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MORPHOLOGICAL AND PHYSIOLOGICAL EVALUATION OF TAMARILLO (*SOLANUM BETACEUM* CAV.) SOMATIC EMBRYOGENESIS-DERIVED PLANTS

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List of abbreviations

2,4-D - 2,4- dichlorophenoxyacetic acid

5-AzaC - 5- Azacytidine

ABA - Abscisic acid

AC - Activated charcoal

BAP – 6 - Benzylaminopurine

cDNA – Complimentary deoxyribonucleic acid

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

EC - Embryogenic *calli*

ES – Embriogénese somática

EtOH - Ethanol

LEA - Late embryogenesis abundant proteins

MS – Murashige and Skoog culture medium (1962)

NAA - 1- Naphthaleneacetic acid

NEP-TC – Non- embryogenic protein

NUC - Neglected and underutilized crop

PCR - Polymerase chain reaction

PSII - Photosystem II

qPCR – Quantitative polymerase chain reaction

RNA - Ribonucleic acid

SE – Somatic embryogenesis

SEM – Scanning electron microscopy

TBE – Tris-borate-EDTA (2-[2-(bis(carboxymethyl))

TP - Murashige and Skoog culture medium supplemented with picloram

TSA - Trichostatin A

Resumo

A embriogénese somática (ES) é uma ferramenta eficaz para clonagem e micropropagação em larga escala para muitas espécies. No tamarilho (*Solanum betaceum* Cav.) a clonagem por embriogénese somática está bem estabelecida e ocorre através de um protocolo bifaseado. A primeira fase passa pela formação de um calo embriogénico através da indução de segmentos de folhas jovens ou embriões zigóticos em meio rico em auxina (2,4-D ou Picloram), suplementado com uma elevada concentração de sacarose (0,3 M). Este calo pode ser mantido nesse mesmo meio por 4-5 semanas, sem perda da sua capacidade embriogénica durante subculturas sucessivas. Depois da sua transferência para um meio sem auxina e com uma quantidade de sacarose mais reduzida (0,1 M), o calo embriogénico desenvolve-se em embriões somáticos que se convertem em plantas. Algumas desvantagens deste processo são anomalias dos embriões e restrições no seu desenvolvimento e conversão. Realizaram-se diversos ensaios para otimizar o desenvolvimento de embriões e regeneração de plantas.

Diferentes linhas de calo embriogénico (EC1, EC2, EC3) derivadas de indução de ES em folhas com tempos diferentes em cultura (1 a 5 anos) foram sujeitas a 3 tratamentos distintos: 1) 0,05 µM tricostatina A durante 2 dias; 2) 20,0 µM 5-azacitidina durante 2 dias; 3) imersão em 0,2% (w/v) de carvão ativado. Na linha EC2 o tratamento com carvão ativado levou a um maior número de embriões por 100 mg de calo (≈ 74,3). No entanto, a conversão ocorreu apenas nos tratamentos com tricostatina A e 5-azacitidina com taxas mais altas para a linha EC1 (10,0% e 5,17%, respetivamente). A taxa de sobrevivência de plantas mais elevada foi obtida em plântulas provenientes do tratamento com tricostatina A. Para avaliar a qualidade dos embriões realizaram-se análises de microscopia eletrónica de varrimento e de expressão de genes. Plantas aclimatizadas provenientes de calo submetidos aos diferentes tratamentos foram comparadas com plantas provenientes de calo sem tratamento e com plantas provenientes de sementes, através de parâmetros de crescimento, de trocas gasosas e fotossintéticos. Os resultados demonstraram que as plântulas obtidas por embriogénese somática têm um desenvolvimento semelhante àquelas obtidas por semente.

Palavras-chave: 5-azacitidina; carvão ativado; desenvolvimento de embriões; performance de plantas; tricostatina A

Abstract

Somatic embryogenesis (SE) is an effective tool for cloning and large-scale propagation as shown for many species, including many trees. In the solanaceous tree tamarillo (*Solanum betaceum* Cav.) plant cloning through SE is based on a well-established protocol involving two steps. In the first one, embryogenic *calli* is formed by induction from young leaf segments or mature zygotic embryos in an auxin-containing medium (2,4-D or Picloram) supplemented with high sucrose levels (0.3 M). These *calli* can be kept in the same medium for 4-5 week, maintaining their embryogenic potential throughout successive subcultures. Following transfer to an auxin-free medium containing lower sucrose levels (0.1 M), embryogenic *calli* evolve into somatic embryos that convert and produce plants. Main drawbacks of this system are the high frequency of abnormal embryos as well as arrested somatic embryo development and conversion. To optimize somatic embryo development and plant regeneration several experiments were carried out.

Different embryogenic lines (EC1, EC2, EC3) derived from SE leaf induction with different times of culture (1 to 5 years) were subjected to 3 different treatments: 1) 0.05 µM trichostatin A for 2 days; 2) 20.0 µM 5-azacycitide for 2 days and 3) a rinse with 0.2 % (w/v) activated charcoal, before transference to auxin-free conversion media. Activated charcoal treatment led to a higher number of somatic embryos reaching ≈74.3 embryos per 100mg of *calli* in the line EC2, but only the trichostatin A and the 5-azacytidine treatments allowed the efficient conversion of somatic embryos into plantlets in the line EC1 (10.0% and 5.17%, respectively). Survival rates were higher for plantlets resulting from the trichostatin A treatment. Scanning electron microscopy and gene expression analysis were made to evaluate embryo quality. Successfully acclimatized plantlets from *calli* previously subjected to the different treatments were compared with plants from non-treated *calli* and with plants from zygotic origin, through growth, gas-exchange and photosynthetic parameters. Results show that SE-derived plants have a similar development to seed-derived plants.

Keywords: 5-azacytidine; activated charcoal; emblings performance; embryo development; trichostatin A

I. Introduction

1. Contextualization of the work

Tamarillo (*Solanum betaceum* Cav.) is small solanaceous tree that produces edible and high nutritious fruits from which other products might be produced, such as smoothies and jams. Nowadays, consumers are always searching for new and nourish products and the fact that tamarillo offers a healthy and appealing option shows the potential of this fruit in the market. Tamarillo's fruits reach a high market value (10-15€/Kg) and the fact that Portugal possesses adequate weather conditions for fruit production led to an increase of interest from the producers. Nevertheless, tamarillo is considered yet a NUC (neglected and underutilized crop).

Tamarillo is also used as a model to study lines of search like SE (somatic embryogenesis). SE is a powerful tool for plant genetic improvement when used with in combination with more conventional breeding and propagation techniques (Loyola-Vargas *et al.*, 2008) and in the future might be used for large-scale cloning of elite tamarillo trees. SE has a well-established protocol for tamarillo developed in the Laboratory of Vegetal Biotechnology of the University of Coimbra yet, an optimization of embryos conversion into plantlets is still required and performance of SE derived plants is still to be studied after acclimatization and transfer to field essays. Thus, it is important to follow up closely embling conversion into plantlets and its acclimatization with morphological e physiological analysis for better understanding its limiting steps to aim high quality performance plants.

2. *Solanum betaceum* Cav.

2.1. Characterization and distribution

Tamarillo, also known as tree tomato or "tomate de la paz", is a small tree belonging to one of the most economically relevant families - Solanaceae (Correia and Canhoto, 2012). Although tamarillo is a lesser known species in the North Temperate Zone, it's very appreciated in the tropical and subtropical regions due to its edible and nutritious fruits (Acosta-Quezada *et al*., 2011). After a fast increase of production and consumption of this fruit, the name tree tomato was officially changed in 1967 to a more commercially appealing name, tamarillo (Atkinson and Gardner, 1993). This change was mostly to avoid it to be wrongly mistaken with the common garden tomato (Morton, 1987).

This species is native of the Andean regions of Peru, Chile, Ecuador and Bolivia, where it is widely found and has been cultivated for a long time (Meadows, 2002). From there it spread to Central America and West Indies. Afterwards tamarillo was introduced to South Europe and to the Portuguese islands Madeira and Azores (Meadows, 2002). By the end of the 19th century, it had extended to China and British Colonies including Australia and New Zealand. Currently tamarillos are grown and cultivated all around the globe in countries like USA, Brazil, India, Sri Lanka, Australia and Kenya (Meadows, 2002) (Fig.1). Most commercial production occurs in Colombia, Peru and Ecuador (Ramírez and Kallarackal, 2019).

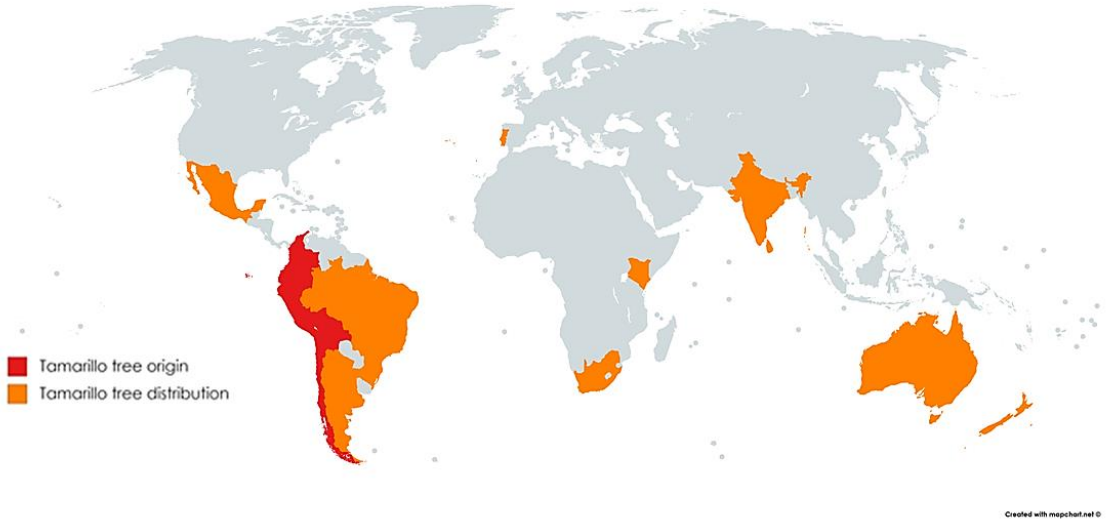

Figure 1 - Tamarillo tree origin and distribution.

Tamarillo is a small perennial tree that can reach 2 to 4 meters (Fig. 2A). Usually the plant presents a single trunk with brittle branches in which deciduous leaves emerge. Flowers, and later on fruits, emerge at the top of the branches. The pale-pink lavender and scented flowers develop in small groups (Fig. 2B) and typically blossom since late summer to the beginnings of fall, nevertheless, exceptions may occur (Bakshi *et al.*, 2016). Pollination is almost always autogamic which may relate to the low genetic variability found in natural populations (Barghchi, 1986). The egg-shaped fruits are suspended by a long stalk and may occur isolated or in groups of 3 to 12 units (Fig. 2C). Generally, the fruits are 5 to 10 cm long and 3 to 5 cm wide and fruit ripening happens between the months of October and April. The fruit's epicarp is smooth and presents a large variety of colours, such as dark red, orange and yellowish or even a mixture of these (Fig. 2D). The pulp is juicy and displays the same variation of colours as the epicarp. The seeds are flat, thin and hard. All the parts of the fruit are edible, but the skin

should be removed before eating, since it's thick and bitter. The seeds can be eaten with the endocarp, which is consistent and has a sweet-sour pleasant flavour.

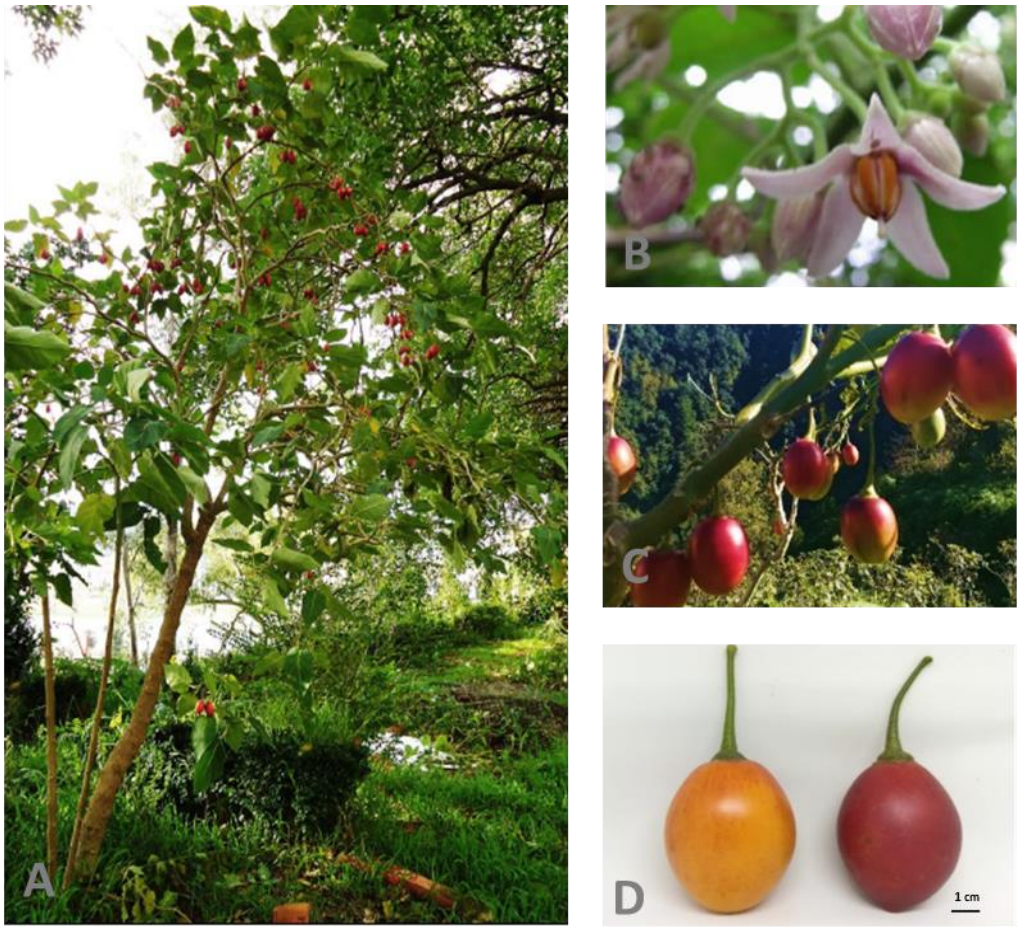

Figure 2 – Tamarillo (*Solanum betaceum* Cav.). (A) - Tamarillo tree growing at the Botanic Garden of the University of Coimbra; (B) - Tamarillo tree flowers; (C)- Tamarillo fruits from a red tamarillo tree variety from Madeira island; (D)- Yellow and red tamarillo fruits.

