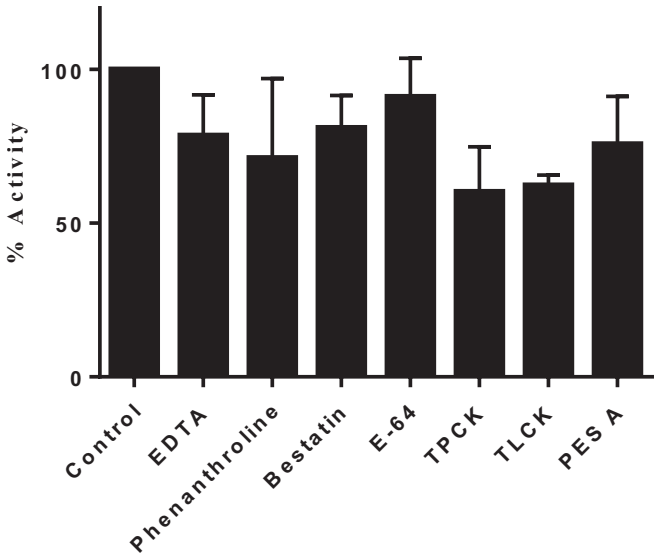

Figure 31. Protease inhibitor assay for NEC2 samples. Results are presented as mean ± SD (n=3) relatively to the control (100%).

The activity profile for NEC2 medium was slightly different, with the inhibitors of metalloproteases showing a higher level of activity and a greater standard deviation, while bestatin and E-64 remain with high values. TPCK, TLCK and Pepstatin A present the lowest values in this assay.

4. Discussion

4.1. Auxin analysis

4.1.1. Auxin polar transport inhibitor assay

In vitro somatic embryogenesis induction protocols can be divided in two stages, one in which somatic cells enter in a dedifferentiated cell state and acquire embryogenic potential and another in which these cells involve into somatic embryos (Yang *et al.,* 2012). These two stages are usually applied *in vitro* by changing the external stimuli, usually stress or PGRs (Fehér *et al.,* 2015). Tamarillo is one of these cases, where the SE process is induced in an auxin-rich medium, and the proembryogenic masses formed during this stage transform into somatic embryos upon transference to an auxin-free medium (Correia and Canhoto, 2012).

The inclusion of the polar auxin transport inhibitor TIBA at lower concentrations in the maturation medium of tamarillo embryogenic callus did not affect the number of somatic embryos formed by mass of tissue. However, this factor, as well as the mass increment was lowered by concentrations of TIBA higher than 5 μM. Abnormal somatic embryos were also recorded, although without a clear relation to the level of TIBA.

Early studies have shown, in both zygotic and somatic embryos, that the endogenous auxin content is important to the developmental program of embryos as well as their germination (Liu *et al.,* 1993). Therefore, the auxin polar transport inhibitor assays made in this work aimed to test whether somatic embryo development of tamarillo was also affected by the mechanism of polar auxin transport, when the proembryogenic masses were transferred to a medium without auxins. In this stage of embryo development the endogenous auxin is greatly responsible for the organized division and specification of cells, or embryo patterning (Möller and Weijers, 2009).

Previous studies have shown that TIBA can inhibit somatic embryogenesis even in the presence of strong auxins such as 2,4-D (Venkatesh *et al.,* 2009). Several other studies have also demonstrated that TIBA affects the maturation of somatic embryos, particularly in the earlier stages of globular and heart-shaped embryos (Liu *et al*., 1993).

The TIBA mechanism of action is based on blocking auxin transport by binding to PIN regulator efflux carriers (Depta *et al*., 1983) without directly antagonizing the response cascade triggered by the auxin, with the auxin polar transport being fundamental for the effective response of the tissue. In fact many studies seem to support the idea that the polarity of cells is achieved by cell-to-cell communication, greatly influenced by auxins (Jenik *et al*. 2007; Möller and Weijers, 2009).