As a subtropical plant, tamarillo grows better in medium and high altitudes in neotropical mountain forests (Bakshi *et al.*, 2016). In colder climates it can grow at lower altitudes. This species requires well-drained soils and sunny locations for a faster development. If exposed to extreme freezing, tamarillo may die but it will recover from light frosts. If cultivated in windy areas, tamarillo should be well protected since it is very sensible to strong winds and can be easily blown away (Bakshi *et al.*, 2016). This species is also very sensible to constantly high temperatures and long-lasting drought (Lopes *el al.*, 2000). Regarding to its life expectancy, tamarillo can be considered a short-lived tree living from 5 to 12 years. To obtain high-quality yields, fruit production should only start in the second year since planting until the seventh or eighth year (Meadows, 2002).

Although tamarillo presents a high tolerance to diseases, it can still be affected by mildew that leads to defoliation. Occasionally it can be attacked by green aphids and

fruit flies and it's susceptible to nematodes and some viruses (Mossop, 1977; Eagles *et al*., 1994).

2.2. Economic relevance

Tamarillo varieties are cultivated mostly due to its edible fruits, which can be eaten raw and used in salads or in processed products like snacks, juices, jams and *smoothies* (Fig. 3). Regarding tamarillo fruits nutritional value, they rate higher as a source of vitamins, minerals and antioxidants when compared with other common fruits and vegetables (Lister *et al*., 2005). Tamarillo nutritional attributes include low fat and subsequently low calories (28 Kcal/100g); low carbohydrate content; high protein content (1.5- 2g/100g); high quantity of vitamins like vitamin C, E and B6 and provitamin A (Holland *et al.*, 1991). Moreover, tamarillo fruits also contain minerals essential to the normal functions of the human body, such as potassium, cooper and manganese.

Figure 3 - Different applications for tamarillo fruits such as jams, salads, smoothies, pizzas (http://www.tamarillo.com).

Some studies have shown that the fruits also offer a high concentration of anthocyanins, carotenoids and phenolics, adding value to them. These compounds have biological, therapeutic and antioxidant properties and may have an important role in human health (de Rosso and Marcadante, 2007; Kou *et al.*, 2008; Hurtado *et al.*, 2009).

According to the New Zealand Tamarillo Growers (2008) the main tamarillo varieties are red, yellow and amber but since the consumers prefer red fruits due to its superior taste and appealing pulp colour, cultivars with red fruits are the most popular and appreciated.Even though this species is already commercially exploited by some countries, like the U.S.A, New Zealand and Colombia, it is still considered a NUC (neglected and underutilised crop). According to the Organization Biodiversity International (https://www.bioversityinternational.org/), tamarillo is a species that has

potential for agricultural use but for several unknown reasons, it has not been properly explored.

In recent years, tamarillo has become more noticeable to producers and consumers and its commercial value as being gradually recognised in Portugal, especially in the Atlantic Islands. Nonetheless this is an onward process and even though the fruits' price at the market place are very appealing to Portuguese producers (10 to 15€ per Kg), large scale cultivation is still not a reality due the plants' low tolerance to frost, which affects the plant development and reproduction.

Since this species is economically very interesting and shows a lot of potential to become commercially successful in our country, production optimization becomes a priority, both in quantitative and qualitative levels.

3. Tamarillo propagation

3.1. Conventional methods in tamarillo

Propagation using seeds, cuttings (Prohens and Nuez, 2001) or grafting are the traditional methods normally used to achieve tamarillo plants.

Tamarillos are easily propagated from seeds. Initially seeds produce very fragile plantlets but after that the plants can reach a height of 1.5 to 1.8 m before branching out (Bakshi *et al.*, 2016). These plants can be very uniform throughout growth, but they do not guarantee genetic uniformity and are ineffective when the aim is propagation of selected genotypes.

Tamarillo cuttings root easily but produce smaller bushy trees compared with seed-derived plants. Grafting onto *Solanum mauritianum* is also a method used with the aim of improving the tolerance to wet soil conditions and to avoid the root to rot (Bakshi *et al.*, 2016). Although these last two techniques allow the propagation of desirable genotypes, they do not ensure freedom from virus infections (Gahakwa *et al*., 2013). Also, these processes take a long time to be successfully concluded which may result in a reduce number of plants.

If the objective is crop improvement, asexual propagation methods are the most suitable but phytosanitary problems (Mossop, 1977 cited in Correia, 2011) are a big issue and interspecific hybridisation is unviable due to its low rates of success (Correia and Canhoto, 2012). Therefore, biotechnological methods such as *in vitro* cloning and genetic transformation are very efficient and offer a lot of advantages (Barghchi, 1998).

3.2. *In vitro* culture methods for tamarillo

Over the past decades several methodologies have evolved to advance in research of plant biotechnology. One of them is micropropagation, a technique that allows *in vitro* cloning trough plant cell tissue culture. This technique surpasses some of the problems presented by the conventional propagation methods, presenting several advantages, such as the ability of propagation in large scale and the production of plants genetically uniform with a superior phytosanitary quality (Canhoto, 2010). Additionally, plants attained from micropropagation show, in general, more vigour, are more uniform between them and present a faster maturation and growth compared with plants attained from seed. There are three techniques of micropropagation distinguishable through the initial material used and the type of response obtained (Canhoto, 2010): axillary shoot proliferation, organogenesis and somatic embryogenesis.

When an axillary shoot is established *in vitro*, in a specific culture medium, it grows and originates a new stem shoot made of a group of phytomers. Since these phytomers regenerate again into new stem shoots this process may repeat itself numerous times. Axillary shoot proliferation is usually the preferable technique for large scale production given that it enables plant exponential multiplication (Canhoto, 2010). This methodology presents the advantages of not requiring induction of new meristems thus reducing regeneration time and not requiring the formation of *callus* guarantying genetic uniformity. In other techniques the formation of *callus* is often necessary which may lead to some chromosomic and genetic anomalies and consequently some variations in the originated plants (Canhoto *et al*., 2005).

Organogenesis is a process where new organs (meristems, roots, shoots) form on an explant tissue (leaves, stems, roots, hypocotyls, petals or sepals), under specific chemical and physical conditions (Canhoto, 2010). This methodology requires the induction of new meristems (adventitious meristems) which may be caused by numerous factors, being the most significant the presence of auxins and cytokinins (in a specific balanced ratio) and of oligosaccharides in the medium culture (Angulo-Bejarano and Paredes-López, 2011). There are two distinct processes in organogenesis. One of them is direct organogenesis where an explant develops meristems and then shoots, and after rooting, originates new plants. The other one, more commonly used, is indirect organogenesis where there is *callus* induction from an explant from which gems develop and then proceed to rooting (Canhoto, 2010). Organogenesis is a technique with a lot of potential given that theoretically a single explant may originate as many plants as the

number of its cells. Some of its disadvantages are occurrence of contaminations, phenols oxidation and recalcitrance to regeneration inherent to some species (Canhoto, 2010).

Obtention of tamarillo plants through axillary shoot proliferation and organogenesis have already been described (Guimarães et al., 1996; Obando and Jordan, 2001 cited in Correia, 2011), and are efficiently used for tamarillo micropropagation (Santos, 2012).

Finally, somatic embryogenesis (SE) in which under certain conditions somatic cells evolve into embryogenic cells capable of developing somatic embryos and consequently plants (Canhoto, 2010). In natural conditions, the zygotic embryo is obtained from sexual reproduction through the junction of the female and the male gamete creating a new entity, the zygote, that after successive divisions and specialization develops into the zygotic embryo (Canhoto, 2010). In SE the fusion of gametes is not required, and somatic embryos are attained through parts of the mature plant. Much like zygotic embryos, somatic embryos present a bipolar structure with a stem and root pole and even develops trough the same developmental phases as the zygotic embryos (globular, heart-shaped, torpedo and cotyledonary). Since the zygotic embryo is surrounded by tissues like the endosperm and the seed coat, this technique allows studying embryo development, especially in the initial stages (Santa-Catarina *et al.*, 2001; Yang and Zhag, 2010; Correia, 2011)

The induction of SE can be achieved from several explants, such as young leaves, zygotic embryos, cotyledons, hypocotyls or roots and it allows large-scale propagation of genetically uniform plants, since it enables the production of an unlimited number of embryos which carry the same genotype as the initial explant (Aslam *et al.*, 2011). Moreover, this method allows the cryopreservation of the embryogenic material providing the opportunity of studying the material in the field to test its quality (Canhoto, 2010; Graça, 2016). Also, once the embryos have a unicellular origin this is the most viable technique to regenerate genetic transformed plants (Montalbán *et al.*, 2010). Nonetheless, somatic embryogenesis presents some limitations. SE is associated with a high number of abnormal embryos, low rates of conversion of embryos into plants and low capacity of attaining embryogenic material from adult explants. As said before, the formation of *callus* may lead to chromosomic or genetic anomalies. If the cultures are maintained for a long period of time they may lose its embryogenic competence and produce a low number of embryos or even stop producing them at all and inhibit the embryos to mature completely which leads to low rates of germination and debilitated plantlets (Pullman *et al*., 2003).

3.2.1. Somatic embryogenesis in tamarillo

Somatic embryogenesis (SE) in tamarillo was first described in 1988 by the Laboratory of Plant Biotechnology from the Univercity of Coimbra (Guimarães *et al*., 1988) through its induction on mature zygotic embryos and hypocotils. Thenceforward the same group established a successful protocol of SE for different kinds of explants and studied different aspect related to SE and somatic embryo development. Thus, this protocol is used, not only as clonal propagation tool, but also as a model system for adquiring more knowledge on the cytological and molecular mechanisms related with somatic embryos' formation and development (Correia *et al*., 2012).

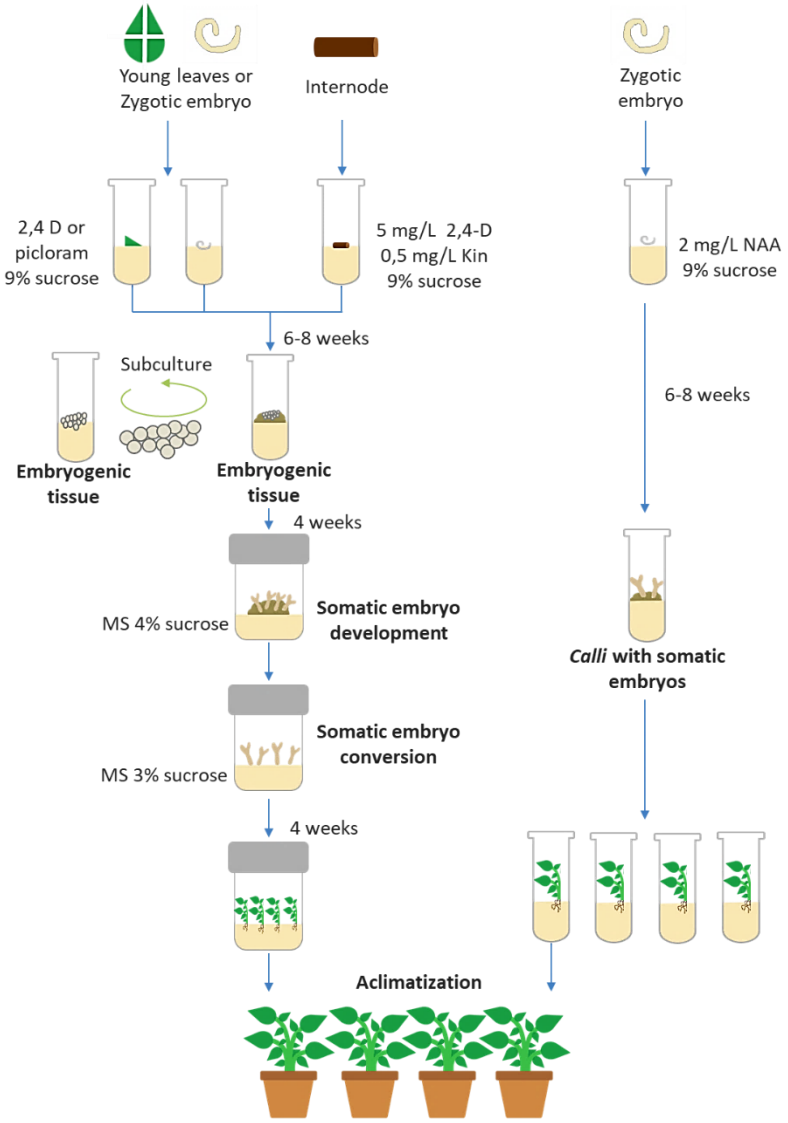

Figure 4 - Somatic embryogenesis in tamarillo (adapted from Canhoto *et al.*, 2005).