The results here presented support this hypothesis, that the cell to cell communication mediated by auxins is fundamental for the development of somatic embryos, as the high concentrations of TIBA affected the number of somatic embryos formed.

Interestingly, the cellular proliferation observed was only marginally affected by the presence of TIBA as it was only significantly lower on the highest concentration of TIBA used. Indeed, the lower concentrations of TIBA presented a slightly higher proliferation, although statistically insignificant, compared to the control. These results show that the cell proliferation phenomena is not as dependent of the auxin gradients as the maturation of somatic embryos. Similar results have been described in cultures of *Carthamus tinctorius*, where certain concentrations of TIBA (between 1 and 6 μM) effectively increased the callogenic response (Ali and Afrasiab, 2014).

4.1.2. Ehrlich reaction

The Ehrlich reagent has been used to measure several indole containing molecules, from tryptamines to ergoloid compounds (Ehmann, 1977), and has been optimized for colorimetric quantifications of IAA (Anthony and Street, 1969) and, in specific conditions, IAA and IBA (Guo *et al.,* 2010). Because of this, it was used as presumptive test in the quantification of IAA in the callus tissues of tamarillo.

Anthony and Street (1969), who first described the application of this analytical reaction to the quantification of auxins with an indolic moiety, have optimized the method for IAA analysis by experimentally testing the optimum concentration of HCl to dissolve the Ehrlich reagent, and the concentration of TCA required to achieve maximum colour intensity while retaining linearity between concentrations. The method showed a linearity range similar to the described when tested with buffered stock solutions of IAA. The dilution described in the methodology was required to avoid the precipitation of proteins present in the tissue when TCA was added. The tests showed that a tenfold dilution allowed for the protein precipitation effect to be neglected while the absorbance value measured was still within the linearity range.

The quantification of IAA in the callus samples showed large standard deviations, pointing to a relatively low specificity of the test, at least when compared to the HPLC analysis. Furthermore, the absolute values of IAA concentrations, as measured by this method, were also systematically higher than the ones quantified by the HPLC analysis.

These results could be explained by secondary reactions of the Ehrlich reagent with other indolic compounds present in the samples, such as precursors of IAA synthesis, resulting in an overestimation of the endogenous IAA values. Indeed, the

comparison between the two methods showed a linear relation, with the value measured by the Ehrlich method higher than the HPLC. By avoiding the secondary reactions the HPLC technique and resolving the measured compounds increases their purity, therefore increasing the specificity of the analysis.

The linear relation shows that the overestimation made by the Ehrlich method is constant in all the samples and although the limit of detection is about ten times higher than the HPLC method (0.25 μg/ml for the HPLC protocol and 2 μg/ml for the Ehrlich method), the LOQ is also significantly higher (25 μg/ml in the HPLC method and 50 μg/ml in the Ehrlich method), with the overall linear range greater than the HPLC method.

Despite the overestimation presented by the method, and its low precision, the differences found in auxin endogenous levels were later confirmed by HPLC with the statistical analysis of these results yielding virtually the same results, with the only different concentrations for the EC2 and EC3 samples, both of them induced in a picloram-rich medium.

Detection of auxins by chromatographic methods is considered the most precise and exact method currently available (Kim *et al.,* 2006), and because the identification of the compound analyzed by analysis of the UV-absorbance spectrum is possible, the HPLC results are considered reference in this thesis. However, the Ehrlich reaction is considerably easier to accomplish, and it yields results that are concordant to the chromatographic method in terms of relative concentrations. Therefore, this technique is a good presumptive test for quantification of endogenous IAA concentration in embryogenic tissues, before investigation with more precise but technically difficult and expensive analyses.

The application of this technique to the tissues also permitted the development of a simple colorimetric procedure to determine the degradation of IAA in the tissue.

4.1.3. Degradation of IAA

Auxins are enzymatically degraded by either oxidation of the side chains by peroxidases or the oxidation of the indolic ring by indole-3-acetaldehyde oxidase (EC 1.2.3.7) or IAA oxidase (Simon and Petrášek, 2011). This degradation is physiologically important because it leads to the permanent inactivation of the IAA (Paciorek and Friml, 2006) present in the tissue, thus it explains, to a certain degree, the low endogenous levels of auxin on some tissues as well as their unresponsiveness to the external medium rich in a synthetic auxin.

Colorimetric reactions can be applied to protein solutions, in specified conditions, to measure auxin degradation and, therefore, indirectly determine the catalytic activity of the enzymes involved in its oxidation, namely IAA oxidase and peroxidases. This type of discontinuous assay has been used to determine the activity of these enzymes (Straus and Gerding, 1963; Pujari and Chanda, 2002) in both purified and rude plant extracts. The authors generally use some type of modified Salkowski reagent to measure the residual IAA after stopping the reaction. In the present work, Ehrlich reaction was used instead to measure the residual IAA because it has been described as more exact than the Salkowski's method (Anthony and Street, 1969; Guo *et al*., 2010) and it was previously used in this work to measure IAA in callus tissue with the operating conditions defined and optimized. Additionally, the required addition of TCA 100 % (w/v) to the reaction mixture guarantees that any enzymatic reaction is immediately stopped.

The tissue rate of auxin degradation was indirectly evaluated by measuring the concentration of residual IAA after a period of incubation by using Ehrlich reaction. An interesting result was the overall lower SD presented in this assay when compared to the Ehrlich quantification of endogenous auxin. This difference could be explained by the concentrated solution of IAA added to the reaction mixture in the beginning (0.2 mM) of the incubation time, originating a solution significantly richer in IAA than the crude extract, witch lead to a greater specificity of the reaction to IAA, reducing the secondary reaction with other indoles. Moreover, as the assays were made in crude extracts, the IAA oxidase and peroxidase influence is not distinguishable, although the presence of an IAA oxidase cofactor ($MnCl_2$) and the absence of both a hydrogen donor and H_2O_2 in the solution likely reduces the activity of peroxidases while optimizes the catalysis by IAA oxidase. Regardless of this consideration, the assay showed a greater rate of auxin degradation in non-embryogenic callus. Therefore, the endogenous level of IAA is inversely related to the degradation rate, i.e., tissues with lower IAA levels presented the highest levels of IAA degradation (NEC1 and NEC2), while the tissues with high values of endogenous auxin presented the lowest degradation rate.

The results indicated that the degradation of IAA in the non-embryogenic callus is at least partially responsible for the low concentration of endogenous auxin in this tissue, caused by a higher biotransformation rate. In this context, the homeostasis of auxin in embryogenic and non-embryogenic calluses of tamarillo seems to be related to the degradation pathway of the complex auxin metabolism. This type of attenuation of the auxin signalling system has been described in *A. thaliana,* where the oxidation of auxin by enzymatic systems unable the activation of certain auxin-responsive genes (Peer *et al.,* 2013).

Analysis of the intracellular protein levels showed that the non-embryogenic callus presented lower concentrations of intracellular protein, a factor that could explain the higher specific activity of these tissues, as a greater fraction of protein could be related to the catalysis. These results express the overall degradation of IAA in the tissue, not being possible to distinguish between non-enzymatic and enzymatic activity.

4.2. HPLC analysis

4.2.1 Method validation

The sample preparation method used a liquid-liquid extraction method based on successive serial partitions against ethyl acetate (Kim *et al.,* 2006), using IPA as an internal standard. The recovering percentage of both IAA and IPA was 89.3 and 92.2%, respectively, in accordance to the value specified by the authors which was over 89% recovery for both compounds.