Induction of SE in tamarillo (Fig. 4) can be achieved from different types of explants, such as mature zygotic embryos, young leaves, cotyledones and hypocotyls. The explants response to initiate an embryogenic culture is a crucial step in order to

advance in the stages of SE. In some species, particularly trees, induction of SE in adult material is often difficult due to its predisposition for recalcitrance (Thorpe and Stasolla, 2001). A way to avoid this problem in tamarillo is to establish plants by axillary shoot proliferation and then induce SE in leaf segments of these plants (Correia *et al.*, 2011).

There are two pathways leading to SE: “one-step” process or a “two-step” process. The “one-step” SE is achieved by exposing explants such as zigotic embryos to MS (Murashige and Skoog, 1962) medium with 1-naphthaleneacetic acid (NAA) and high sucrose content. After 4 to 6 weeks of culture, the zigotic embryo’s hypocotyl region forms a reduced *callus* with somatic embryos (Guimarães *et al*., 1996). This process has been successful for other woody species like *Feijoa sellowiana* (Canhoto and Cruz, 1996) and *Myrtus communis* (Canhoto *et al.*, 1999).

The second process designated as “two-step” process, requires two different media to attain full somatic embryo differentiation (Williams and Maheswaran, 1986). The “two-step” SE in tamarillo is achieved by exposing explants such as zigotic embryos (Fig. 5A) or young leaves segments (Fig. 5B) to MS medium with 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram, respectively, and high content of sucrose. After 4 to 6 weeks of culture a growing non-embryogenic *callus* is formed. Then that *calli* expands and also some slow growing whitish clusters of embryogenic *calli* appear and continue proliferating after the 8^{th} - 10^{th} weeks (Guimarães *et al*., 1996; Canhoto *et al.*, 2005). Non-embryogenic and embryogenic *calli* are easily distinguishable (Fig. 5C). Embryogenic *callus* is formed by globular whitish opaque nodules, small isodiamentric cells and shows a slower growth compared with the non-embryogenic one (Correia *et al*., 2009) (Fig. 5D) while non-embryogenic *callus* is more friable and presents a darkish color (Guimarães *et al*., 1988) (Fig. 5E). The main advantage ensured by the “two-step” tecnique is the fact that the embryogenic *callus* can be maintained in subcultures for a relative long period of time in an auxin-rich medium without the loss of its embryogenic competence (Canhoto *et al.*, 2005). In spite of that, some lines of long-term embryogenic *callus* are often related to some degree of destabilization of the genetic and epigenetic program and may suffer some variations in its DNA quantity and methylation (Currais *et al.*, 2013); number of chromossomes and transposon activation (Smulders and Klerk, 2011). Chromossome abnormalities, including occurence of tetraploid plantlets, were detected through chromossome counting (Lopes *et al.*, 2000). The longer the culture period is the the higher is the probability of these changes to occur. Studies performed with tamarillo shown that plantlets attained from recent (1 year) embryogenic tissue display less anomalies when compared to those attained from long-term (5 year) embryogenic tissue. The results also seem to suggest that these chromossome

abnormalities took place while the *callus* remained in culture and are not a pre-existing condition of the cultured tissues (Currais *et al.*, 2013).

In a second stage, the embryogenic tissue is placed in a medium devoided of auxins and with a lower content of sucrose inducing the formation of somatic embryos (Fig. 5F-G). Other factors like the nutritional and osmotic environment are important but regardless the conditions and explant tissue, the embryo formation only occurs in a auxin free medium (Canhoto *et al.*, 2009; Correia and Canhoto, 2010).

For both tecniques, the somatic embryos are transferred to an auxin-free medium with 2% sucrose (w/v) and placed in light conditions in order to enable its development and allow an efficient supply accumulation (Canhoto *et al.*, 2005; von Arnold *et al.*, 2002; Fig. 5H-J). These steps require optimization since they are often limitant for a successful embryo convertion and may lead to low rates of embryo germination into plants. Previuos data from assays with tamarillo somatic embryos development indicate that addition of abscisic acid (ABA) in the medium before germination and manipulation of light conditions may lead to a rise of morphologically normal somatic embryos (Correia *et al*., 2012). Studies using scanning microscopy shed some light about the morphological and anatomical characterization of somatic tamarillo embryos (Correia *et al.*, 2011).

Some other limitations of SE, also verified for tamarillo, like the asynchronous embryo development, the anomalous embryos and the continuos proliferation of *callus* make it very difficult to establish a quantification method to evaluate plant regeneration trough SE (Correia and Canhoto, 2012).

The colyledons shift to a greenish colour and the formation of radicles are the first signs of embryo germination (Canhoto *et al.*, 1999). Preferably these events are balanced, meaning that the stem and the root have a simultaneous development leading to a successful acclimatization. The frequency of convertion of somatic embryos to plantlets is very low in many woody species (Park *et al.*, 1994; Correia *et al.,* 2012). This is one of the main reasons SE is still not a commercially viable process for many species.

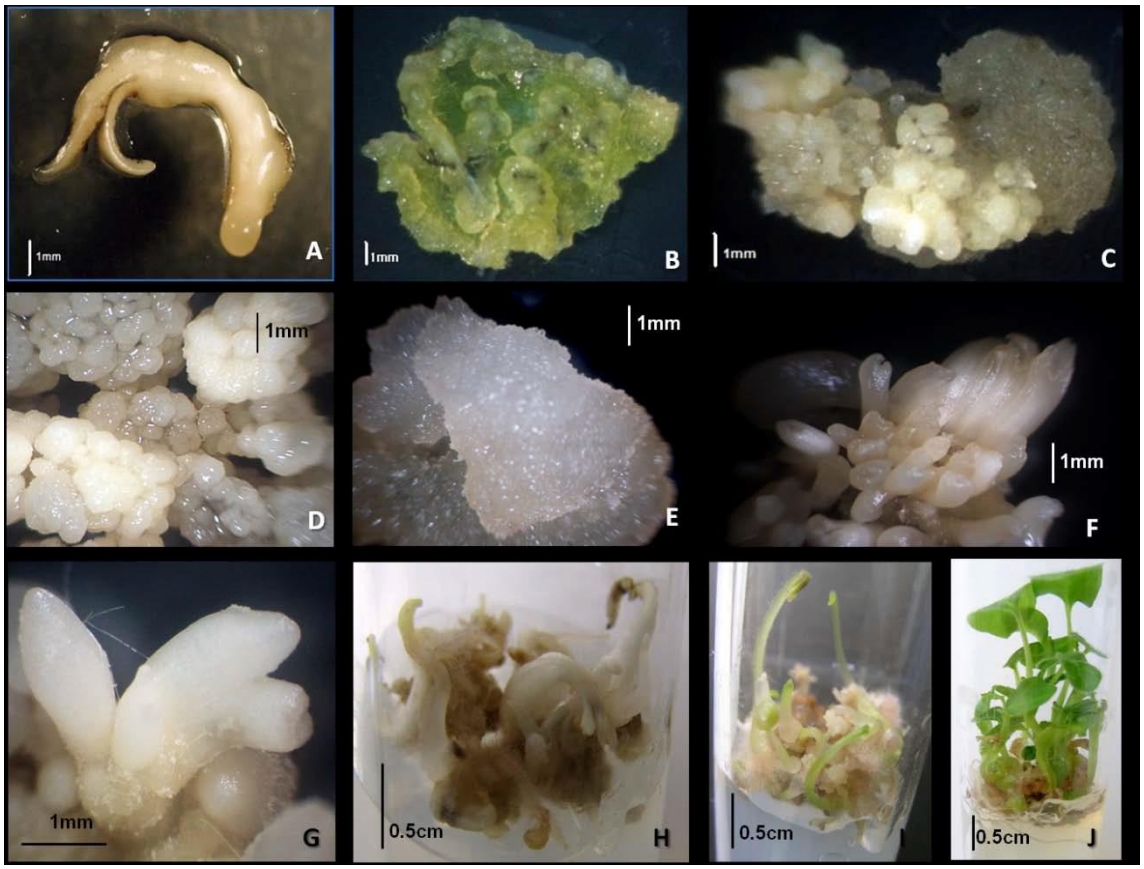

Figure 5 – Somatic embryogenesis in tamarillo. (A) - Zygotic embryo on TD medium; (B) – Leaf explant on TP medium; (C) – Embryogenic and non- embryogenic (darker) zones formed on a leaf explant; (D) - Embryogenic tissue; (E) – Non-embryogenic tissue; (F) and (G) – Somatic embryos at different developmental stages induced at an auxin-free MS medium; (H) to (J) – Somatic embryo conversion and plantlet development on MS medium (adapted from Correia and Canhoto, 2012).

The acclimatization stage, in which the plantlets are transferred to the greenhouse, is considered a preparation for the final relocation in the field with *ex vitro* conditions. The plants survival and growth is related to the development and conversion of the embryos in previous stages. Morphologically normal somatic embryos that conclude their development into mature stage survive desiccation and will probably develop into normal plants that reach acclimatization (Canhoto *et al.,* 2005). Nonetheless, cases where plantlets with morphological anomalies in the initial stages grew into normal phenotype have been reported. Usually, abnormal plantlets development leads to the high mortality rates observed in the acclimatization stage (Canhoto *et al.*, 2005).

3.2.2. Molecular analysis of tamarillo somatic embryogenesis

As stated before, plant cloning in tamarillo through somatic embryogenesis is based on a well-established protocol (Guimarães *et al*., 1988). Assays related to the proteomics of SE were carried out to find specific-embryogenic proteins by comparison between the proteome of tamarillo's embryogenic and non-embryogenic *calli* obtained from different origin explants (young leaves or zygotic embryos) and with different auxins (2,4-D or picloram) (Correia *et al.*, 2019). The results showed an increase of metabolism, protein synthesis and the prevalence of stress related proteins, also demonstrated that the embryogenic cells have a better capacity to regulate metabolism under stress conditions. Proteins, exclusively or predominantly expressed in embryogenic cells, included metabolism-related proteins, such as enolases and treonine synthases, which suggests the relevance of primary metabolite production, such as amino acids and fatty acids, and fermentation as an alternative source of energy at the early steps of somatic embryo development.

The accumulation of storage compounds has also been described of critical importance for embryo development and efficient plant conversion (Canhoto *et al.*, 2005), and also verified for tamarillo (Correia *et al*., 2012).

The late embryogenesis abundant (LEA) proteins belong to another class of proteins related to stress conditions, such as desiccation, low temperature, light, and osmotic stress, and have been described has occurring during the late stages of embryogenesis in seeds during desiccation (Yang and Zhang, 2010) and are well-known markers of somatic embryo development (Pais, 2019). Proteins of the LEA family have also been identified in RNA sequencing libraries of tamarillo embryogenic cells (data not published), constituting possible good indicators of embryo quality.

Previous studies reported and reviewed physiological, biochemical and molecular markers associated with SE, including hormone responsive genes and genes related with signal transduction (Yang and Zhang, 2010). The importance of epigenetic mechanisms such as DNA methylation and histones modifications that may lead to chromatin compaction have also emerged as critical factors during SE regulation. Since the repression/expression of genes depends on the organization of chromatin, specifically its compaction (De-la-Peña *et al.*, 2015).

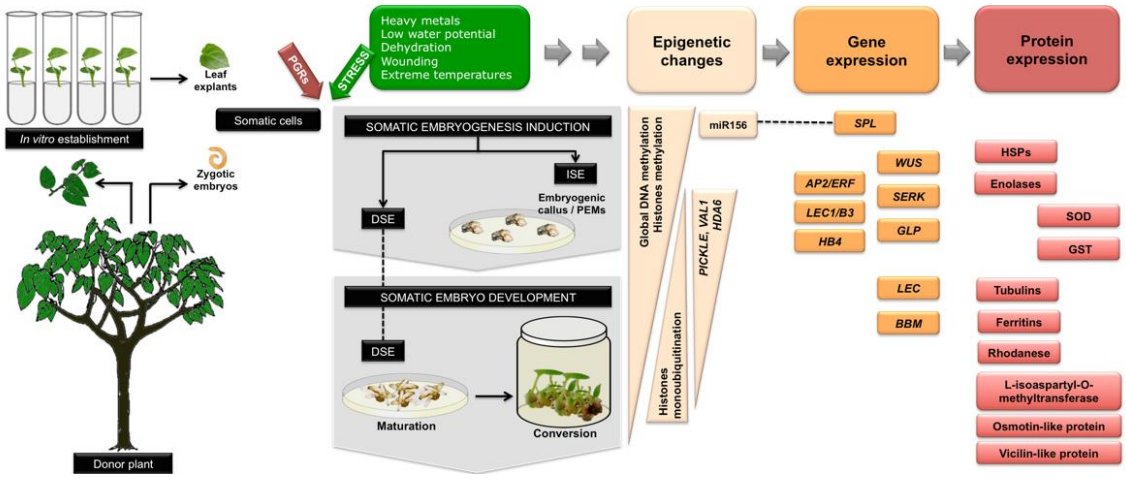

Figure 6 - Molecular biology of somatic embryogenesis in hardwoods (Correia *et al.*, 2016).

Also, in other assays comparing tamarillo's embryogenic and non-embryogenic *calli* protein electrophoretic patterns a 26.5 KDa protein (NEP-TC, GenBank accession: AFI57511.1), was constantly found in non-embryogenic *calli* derived from numerous explants, implying that this protein might be considered a good marker for non-embryogenic *calli* (Ferreira *et al*., 1998). Furthermore, recent studies showed that this protein is a rRNA methyltransferase, apparently with an inhibitor role during tamarillo's SE (Correia *et al*., 2019). The analysis of the epigenetic mechanisms involved in the regulation of SE in tamarillo have not only lead to the identification of this protein involved on a post-transcriptional regulation process, but have also focused on the comparison between global DNA methylation levels between embryogenic and non-embryogenic *calli*, to better elucidate pre-transcription processes that might also be involved (Sanches, 2017). These results revealed a general tendency of the embryogenic cell lines to proliferate more and lose embryogenic competence, associated with accumulation of DNA methylation when subcultured for a long time, and that an initial hypomethylation moment, upon auxin removal, might be essential for triggering somatic embryo conversion.

Although SE has been intensively studied and a lot of assays have been done also for tamarillo regarding SE, less is known about the processes underlying the progression from somatic cells to embryo morphogenesis (Yang and Zhang, 2010). Therefore, increasing attention to the synchronisation of embryogenic cultures making it more appropriate for molecular analysis and for studies of embryo conversion into plantlets is crucial to avoid under- developed embryos to germinate prematurely leading to an increase of plant regeneration levels (Correia and Canhoto, 2012).

4. Objectives

Somatic embryogenesis induction has efficient and well-established protocols for the solanaceous tree, tamarillo. However, the development of somatic embryos and the germination of the emblings that result from this process still require an optimization to aim the increase of high-quality plant production with a superior field performance.

This work is included in a project developed at the Plant Biotechnology Laboratory of CFE (Centre of Functional Ecology) with the main goal of evaluating the performance of somatic embryogenesis-derived tamarillo plants. For that, several assays were conducted by varying and modifying culture conditions to increase the yield and optimize the process of obtaining tamarillo's somatic embryos and emblings from different embryogenic *calli* previously established *in vitro*. Another aim of this project was the morphological and physiological analysis of the obtained emblings after acclimatization. For further enlightenment scanning electron microscopy and molecular analysis during embryo development were also carried out.

II. Material and Methods

1. Plant Material

Tamarillo shoots were established from seeds collected from tamarillo fruits red trees growing at the Botanic Garden of the University of Coimbra.

Primarily, the seeds were sterilised with a 7% (w/v) calcium hypochlorite solution for 15 minutes. Then, the seeds were rinsed tree times with sterile distilled water. For germination, some seeds were placed in Petri dishes with a cotton disk soaked with distilled sterile water and others placed into MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, the pH adjusted to 5.7 and agar was used as solidifying agent at 0.7% (w/v). This medium was sterilized by autoclaving for 20 minutes at the temperature of 121 °C. Both conditions, seeds placed on petri dishes and placed on MS media, were exposed to dark conditions and a 25 °C for 30 days.

After this, the plantlets attained were transferred to new MS media supplemented with 0.2 mg/L BAP (6-benzylaminopurine), for axillary shoot proliferation, and were kept in the growth chamber at 25 °C under a 16h light /8h dark photoperiod. Shoot cultures established from the plantlets were subcultured once a month to fresh media and exposed to the same conditions.

2. Somatic embryogenesis induction from leaf explants of seedling-derived shoots

Leaves from micropropagated shoots were used to induce SE. Somatic embryogenesis induction in leaf segments required TP medium that contained the established MS basal medium (Murashige and Skoog, 1962) supplemented with 9% (w/v) sucrose, 5 mg/L of picloram, Phytagel (Sigma®) as a solidifying agent at 0.25% (w/v) and pH adjustment at 5.7 before sterilization for 20 min. at 121 °C in the autoclave.

Each leaf (young apical leaves) was cut in four segments and each segment was sting, on the abaxial side, with a bisturi and placed in a test tube with 15 mL of TP medium. The cultures were incubated in the dark at 25 °C. After 12 weeks of incubation the embryogenic tissue formed was separated from the non-embryogenic tissue and was transferred to the same medium. The embryogenic *callus* was subcultured each 4-5 weeks in TP medium with the same conditions in which it was induced.

3. Embryogenic *calli* establishment and maintenance

Three lines of embryogenic *calli* (EC1, EC2, EC3) used in this work were previously induced at the Laboratory of Plant Biotechnology and maintained in subculture for different periods of time. The lines EC1 and EC2 were maintained in culture for a year while EC3 was maintained for 5 years. This enabled the study of the relation between the somatic embryos' conversion to plants and factors such as the age in culture of the embryogenic *calli*. These three lines were subcultured in TP medium each 4-5 week and maintained in the dark at 25 °C.

4. Assessment of embryogenic lines and optimization of somatic embryo development

To compare the embryogenic capacity of the three embryogenic lines (EC1, EC2, EC3), 100 mg *callus* of each line were placed into somatic embryo development medium. The development medium consisted of MS medium (Murashige and Skoog, 1962) with a content of sucrose of 4% (w/v), agar at 0.7% (w/v) as a solidifying agent and pH adjustment at 5.7 before sterilization for 20 min at 121 °C in the autoclave. The *calli* were maintained in the development media for 4-5 weeks until the development of somatic embryos was noticeable. The number of somatic embryos obtained per mass of embryogenic tissue was registered.

For the optimization experiment 40 mg of embryogenic *calli* from each embryogenic line (EC1, EC2, EC3) were placed in 20 ml of liquid TP medium for 3 weeks in an incubator shaker, at 100 rpm, in the dark at 24 °C, before being transferred to development media. All EC lines were exposed to three experimental conditions, that included treatments with: 1) 0.05 µM trichostatin A (TSA) for 2 days; 2) 20 µM 5-azacycitide (5-AzaC) for 2 days and 3) a rinse with 0.2 % (w/v) activated charcoal before being filtrated through a vacuum filtration system and then transferred to auxin-free development medium.

The solution concentration of TSA was based on the previous experiments described in literature (Li *et al*., 2014). Since TSA had to be dissolved in ethanol (EtOH), the control for this treatment was made by the solely application of EtOH in the same volume as the TSA solution was. The solution concentration used for the 5-azaC assay was also determined regarding the current literature and its applications in *Theobroma cacao* L. (Quinga *et al*., 2017). Dimethyl sulfoxide (DMSO) was used to dissolve 5-azacytidine. Following the same logic as the previous treatment the control used in this

treatment was solely the application of the same volume of DMSO without 5-AzaC. The treatment with activated charcoal varies from the other two since the *calli* were simply washed with liquid MS media (Murashige and Skoog, 1962) with a concentration of 0.2% (w/v) activated charcoal after filtration. For this treatment the control was simply to not perform the rinse and place the *calli* in somatic embryo development medium right after filtration. After 4-5 weeks the number of developed somatic embryos obtained per mass of embryogenic tissue was registered.

5. Scanning electron microscopy analysis during somatic embryo conversion

For scanning electron microscopy (SEM) analysis embryogenic *calli* from the line EC2 and somatic embryos at different stages of development derived from that same line were collected. Then, the fresh samples were carefully placed in an aluminium stub with a carbon sticker. The observations were performed in a Scanning Electron Microscope FlexSEM 1000 operating at 10.0Kv. Some zygotic embryos germinated in MS medium at 3% of sucrose (w/v) were also examined for comparison.

6. Mass increment measurement

Over the course of the optimization experiment the mass of the three lines of embryogenic *calli* (EC1, EC2, EC3) was measured in three moments in time: an initial measurement occurred to place the embryogenic *calli* in liquid TP medium (t0); a second measurement ensued after the exposure of the *calli* to the treatment and its filtration but before its transference to the development medium (t1); and third mass measurement was performed after the 4 weeks of the *calli* in development medium (t2) as represented in figure 7. The mass increment was then attained by determining the difference between the quantity of the mass measured in each moment. Mass increment = mass (t1) – mass (t0) and then mass (t1) - mass (t2), creating a graph with three points.

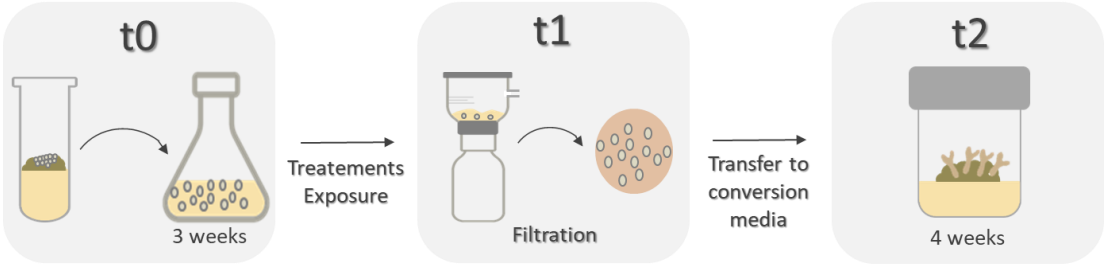

Figure 7- Schematic representation of the moments in which the mass measurements were carried out.

7. Somatic embryo conversion and plant acclimatization

The somatic embryos obtained from all the lines and all treatments were then placed in an auxin-free medium that contained the established MS basal medium with lower content of sucrose (2.5% w/v), agar at 0.7% (w/v) as a solidifying agent and pH adjustment at 5.7 before sterilization for 20 minutes at 121 °C in the autoclave. The somatic embryos were exposed to a 16h light / 8h dark photoperiod for 4 weeks and the number of derived plantlets registered. Afterwards, the conversion rate was calculated by dividing the number of plantlets attained by the number of embryos registered.

$$\text{Conversion rate} = \frac{\text{Number of plantlets}}{\text{Number of embryos}} \times 100$$

The plantlets were then transferred to a mixture of peat and perlite (2:1) in pots and maintained in a climatic growth cabinet chamber under 16h light/8h dark photoperiod and with a 24 °C temperature and 70% relative humidity. Acclimatized plants were transferred to a greenhouse. To calculate the survival rate of the tamarillo plants in the acclimatization stage, the final number of plants was divided by the initial number of plantlets transferred to the mixture of peat and perlite.

$$\text{Survival rate} = \frac{\text{Final Number of plants}}{\text{Number of plantlets transferred}} \times 100$$

8. Molecular analysis during somatic embryo development

A mass of 100 mg of each line of *callus* (EC1, EC2, EC3) was placed in development medium. Throughout the development of embryogenic *calli* to somatic embryos, samples of each line were collected each week, for 5 weeks, until the emblings formation (w1, w2, w3, w4, g1). Samples from each line of *calli* in TP medium were also collected to be used as an initial stage (w0). Mature zygotic embryo' samples were also collected from tamarillo seeds to use as comparison for the stage w4. Embryogenic *calli* from the line EC1 that endured the trichostatin A treatment was also collect as well as a sample of the resulting somatic embryos.

The *calli* samples were grinded into a fine powder in liquid nitrogen, using a mortar and a pestle. Total RNA was extracted with NucleoSpin® RNA plant Kit according to the manufacturer instructions. The concentration of RNA from the samples was

measured with a NanoDrop Value Plus™ Spectrophotometer, at 260 nm. The ratio of absorbance at 260 nm and 280 nm was used to determine the purity of RNA, and only samples with a 260/280 nm ratio of ≈ 2 were used for further analysis.

After asserting RNA quantity and quality, the reverse transcription to cDNA was proceeded with NZY First-Strand cDNA Synthesis Kit from Nzytech® accomplished on a Thermal Cycler (Bio Rad). Primer pairs were designed and chosen for four tamarillo gene sequences (table 1), *LEA14*, *LEA34*, *BABY-BOOM* (*BBM2*), *ENOLASE*, that were previously identified in RNA sequencing libraries of tamarillo embryogenic cells (data not published) and that are referred in literature as related with embryo development (Yang and Zhang, 2010). *ELONGATION FACTOR1-α* (*EF1-α*) (Nicot *et al.*, 2005) was used as reference gene during quantitative real-time PCR (qPCR) analysis. To attest if the primers were efficient, PCR reactions were carried out with different master mixes for each primer with a final volume of 25 µL. Including 12.5 µL of NZYTaq® II 2× Green Master Mix, 9.5 µL of nuclease-free water, 2 µL of the primers (1 µL forward, 1 µL reverse) and 1 µL of cDNA template from each of the *calli* samples.

Table 1 - Primer pairs information designed for four tamarillo transcripts *LEA14, LEA34, BBM2, ENOLASE* and primer pair information for the reference gene *EF1-α*; F stands forward and R stands for reverse

LEA14	F	CGCCACTATGAGAAAGCGGA
	R	AGTTGCCGTCAAGAACCCTT
LEA34	F	TTGAGGCAGGACCAACCTTTT
	R	GCAGCCCCTAAAATACGGAGA
BBM2	F	GCAGTGGTTTCTCTCGTGGT
	R	TCTCAAAGTTGGTCACGGCA
ENOLASE	F	TCCGGAAAGGTGGTCATTGG
	R	CTGCAATAGCCTTGGCAACC
EF1-α	F	ACAAGCGTGTCATCGAGAGG
	R	TGTGTCCAGGGGCATCAATC

The cDNA amplification was performed using the following cycle parameters: initial denaturation (3 min at 95 °C), followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 60 °C and an extension period for 25 s at 72 °C. At the end of the cycles, occurred a final extension for 5 min. at 75 °C. The PCR reaction products were then separated by agarose gel electrophoresis. In each well 10 µL of sample the PCR product were loaded. The gel at 2% (w/v) agarose with 1x TBE buffer was stained with Midori Green Safe (3 µL/100 mL of gel). After the run, the gel was observed and

documented through Gel Doc XR+ with Lab™ Software (Bio Rad). As a standard, a DNA size ladder was also loaded in the gel along with the PCR products.

After attesting the primers efficiency, a qPCR was performed to quantify each gene expression levels. Amplifications reactions were carried out with 1 µL of the previously synthesise cDNA for all the samples, in a final reaction volume of 20 µL, containing NZY qPCR Green Master Mix (2x) ®, 1.6µL of the primers (0.8 µL forward, 0.8 µL reverse) and 7.4 µL of nuclease-free water. Once again using *EF1-α* as reference. The annealing was performed at 95 °C for 10 min followed by 40 cycles of denaturation for 15 s each and finally an extension period of 45 s at 60 °C. The amplification was performed in a BioRad CFX96 Touch™ Real Time- PCR Detection System. This experimental procedure is schematized in figure 8.