The HPLC gradient described by these authors was, however, altered to an isocratic mobile phase of methanol-water (described in methodology) as it was observed to be enough to resolve both compounds. A similar mobile phase associated with this extraction protocol has been reported to separate IAA and IBA with retention times of 6.2 and 10.6 min, respectively (Nakurte *et al.,* 2012). These results differ from those presented in this work with in which retention times of 5.34 min. were obtained for IAA and 6.01 min. for IPA. The discrepancy can be explained by the distinct reverse-phase columns used and the acidity of the aqueous phase, here achieved with orthophosphoric acid to a final pH of 2.3, while Nakurte and co-workers (2012) used 0.1% acetic acid.

The linearity of the method was achieved between 0.25 and 25 μg/ml for IAA, with an equation with a slope of 3×10^7 and a correlation coefficient of 0.9984, asserting the sensitivity of the method.

Furthermore, the UV-spectrum analysis of IAA after electrophoretic analysis was consistent with the analysis made by Kim and co-workers (2006). The LOD and LOQ were not directly comparable because detection used by the authors was based on a fluorescence detector.

4.2.2. Quantification of IAA in callus tissue

The quantifications made by HPLC varied, in absolute values, to those made by the Ehrlich reaction. However the overall relationship between the two types of callus was similar, with the embryogenic callus displaying a higher endogenous IAA concentration than the non-embryogenic one.

Furthermore, another set of comparisons was made according to the embryogenic competence of each tissue, despite the fact than EC1 and NEC1 were induced in presence of a different auxin than EC2, EC3 and NEC2. These comparisons have shown a higher level of endogenous IAA in the embryogenic tissue induced in 2,4-D (EC1), whereas the difference between non-embryogenic calluses (NEC1 and NEC2) was not considerable.

Overall, the endogenous IAA level was markedly higher in embryogenic callus, and this relation was independent of the auxin used in the induction phase. These differences have been observed in other SE plant models such as carrot (Michalczuk *et al.,* 1992) and alfalfa (Pasternak *et al.,* 2002), systems where 2,4-D is also the auxin used to triggers somatic embryogenesis. This compound is necessary not only for

initiation of the somatic embryogenesis process but for the mainetance of embryogenic competence *in vitro*. The mechanism of action is not completely understood, but clearly involves the expression modulation of several genes related to the metabolism of auxins, mainly IAA biosynthesis, degradation and transport (Yang *et al.,* 2012). The role of 2,4-D as an auxin (directly or indirectly) is still in dispute, with some authors hypothesizing that the dedifferentiation process that the cells experience is a response to the stress caused by the herbicide action of this compound (Grossmann, 2000).

After the recognition of the differences between embryogenic and non-embryogenic calluses, the auxin kinetics was investigated along with the induction phase of SE in leaf segments. These have shown a greater embryogenic yield than zygotic embryos, the other explant commonly used on induction protocols (Alves, 2012). Another important factor to consider is that the leafs were gathered from *in vitro* cloned plants sharing the same genotype, and because of this the genetic variability factors were less determinant, although genetic variations in plants regenerated from embryogenic embryos have been reported (Canhoto *et al.,* 2005).

Before the 8th week of induction, leaf samples were removed and assayed for IAA endogenous concentration by HPLC. However, the quantification proved impossible due to the presence of other compound with the same retention time as IAA and IPA (internal standard). The presence of this compounds was persistent until an advanced stage of cell dedifferentiation (in the 8th the presence was residual and the quantification was possible).

The UV-spectrum analysis of these peaks lead to putative identification of these compounds as ferulic acid and caffeic acid derivatives. Indeed, several HPLC protocols to analyse ferulic acid (Kareparamban *et al.,* 2013) and caffeic acid derivatives (Rivelli

et al., 2007) with similar mobile phase conditions have been described. These compounds are likely glycosylated, however it was not possibly to determine their exact structure with UV-spectrum analysis alone. Both compounds have reported antioxidant activity, with possible pharmaceutical applications (Lin *et al.,* 2005; Gülçin, 2006). Ferulic acid is an important compound in plant cell walls and caffeic acid, like other anthocyanins, can have several functional roles in leaves, such as protection of chloroplasts under brief exposers to high intensity sunflecks (Gould *et al.,* 2000). Although not directly related with the present work, it is interesting to note that phenolic compounds can also interfere with somatic embryogenesis induction as showed in pineapple guava (Reis *et al*, 2009). This possible interaction between auxins and phenolic compounds in the control of somatic embryogenesis induction deserves further research.