The method used to analyse the qPCR data was the relative quantification method, or $2^{-\Delta\Delta CT}$ method, where the ΔΔCT value = [(CT1Target – CT1Reference) – (CT0Target – CT0Reference)] (Livak and Schmittgen, 2001). The mean CT values for both the target and internal reference genes were determined and the fold change in the target gene normalized to *EF1-α* and relative to the expression in the control sample.

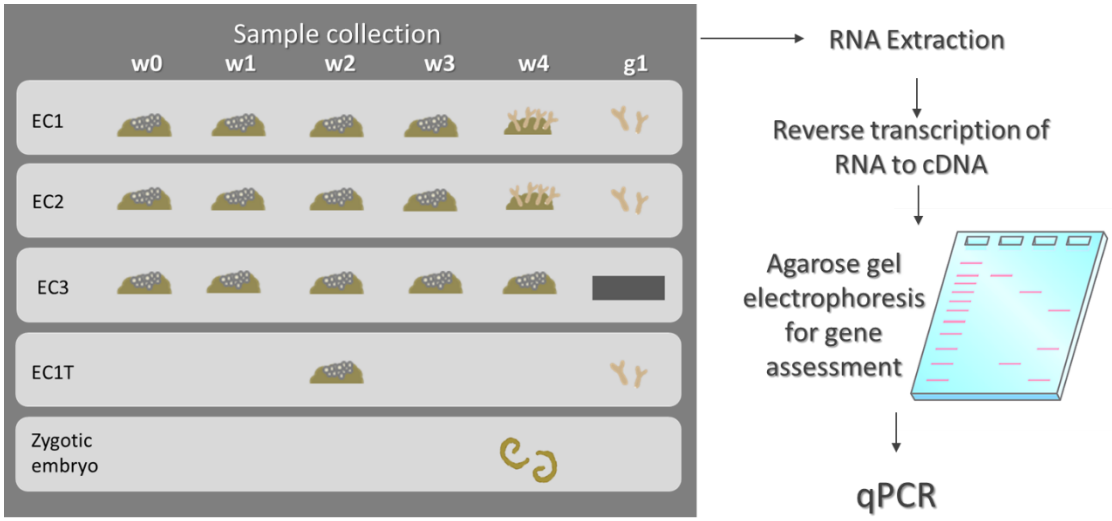

Figure 8- Schematic representation of the steps followed to perform the molecular analysis during somatic embryo development: 1) Sample collection from the lines EC1, EC2 and EC3 and from the EC1 with treatment TSA treatment (EC1T) during the somatic embryo development stage and zygotic embryos; 2) RNA extraction; 3) Reverse transcription of RNA to cDNA; 4) Agarose gel electrophoresis for gene assessment and 5) qPCR.

9. Analysis of physiological parameters

From the acclimatized emblings, after approximately 9 weeks at an exterior greenhouse conditions, 5 per each group/treatment were selected to be analysed. The five groups/treatments were: 1) G - seed-derived plants; 2) EC1 - plants derived from line EC1, without any type of treatment; 3) EC2 - plants derived from the line EC2, without any type of treatment; 4) EC1E - plants derived from the line EC1 with the control treatment of trichostatin A (ethanol); 5) EC1T - plants derived from the line EC1 with treatment of trichostatin A.

Leaf gas exchange measurements were performed *in situ*. Net CO_2 Assimilation rate (A, µmol m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and intercellular CO_2 concentration content (Ci, ppm) were measured using a portable infrared gas analyser (LCpro-SD, ADC BioScientific Ltd., UK) equipped with the broad leaf chamber. The equipment was operating under the following conditions: irradiance of 350 µmol m^{-2} s^{-1}, air flow of 200 mol s^{-1}; block temperature at 25 ºC, and atmospheric CO_2 and H_2O concentrations. When the measurement parameters stabilized the data was recorded (Fig. 9A).

Chlorophyll a fluorescence was also determined *in situ* on the same leaves as used for the gas-exchange measurements (Fig. 9B) with a portable fluorometer (Mini-PAM; Walz, Effeltrich, Germany) (Fig. 9C). Light adapted components of chlorophyll fluorescence were measured as described in Jesus *et al*. (2015): steady-state fluorescence (F), maximal fluorescence (F'm), variable fluorescence (F'v, equivalent to F'm-F) and quantum yield of PSII photochemistry [ϕPSII, equivalent to (F'm-F)/F'm)]. Leaves were then dark adapted for at least 20 min to obtain F0 (minimum fluorescence), Fm (maximum fluorescence), Fv (variable fluorescence, equivalent to Fm-F0) and Fv/Fm (maximum quantum yield of PSII photochemistry).

Water potencial (Ψ_w) was determined with a Scholander-type pressure chamber (PMS instrument Co., Albany OR. USA) (Fig. 9E-G). The height of the plants was also recorded by measuring the plant shoots with the help of a digital caliper. Biomass was further assessed after separating the shoot part (Fig. 9D) and root part (Fig. 9H) of the plants and placing them for a period of 8 days in a heat chamber at 40 °C (Fig. 9J).

Figure 9 - Plant performance analysis. (A) - Gas exchange parameters assessment; (B and C) – Photosynthetic parameters assessment and portable fluorometer (Mini-PAM; Walz, Effeltrich, Germany), respectively. (D) – Measurement of the plant height; (E, F and G) – Water potential measurement with a Scholander-type pressure chamber (PMS instrument Co., Albany OR. USA); (H) – Plant root after washing; (J) – Dry plant shoot and root for biomass assessment.

All the experimental procedures described in the material and methods are represented in the following figure (Fig. 10).

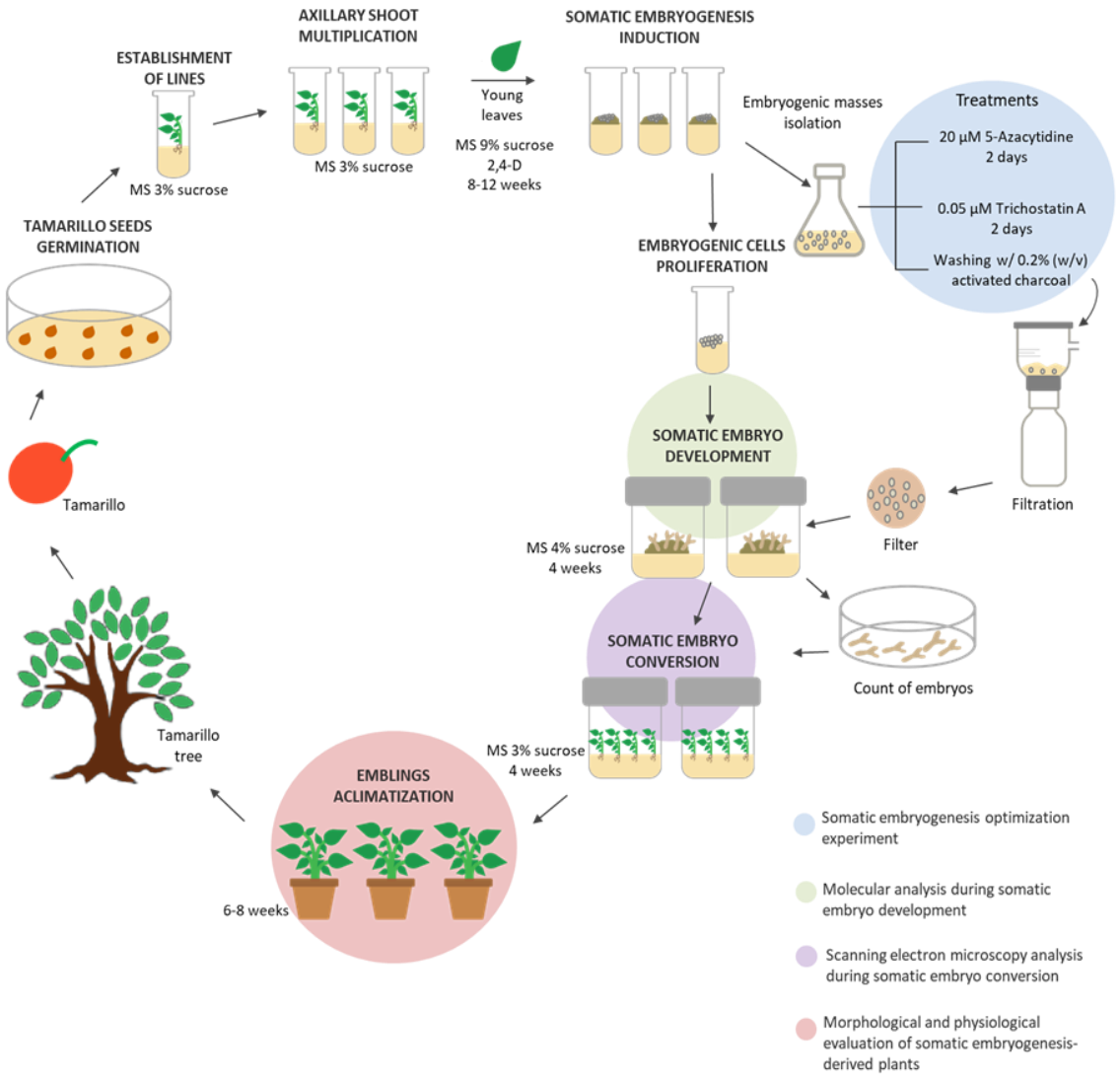

Figure 10- Schematic representation of somatic embryogenesis induction with the treatments and analysis carried out in this experiment.

10. Statistical analysis

The somatic embryogenesis optimization treatments from each *calli* line were made with at least 3 replicates, those values were given means. The data resulting was analysed by a two-way ANOVA, followed by a Tukey´s multiple comparison test to identify significant differences between means ($P < 0.05$).

The data resulting from the morphological and physiological evaluation of the plants were analysed by a one-way ANOVA comparing the means of each group, followed by a Tukey´s multiple comparison test ($P < 0.05$). The statistical analysis was performed through GraphPad Prism for Windows v. 6.01.

III. Results

1. Assessment of the embryogenic capacity

After the *calli* from the three embryogenic lines were maintained for 4 weeks in development medium their embryogenic capacity was evaluated by comparing the number of somatic embryos per 100 mg of *calli*. In figure 11A can be observed that the line EC2 has a higher capacity of developing somatic embryos and it is noticeable the lack of competency of the embryogenic line EC3 (long term established). It was also observed that the development was not synchronized and the embryos resulting from the embryogenic lines were elongated and did not present any sort of cotyledonary shape. Abnormal phenotypes were often observed and some of them formed tight clusters (Fig. 11B).

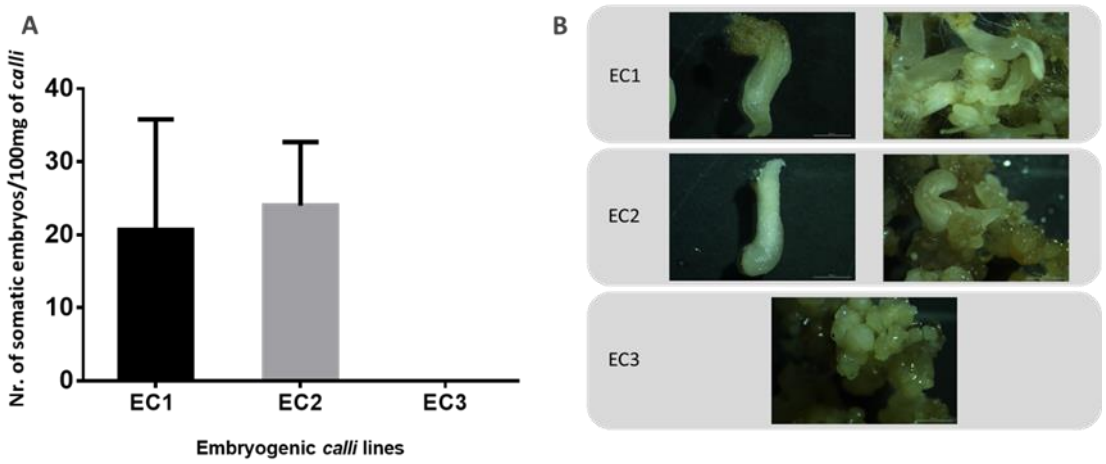

Figure 11 - Assessment of embryogenic lines and morphological aspects of embryo development (A)- Number of somatic embryos per 100 mg of *calli* of the three *calli* lines EC1, EC2, EC3. Means ± SDs, n=3, no significant differences between the number of embryos resulting from EC1 and EC2 were found, according to the Tukey's test ($p<0.05$). (B) - Embryos resulting from each line after 4 weeks development medium.

The development of the somatic embryos takes about 4 weeks (Fig.12A). Following somatic embryo counting (Fig. 12B) from the lines EC1 and EC2 they were placed in the conversion media for 4 weeks and exposure to a 16h light /8h dark photoperiod (Fig.12C). The conversion rate was calculated and revealed a higher percentage for the line EC1 of 48.15% than the EC2 (25.93%).

During the conversion process (Fig. 12D-E) it was possible to observe shoot apical meristem (SAM) development through a sheath-like structure and the greenish colour that the shoots gained after being placed under germination conditions. Often the shoots did not develop any kind of foliage and remained in that stage, then turned into a brown colour and died without converting properly. Root development was also observed with several adventitious roots arising at the base of the shoots, without developing a

main one. Frequently, the roots showed a faster development then SAM, therefore the processes were not simultaneous.

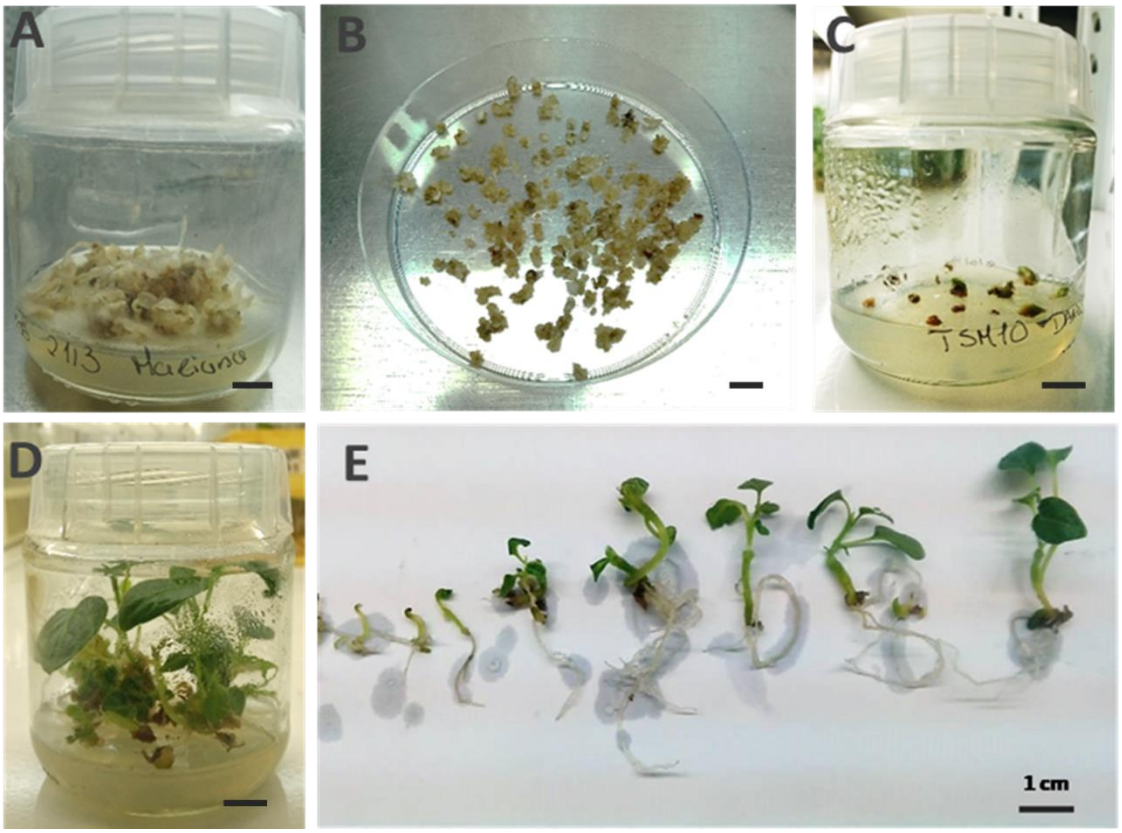

Figure 12 – Somatic embryo development and conversion into plantlets. (A) - *Calli* after 4 weeks in development media already with formed somatic embryos (flask size is 6 cm x 7 cm) ; (B) - Embryos in a Petri dish 100 mmx 20 mm for counting; (C) - Somatic embryos in the conversion medium; (D) - Plantlets resulting from somatic embryos after 4 weeks in the conversion medium; (E) – Somatic embryogenesis- derived plantlets in different stages of development.

The tamarillo emblings that already presented roots were then separated and the roots were washed to remove the medium leftover and avoid contamination by microorganisms (Fig.12E). Plantlets did not show a similar development, meaning that some were more evolved then others and some showed irregular phenotypes, such as, twisted stems, stems with bulky appearances, that seemed to be the result of the embryos' clusters, shoots with reduced growth and with thick small leaves when compared (Fig. 13A) with plantlets obtained from seeds (Fig. 13B).

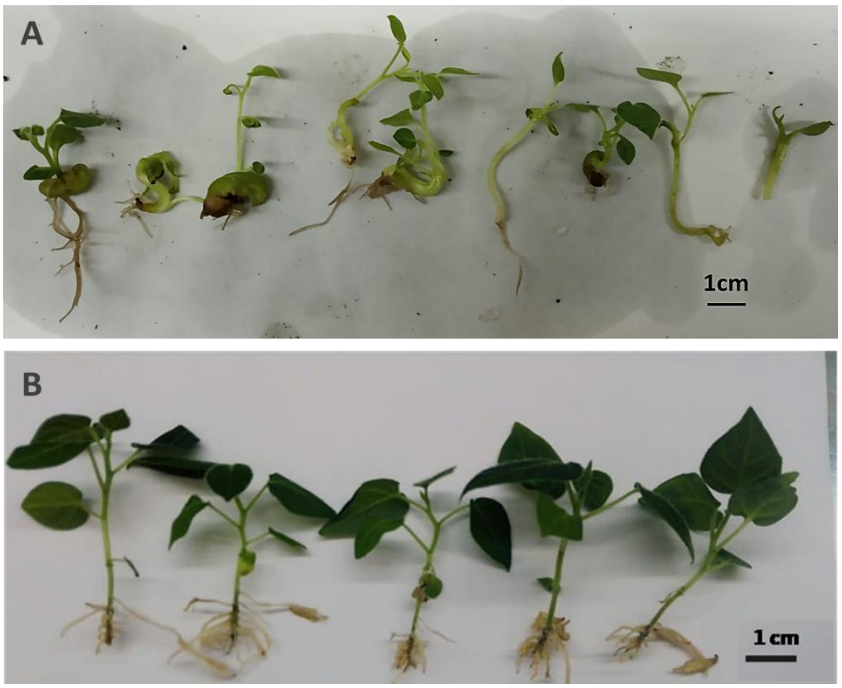

Figure 13 – Somatic Embryogenesis derived emblings (A) and seedlings derived from seeds (B).

Finally, they were transferred to a mixture of peat and perlite (Fig. 14A). Following further growth plants were transferred to bigger 1L pots (Fig. 14B). After the acclimatization phase of the plants they were placed in a greenhouse (Fig. 13C) The data, obtained after about 12 weeks, showed a survival rate of 81.25% for SE derived plants and 100% for seed derived plants.

Figure 14 – Plant acclimatization. (A) – Emblings (right) and seedlings (left) in a mixture of peat and perlite; (B) – Developed plants in 1L pots; (C) - Plants in the greenhouse.

2. Scanning electron microscopy analysis during somatic embryo conversion

SEM analysis revealed that the transfer of *calli* from TP medium (auxin containing medium) to development medium induced notorious changes in the aspect of the *calli* due to differentiation of the pre-embryogenic masses (Fig. 15A,E and F) into somatic embryos (Fig. 15B-D and G-J). A particular feature of the somatic embryos is that they do not show a typical cotyledonary stage, rather formimg a cup shaped structure at the shoot zone (Fig. 15G-J) that seems to wrap the shoot apical meristem developing inside.

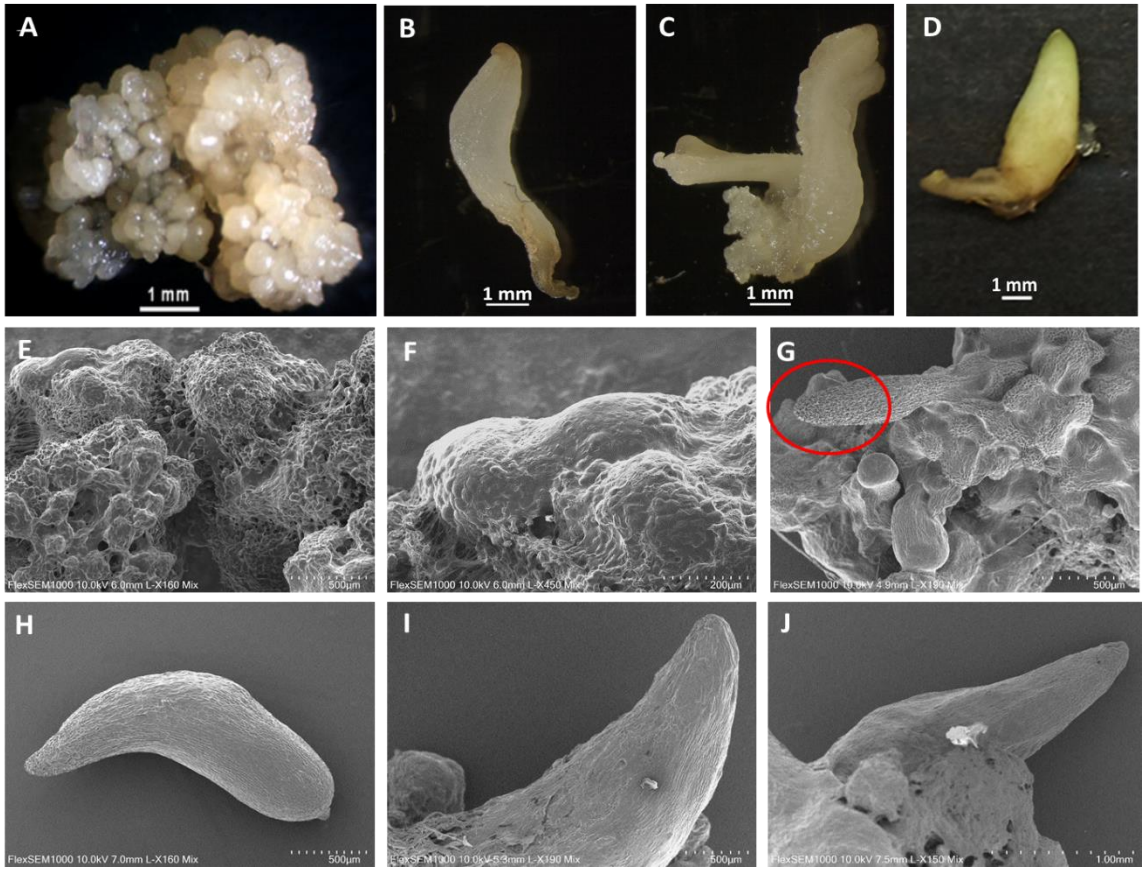

Figure 15 – Development of somatic embryos from pre-embryogenic masses (A)- Embryogenic *calli*; (B and C) – Fully developed somatic embryos; (D) – Somatic embryo in an early stage of conversion already in conversion medium; (E)– SEM image of embryogenic calli on TP medium; (F)- SEM image of embryogenic calli on development media; (G) – SEM image of embryogenic calli with differentiated somatic embryos (some cup-shaped); (H) – SEM image of fully developed somatic embryo; (I and J)- SEM image of somatic embryos in early stages of conversion.

In the photos is apparent the green colour and the growing of the emblings and a developed root (Fig. 16 A, B). One of the images shows an embling that did not convert into a plantlet (Fig.16 C), one of the most recurrent issues with SE. Generally, these emblings reach this swollen form then stop evolving. Other shows the plantlets derived from SE (Fig. 16D) that were removed from the conversion medium at the same time

revealing the completely different levels of development of plantlets despite sharing the same process up until this point.

The SEM observations of the initial conversion of the emblings into plantlets, showed the presence of trichomes in the leaves of developing shoots (Fig. 16E-H). Even though the emblings showed an abnormal foliage development in contrast with the seedlings' development (Fig. 16G), trichome were also observed in seedlings resulting from the germination of zygotic embryos as seen in figure 17, showing a similar development between the emblings and seedlings.

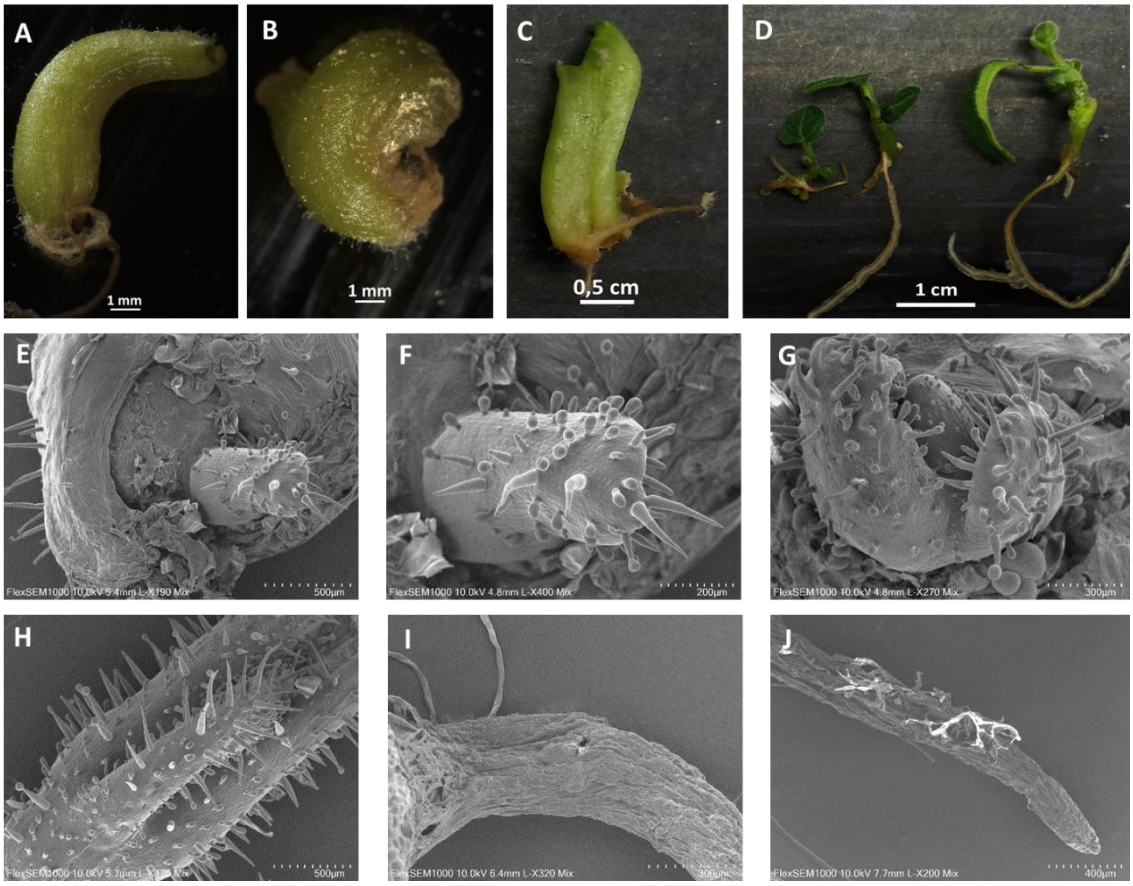

Figure 16 - Development of somatic embryos into plantlets. (A and B) – Somatic embryos in conversion into plantlets; (C)- Somatic embryo that did not convert into a plantlet; (D) – Three tamarillo's plantlets. (E and F) – SEM image of an initial stages of an embling's shoot; (G) – SEM images of an embling's shoot already with primordial leaves; (H) – SEM image of an embling's s primal stalk; (I and J) – SEM image of an embling's initial and ending segments of the root, respectively.