In spite of these considerations, the endogenous IAA was quantified from the 8th week onwards, with the IAA concentration increasing steadily. However, by the 12th week the concentration was still about 12-fold lower to that of the embryogenic calluses. These results are in accordance with other studies of auxin variation during the SE process, namely those of Yang an co-workers (2012), who found, in the somatic embryogenesis process of cotton, a profile of endogenous IAA concentration that decreased in the first stages of cell dedifferentiation and increased in the end of the induction phase, with the final embryogenic tissue presenting values 11-fold higher than the initial explants. In the present work, the endogenous level of IAA in the early cell dedifferentiation periods was not possible to determine, however, the results indicated that the induction phase is characterized by a lower level of IAA, while the mainetance of embryogenic competence is largely dependent on high concentrations of IAA. In fact,

tissues with minor embryogenic competence (such as the callus tissue EC3) presented a significantly lower concentration of IAA.

Recent studies have shown that the endogenous auxins are responsible for the activation of a complex pathway, which leads to the activation of several genes, either related with the metabolism of auxin or metabolic pathways, such as basic metabolic pathways and the biosynthesis of secondary metabolites (Zhu *et al*., 2008; Yang *et al.,* 2010). Additionally, Yang and co-workers (2010) also found distinct transcription profiles in embryogenic and non-embryogenic tissues, related with the auxin signal pathway. Comparative proteomic studies in tamarillo have also shown differently expressed proteins in embryogenic and non-embryogenic callus, despite the type of auxin used for induction (Correia *et al.,* 2012).

Given the results here presented, and the analogy to other species, it can be assumed that the endogenous auxin level is influencing the proteome of the different cell lines.

4.3. Extracellular protein analysis of suspension cultures

4.3.1. Protein excretion and concentration

To measure the growth of the suspension cultures, two parameters were determined, the mass increment and the absorbance at 280 nm (A_{280}). The first relates to the propagation of the cells, being a control parameter, i.e., to confirm the proliferation of the cell suspension during the growth period, and it was found to be in accordance with previous results established for suspensions of tamarillo callus, with values between 100 and 400 mg, from an initial mass of 40 mg of callus per 20 ml of culture medium (Alves, 2012). The last parameter relates to the protein excretion, as proteins

have an UV-abortion maximum in this wavelength, thus allowing a crude spectrometric estimation of the amount of protein (Aitken and Learmonth, 2002). A culture medium without auxin was used as a blank, as it was observed that both picloram and 2,4-D had considerable absorptions at 280 nm. The control of these parameters was necessary to assure a successful concentration of proteins from the cellular suspensions culture media, as previous work has shown to be a decisive step in order to achieve successful detection of proteins (Alves, 2012).

In terms of A_{280}, all the samples showed an increment in the first 2 weeks of growth followed by a small decrease in the last week. As further analysis showed protein secretion in all the samples, it is possible that this decrease can be explained in terms of consumption of an absorbent species present in the culture medium, possibly the synthetic auxin, and not be related to the protein itself.

Following protein concentration by ammonium sulphate, proteins were detected in all the samples by SDS-PAGE and direct spectrophotometric quantification using the Bradford protein assay, with greater amount of protein present in culture media from non-embryogenic callus. The method of protein concentration by ammonium sulphate proved to be effective to achieve detection in both electrophoretic and activity assays, although an extra dialysis step is necessary to remove the excess salt from the protein concentrate. These results are in accordance with previous data from other plants (Pavoković *et al.,* 2012) as well as from tamarillo suspension cultures (Alves, 2012). Moreover, the profile of absorbance at 280 nm was coincident in these finding, i.e., the greatest increments of A_{280} (EC2 and NEC2) corresponded to the greatest protein values found. Therefore, in the conditions described, this parameter can be used to follow the protein secretion of the cell suspension cultures.

The SDS-PAGE has shown a extracellular profile with high molecular weigth proteins (approximately 127 kDa) in the non-embryogenic callus medium and in the low competetent embryogenic callus (EC3) medium and two low molecular weight bands present in all the samples (approximately 15 to 19 kDa). These results are in accordance with data previously reported for tamarillo (Alves, 2012).

The intracellular protein content was also evaluated by SDS-PAGE. The embryogenic and non-embryogenic profile showed distinct profiles, with the abundance of protein bands much higher in embryogenic tissue. The electrophoretic data of intracellular protein supports the total protein quantification made on the intracellular tissue.

As described in the results, it was observed that the embryogenic tissue presented two observable, apparently non-miscible separate phases upon centrifugation, however, after SDS-PAGE analysis no distinct protein compositions were found between these two phases. This additional phase may be caused by a higher concentration of fatty acids and lipids in the embryogenic callus, as significant differences in these compounds have been described in the concentration embryogenic and non-embryogenic callus (52.785% and 24.789%, respectively) of *Eurycoma longifolia* (Iriawati *et al*., 2014).

The few bands easily visible in NEC2 were in the same range of molecular weight of the low molecular bands that appeared in the analysis of the extracellular medium (20 – 25 kDa). These bands were also present in the other tissues tested as well as in the extracellular medium. These type of low molecular weight peptide chains in embryogenic and non-embryogenic media have already been identified in other plants, such as sugarcane (*Saccharum* sp., Oropeza *et al.,* 2001). Others studies in other plants,

such as *Dactylis glomerata* and gravepine, have also identified differences in the extracellular protein profiles, both qualitative and quantitative (Maës *et al.,* 1997; Rakleova *et al*., 2010).

The analysis of the extracellular profiles made here also presented differences, both quantitatively, with the non-embryogenic tissue presenting a greater amount of extracellular protein, and qualitatively, as different bands were visible in in the non-embryogenic tissue, with similar results as previous studies on tamarillo cell suspensions (Alves, 2012).

4.3.2. Assays of enzyme activity

The first activity test made was the analysis of the extracellular medium by zymography in a polyacrylamide gel co-polymerized with gelatine. In this technique there are no reducing agents present in the buffer and the samples are not boiled to avoid denaturation and subsequently loss of activity. To better visualize the bands showing enzymatic activity and to compare them with a molecular weight marker, an additional SDS-PAGE was made without reducing agents or boiled the samples, in the same buffer used for the zymography, to maintain the natural tri-dimensional conformation.

These results showed proteolytic activity in a high molecular weight range, from 130 to 250 kDa. This apparent molecular weight range is, however, relative to non-denaturing conditions, and cannot be directly compared with the SDS-PAGE previously made. However, the proteolytic activity of the proteins was demonstrated. The fluorometric assays allowed further characterization of the proteolytic activity, including studies with specific protease inhibitors.

The fluorometric assays using a protease substrate (Phe-AMC), showed a similar specific activity between the embryogenic tissue and non-embryogenic tissue (EC2 and NEC2), with the embryogenic tissue with low embryogenic yield (EC3) presenting a lower activity. The NEC2 media presented a much higher total activity (data not shown). However, when the specific activity was determined for each sample, taking into account the total protein of the medium, this value decreased. This data suggest that the protein present in NEC2 is less pure than in the EC2, i.e., a smaller fraction of the protein present is indeed proteolytic (Scopes, 2002).

These results support the data previously obtained by zymography and SDS-PAGE analysis. Protease activity has been previously described in both embryogenic and non-embryogenic culture media of *Dactylis glomerata* (Rakleova *et al*., 2010) where the same isoforms of cysteine protease were found.