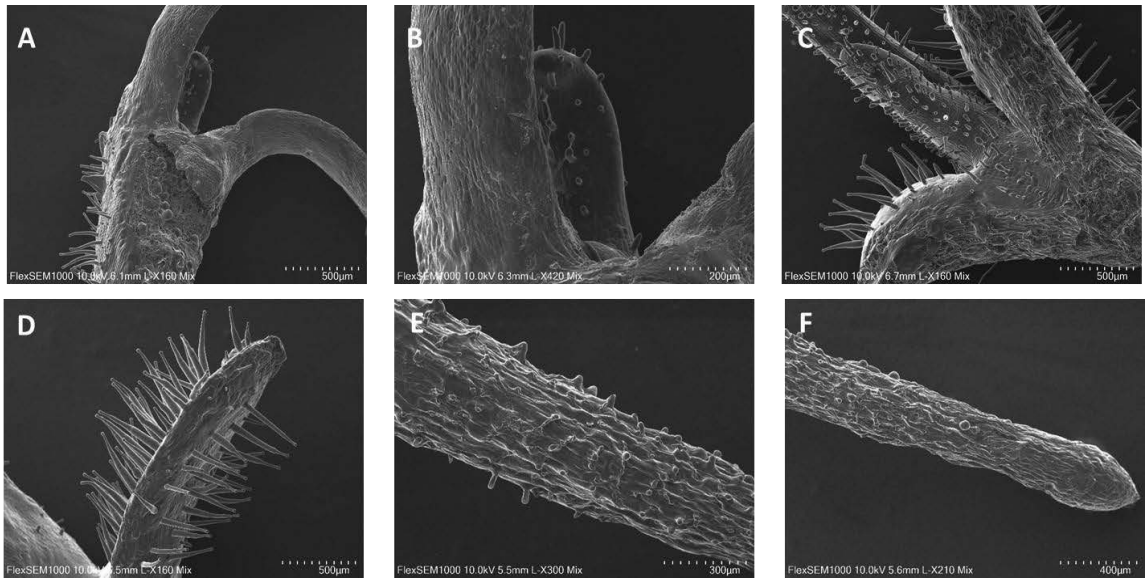

Figure 17 - SEM observations of seedlings resulting from zygotic embryos germination. (A, B and C) – Early stages of a seedling shoot already with leaf primordia; (D) – Young leaf; (E) – Stem of a seedling; (F) – Initial root of a seedling root.

3.1. Mass increment results

The mass of each line was measured at three points: t0 – at the time the *calli* was placed in TP liquid medium; t1- after 3 weeks in liquid medium and t2- after 4 weeks of the *calli* in development medium at a point in which somatic embryos were already differentiated from the pre-embryogenic masses.

The results obtained are given in figure 18. The line EC1 showed little mass increment from t0 to t1. Only on t2 noticeable differences between treatments could be observed, leading a mass increment, and showing that the treatment with 5-AzaC led to a reduced (0.0633 g) growth in a pronounced contrast with the control (DMSO) in which mass increment reached 1.177 g. The treatments with TSA and AC did not show any relevant differences between them (Fig. 18A).

Similarly to the line EC1, the line EC2 showed poor mass increment from t0 to t1 and the differences between treatments were only perceptible in t2. The treatment with 5-AzaC led to a reduced mass increment (0.89 g), but the control (DMSO) led to the lowest increment of 0.77 g, unlike the line EC1. The treatments with TSA and AC remained without any relevant differences between them. The control showed the highest value in t2 (1.77 g) (Fig. 18B).

The line EC3 showed the highest mass increment in all periods of time compared to the other two lines. The period from t0 to t1 showed little mass increment but still higher in comparison with the other lines. Some differences were noticeable after the

calli placement in development media. The TSA treatment showed the lower increment value and AC treatment the highest, but still not relevant when compared to the other *calli* lines (Fig. 18C).

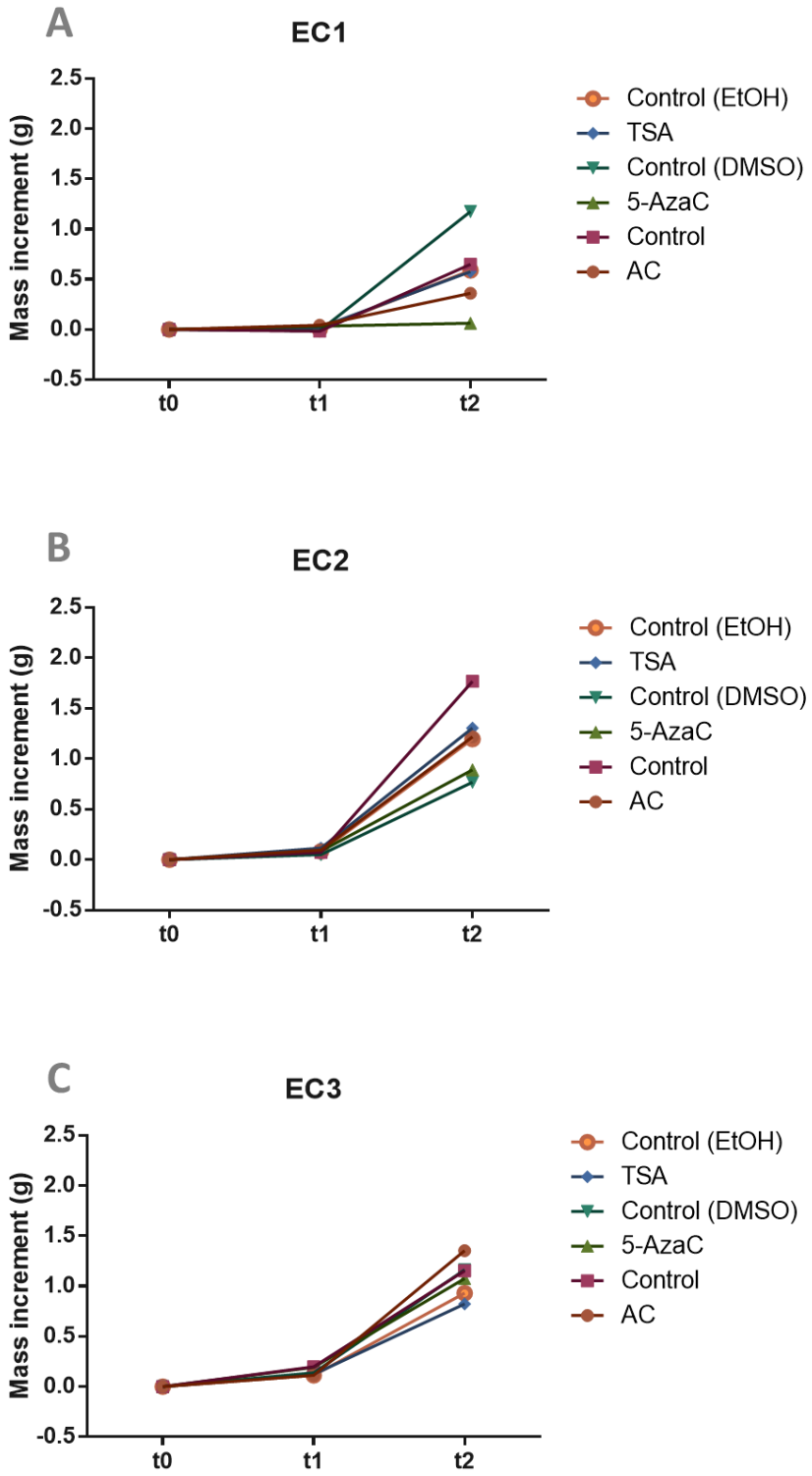

Figure 18 - Mass increment registered over the development optimization experiment. (A) - Line EC1 mass increment; (B) - Line EC2 mass incremet; (C) - Line EC3 mass increment. With t0, t1 and t2 standing for the points in time of mesurement of the mass, *n*=3.

3.2. Optimization of somatic embryo development

To optimize embryo development and ultimately their efficient conversion into viable plants, several assays were conducted, with the use of TSA, 5-AzaC and activated charcoal, and the efficiency of each treatment was evaluated by the count of embryos per 100mg of *calli* of each line and the capacity of conversion of the resulting embryos into plantlets.

TSA treatment led to a higher number of somatic embryos for the line EC2, followed by the line EC1 whereas the line EC3 only presented a low number of embryos in the control (Fig. 19A.1). The morphology of the somatic embryos was very similar to what was described in the section of the assessment of the embryogenic capacity. From the control (EtOH) treatment the somatic embryos formed were very translucent in the lines EC1 and EC2 showing a big contrast with the ones resulting from the TSA which displayed an opaque whitish colour (Fig. 19A.2).

5-AzaC treatment resulted in a higher number of somatic embryos for the line EC1, ensued by the line EC2. No somatic embryos could be obtained from the line EC3 (Fig. 19B.1). The control (DMSO) led to a formation of oxidized *calli* with a dark colour for EC1 and EC2. Also, the embryos presented the phenotypes like those analysed in section of the assessment of the embryogenic capacity. The 5-AzaC treatment led to the formation of embryos with different aspects, some very translucent and with irregular shapes and some opaque but very small (Fig. 19B.2).

The rinse with AC suspension led to higher number of somatic embryos per 100mg of *calli* in the line EC2, followed by the line EC1 and a very reduced number of embryos for EC3 (Fig. 19C.1). The somatic embryos resulting showed the same aspects as the somatic embryos described in the topic of assessment of embryogenic capacity, no cotiledonary shape and abnormal phenotypes. Clusters of embryos were also observed. Still, the treatment with activated charcoal showed a little difference in comparison to the control. In the resulting embryos from the control was possible to see little root hairs-like structures, that in the treatment with AC were not present (Fig. 19C.2).

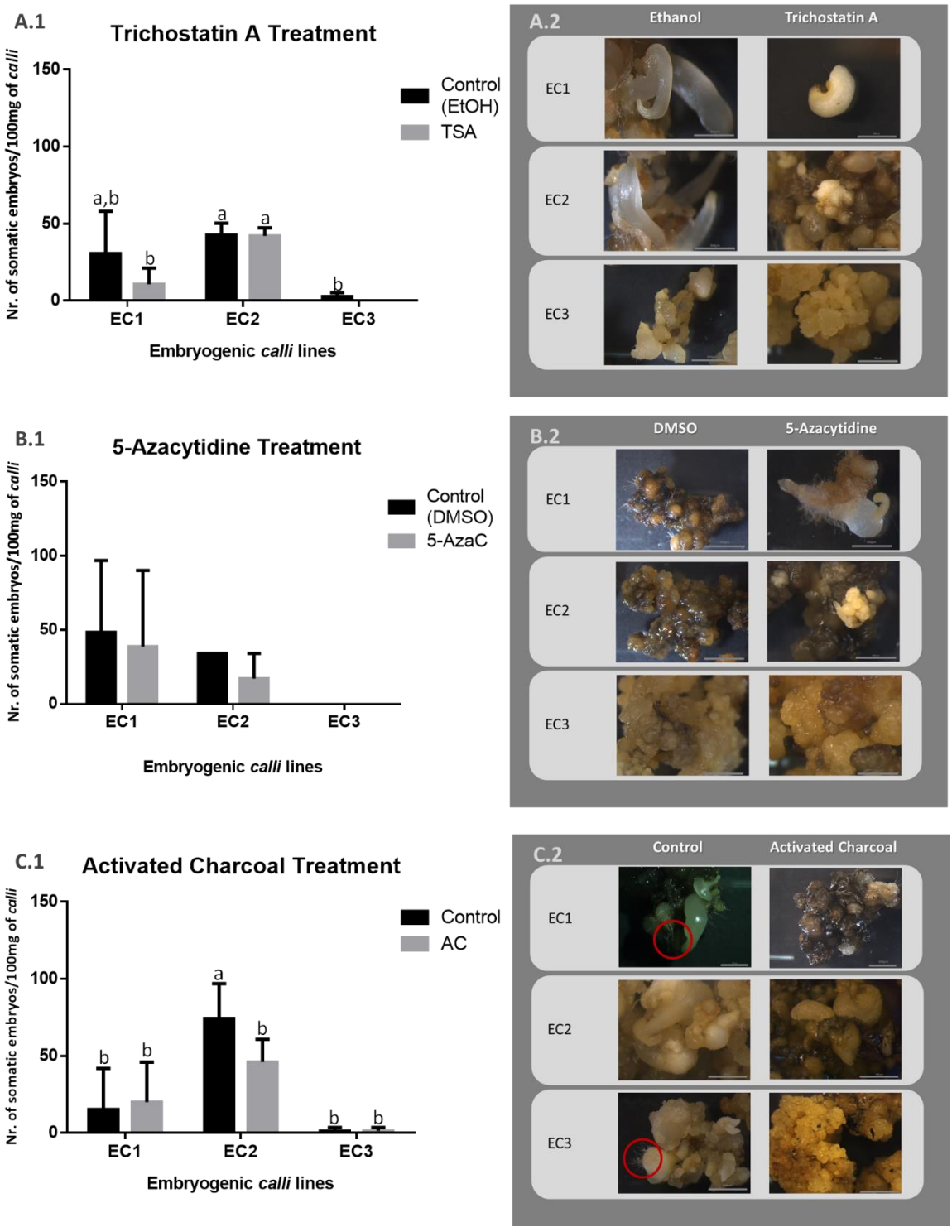

Figure 19 - Somatic embryo development optimization experiment results. Nr. of somatic embryos per 100mg of *calli* resulting from the lines EC1, EC2, EC3 with: (A.1) - TSA treatment; (B.1) – 5 -AzaC treatment; (C.1) - AC treatment; Aspect of the somatic embryos from the lines EC1, EC2, EC3 with: (A.2) - TSA treatment; (B.2) – 5- AzaC treatment; (C.2) – AC treatment (the red circles point the root hairs- like structures). The grey bar corresponds to 500µM. Means ± SDs, n=3, different letters indicate significant differences between treatments, according to the Tukey's test ($p<0.05$).