The glycosidase affinity tests have shown a difference in the extracellular media, with the extracellular culture media of embryogenic callus showing a greater specific activity in both substrates tested. The importance of extracellular glycosidases in the somatic embryogenesis process has been studied, with some data pointing to their importance (van Hengel *et al.*, 2001). In these studies, it was shown that these enzymes were secreted to the liquid medium. The biochemical importance of these enzymes to the process of somatic embryogenesis is not fully understood. Yet, recent studies have shown that the cell wall of zygotic embryos and somatic embryos of *Daucus carota* is different, with cell wall composition of somatic embryos being further dependent on the culture medium, particularly if they were grown in a solid or liquid medium (Dobrowolska *et al.,* 2012).

The results presented in this thesis show a different activity in the several media tested, with the embryogenic medium presenting a significantly higher activity in both substrate tested. This expression can be a response to liquid culture medium or even the carbon source present, as previous works have demonstrated that this factor can influence the extracellular expression profile of the cells (Blanc *et al.,* 2002). Collectively, these and other works suggested that the wall composition of the embryogenic cells is an important factor for embryogenesis. Furthermore, the presence of certain enzymes in the liquid growth medium of certain terrestrial plants can be the result of a response to an abnormal environment (Dobrowolska *et al.,* 2012).

The search for molecular markers of embryogenesis, particularly protein markers, has been intense and conducted in several plants (Tchorbadjieva, 2005). The specific activity of both substrates, particularly MU-G is higher in extracellular medium of embryogenic tissue, regardless of the synthetic auxin used in the induction phase. Therefore, the enzyme responsible for these cleavage, if purified and characterized, could be a marker of embryogenic competence in tamarillo callus cultures.

4.3.4. Proteolytic enzymes inhibitors

The assays in which the dominant classes of proteolytic enzymes were analysed by incubation with several specific protease inhibitors showed that the EC2 and NEC2 extracellular media presented qualitative differences.

In the case of the extracellular medium of EC2, the high activity observed in presence of bestatin, E-64 and pepstatin A as well as the large standard deviations observed seem to rule out the presence of cysteine proteases and aspartic proteases, respectively, although in this second case only one type of inhibitor was tested. On the other hand, EDTA and phenathroline present lower activity values, as well as TPCK,

TLCK. Therefore, it is possible that in this callus, the major classes of extracellular proteases present are metalloproteases (inhibited by EDTA and phenathroline) and serine proteases (inhibited by TPCK and TLCK).

The analysis of the NEC2 medium revealed the same high activities in the presence of bestatin, E-64, pepstatin A as well as EDTA and phenathroline, while the activity values of both TPCK and TLCK remain lower. In this case, the serine protease class seems to be dominant.

These results are in accordance with earlier mass spectrometry analysis for the identification of the proteins present in the tamarillo suspension cultures extracellular media, conducted previously in our laboratory (data not published). These studies have shown the presence of a putative metalloprotease in the embryogenic medium as well as a subtilisin-like protease (serine class) in the non-embryogenic tissue.

The main classes of proteases identified by these assays are distinct from those described in other works with embryogenic suspensions of other plants, namely *Dactylis glomerata* where the main class identified was cysteine proteases (Rakleova *et al.,* 2010). The discrepancy in the results can be related to the taxonomic distance between the two species.

5. Conclusion and future perspectives

Somatic embryogenesis is an important biotechnological tool for the development of efficient *in vitro* protocols of culture and improvement of plants, as well as a model for biochemical and physiological studies, particularly in the developmental stages of embryos.

Protocols for the regeneration of tamarillo plants from adult explants as well as for the proteomic analysis of induced explants have been already described. This work was a step forward in the characterisation of tamarillo somatic embryogenesis.

The first factor studied was the endogenous level of auxin, as these plant growth regulators are present in the majority of induction protocols, normally by adding a synthetic auxin, such as picloram or 2.4-D, to the culture medium. Several studies have concluded that these compounds, either directly acting as an auxin, or by triggering specific stress responses in the cells, originate the production of endogenous auxins, particularly IAA, which in turn is necessary for the maintenance of embryogenic competence in the proembryogenic masses.

The determination of endogenous levels of plant hormones is technically difficult, with compounds existing in vestigial amounts bellow the limit of quantification of a given method or otherwise difficult to extract from the complex biological matrix. For the presumptive determination of IAA in embryogenic and non-embryogenic callus, the Ehrlich reaction has proven to be adequate to distinguish the major differences between the different types of callus. For further analysis, the HPLC method has proven sensitive and exact, despite the fact that the isocratic phase described does not separate certain phenolic compounds present in tamarillo leaves. This method could be adjusted with specific HPLC conditions, such as an optimized gradient of the mobile phase suited for the resolution of these compounds or other operating conditions (different column, different organic solvents).

The HPLC methods are also adaptable for the simultaneous quantification of other classes of PGRs, such as abscisic acid, another plant hormone controlling SE. Therefore, in future studies, this quantification method can be adapted and employed in the type of assays here described.

The results presented here showed that the endogenous level of IAA is different in embryogenic and non-embryogenic callus, and that the profile of IAA tends to increase as the dedifferentiation of the cells in the original explant progresses. Additionally, the auxins are also relevant to promote the formation of somatic embryos from these proembryogenic masses.

The assays of auxin degradation also demonstrated a difference in the several types of callus studied, with the embryogenic masses presenting a lower degradation rate. This results point to a different activity in the oxidation mechanism of auxins. Therefore, this quantification can be refined in the future by using purified extracts of the principal enzymes involved in the oxidation of auxins, peroxidases and IAA-oxidase, in order to better characterized the auxin oxidation mechanism expressed in these types of cells. This study could also be complemented with biosynthesis studies, in order to evaluate the major pathway used and the types of enzymes expressed.

A necessary complement to the data presented is relating the endogenous level of auxin with its cellular distribution, for example by immunodetection of the PIN proteins. These type of studies are important in order to relate the biochemical parameters with the physiological functions they influence.

Another potentially important finding of this work, although outside the original objectives, were the phenolic compounds found in the tamarillo leafs. The type of molecules

identified, although widely distributed in nature, are of great interest in several applications, and their isolation and characterization could prove to be interesting.

The second part of this project focused on the study of the extracellular protein profile of liquid-grown callus. The fact that the proteins secreted by the proembryogenic masses can decisively influence the SE project has been recognized for some time, and previous work in our laboratory had established a protocol to grow tamarillo callus cells in suspension. In the present work it was shown that the absorbance of the medium at 280 nm can effectively follow protein excretion secretion during the plant growth and with the initial mass to volume conditions used, at the end of the 3-week period previously established, there is a decline in absorbance likely due to the consumption some media compound, possibly the synthetic auxin used.

The proteolytic profiles and activity assays showed qualitative (different classes of proteases) and quantitative (quantity of extracellular protein and specific activity) differences between the embryogenic and non-embryogenic tissues, confirming previous data for tamarillo and other species. The mechanism by which these extracellular proteins influence the embryo formation is not yet clear and deserves further research. The non-embryogenic callus showed high amount of extracellular protein and a low specific activity, probably due to diversity of proteins present in the extracellular medium.

Additionally, activity assays with specific glycoside substrates have shown a significant difference between embryogenic and non-embryogenic tissue, which further reinforces the idea that the extracellular proteins are important for the embryogenic studies. The use of these types of specific activities as a marker of embryogenic competence can also be considered, as the differences found were significantly different and apparently relatable with this characteristic.

Overall, the results presented here contribute to a better understanding of some biochemical aspects underlying somatic embryogenesis of tamarillo and, hopefully, other species. Although the data does not fully describe the phenomena of auxin homeostasis and extracellular protein excretion, they confirm some data described in the literature, and lay methodological foundations for future biochemical and physiologically studies in this complex biological phenomena.

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