3.3. Conversion and plant survival

Overall the embryo conversion rates were low for all the treatments and respective controls (Table 2). The conversion rate revealed that the low number of embryos resulting from line EC3 with TSA and AC treatments did not have capacity to convert into plantlets. In general, the treatments led to a higher conversion rate for the line EC1, being that the TSA treatment showed the highest percentage (after its control EtOH and the 5-AzaC treatment control with DMSO). The line EC2 showed a higher percentage for the 5-AzaC treatment (after the control for the AC treatment).

The survival rate of the plantlets resulting from the embryos revealed that all the plantlets resulting from the TSA treatment survived the acclimatization stage in both lines EC1 and EC2. For the line EC1 none of the plantlets from the 5-AzaC treatment control (DMSO) and from the AC treatment and its control survived, but the 5-AzaC treatment showed a high percentage of 83.33 % followed by the EtOH control of 60%. The line EC2 revealed 0% survival for almost all treatments, except for TSA treatment as stated before and the control for the AC treatment as shown in table 2.

Table 2 – Conversion rate of somatic embryos into plantlets resulting from the lines EC1, EC2, EC3 under TSA, 5-AzaC and AC treatments.

Treatment	Embryo conversion rate (%)			Plantlets Survival rate (%)	
	EC1	EC2	EC3	EC1	EC2
Control (EtOH)	44.26	2.35	0	60.00	0
TSA	10.00	1.59	0	100.00	100.00
Control (DMSO)	15.46	0	-	0	0
5-AzaC	5.17	3.92	-	83.33	0
Control	0	8.52	0	0	52.63
AC	8.33	0	0	0	0

4. Molecular analysis during somatic embryo development

The gene expression *of LEA14* (Fig. 19A)*, LEA34* (Fig. 19B), *BBM2* (fig. 20A) *and ENOLASE* (Fig. 20B) was analysed through qPCR in all three embryogenic lines EC1, EC2 and EC3 during the somatic embryo development stage throughout 5 weeks: w1, w2, w3, w4 and the last week - g1, that concerns the early stage of conversion. In concern to g1 it is important to mention that this stage was only analysed in the EC1 and EC2 lines since the EC3 line did not produce somatic embryos.

Despite the fact that it was not possible to observe a general tendency for gene expression throughout the analysed stages, for *LEA14, LEA34, BBM2* genes analysed and particularly for the most competent line (EC1), there was an increase in gene expression when the masses were transferred from TP medium (w0) to development medium without auxins, immediately after the first week in culture (w1). Then, those expression levels decreased, after week 2 (w2) or week 3 (w3), until reaching the lowest values at the end of the development stage (week 4, w4), and staying at those same levels when the embryos started to convert into plantlets (g1).

The analysis of *LEA 14* (Fig. 20A) showed a strong increase from w0 to w1 and a decrease of expression from w1 to w3 then a small increase in w4 for the lines EC2 and EC3. EC1 line only differs from the previous in the fact that from w0 to w1 the expression decreases.

LEA 34 (Fig. 20B) showed the lowest levels of expression of all the genes and for all the lines. EC1 suffered a small decrease from w0 to w2 followed by an increase in w3 and right after a decrease in w4 and revealing its highest expression value in g1. EC2 suffered a high increase from w0 to w1 then a decrease in w2 and the same discrepancies as EC1 but with much higher levels for w3.

For *BBM2* (Fig. 21A) expression, the values were also low, but with much higher levels for the EC2 and particularly in the w1 and w2 stages.

ENOLASE (Fig. 21B) was the gene with the highest expression levels. Overall the same happened for all the lines, with an expression increase from w0 to w1, but with the values remaining high also in w4. The g1 stage revealed lower expression for *ENOLASE* compared with w4. With a big difference for the EC2 line.

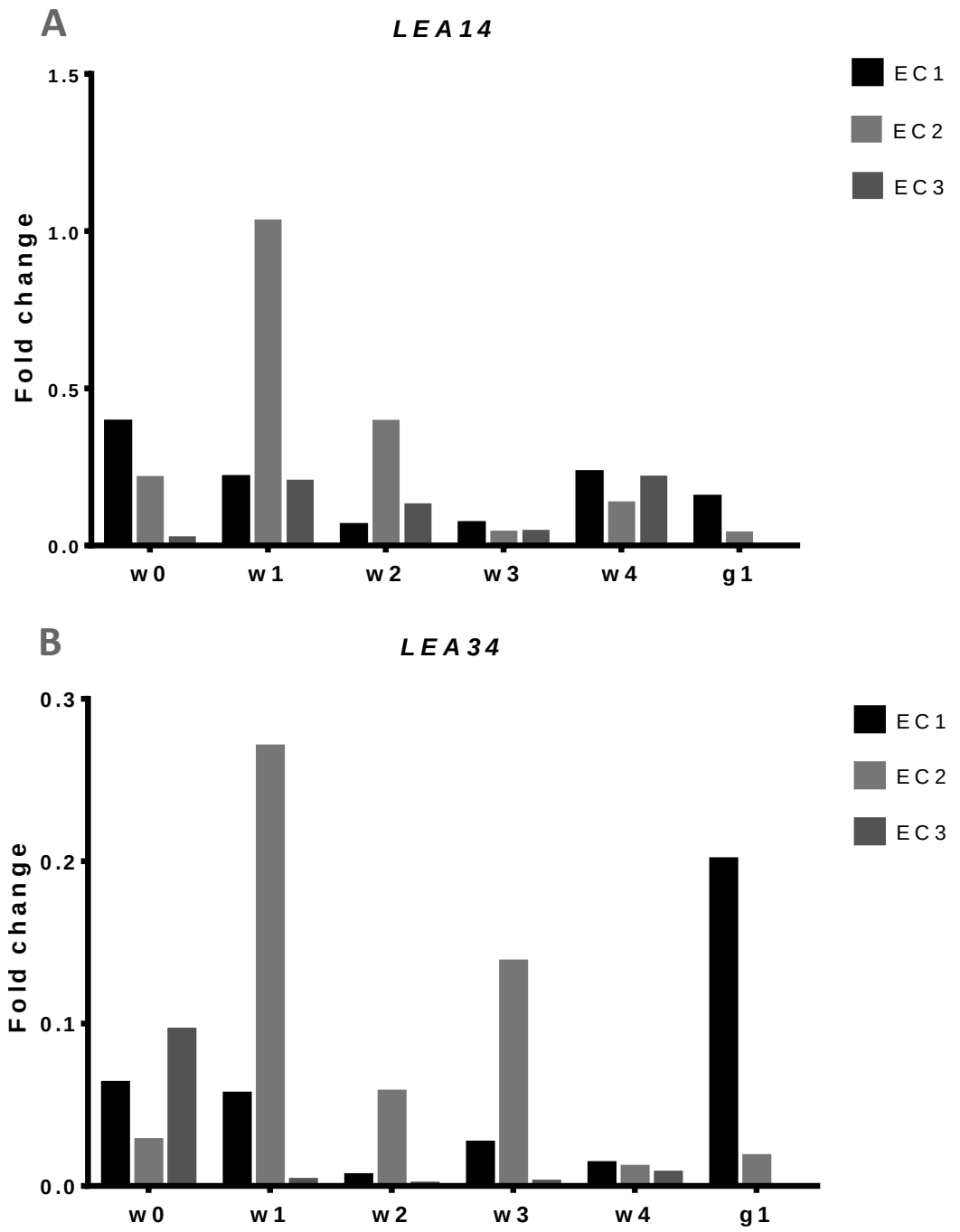

Figure 20 - *LEA14* (A) and *LEA34* (B) expression trough the somatic embryo development stage in the lines EC1, EC2 and EC3.

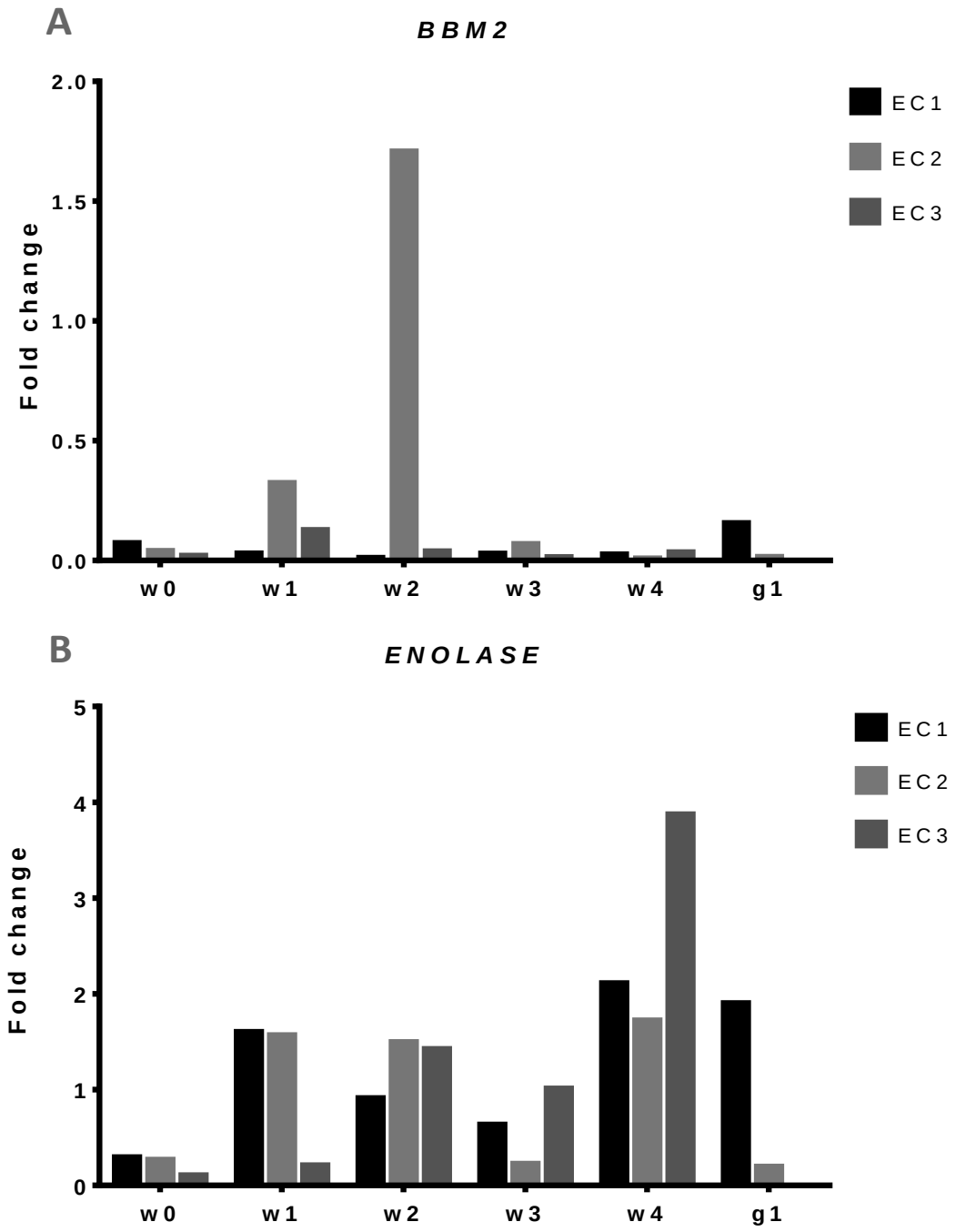

Figure 21 – *BBM2* (A) and *ENOLASE* (B) expression trough the somatic embryo development stage in the lines EC1, EC2 and EC3.

The levels of expression of the genes were compared between the zygotic embryo and week 4 of somatic embryo development. The zygotic embryo presented the lowest levels of *LEA14* and *ENOLASE* (Fig. 22A and D, respectively) when compared with other lines. The line EC1 presented the highest level of *LEA14* in w4 of embryo development and EC3 the highest for *ENOLASE*. For *LEA34* (Fig. 22B) the zygotic embryo revealed the highest level of expression followed by the line EC1, the line EC2 and the line EC3. Establishing a pattern between the lines, since the line EC1 had a higher percentage of conversion of embryos into plantlets followed by the line EC2 that had an inferior rate and finally the line EC3 that could not even produce somatic embryos anymore. *BBM2* showed higher levels for w4 EC3, similar levels for ZE and w4 EC1 and the lowest expression for EC2 line (Fig. 22C).

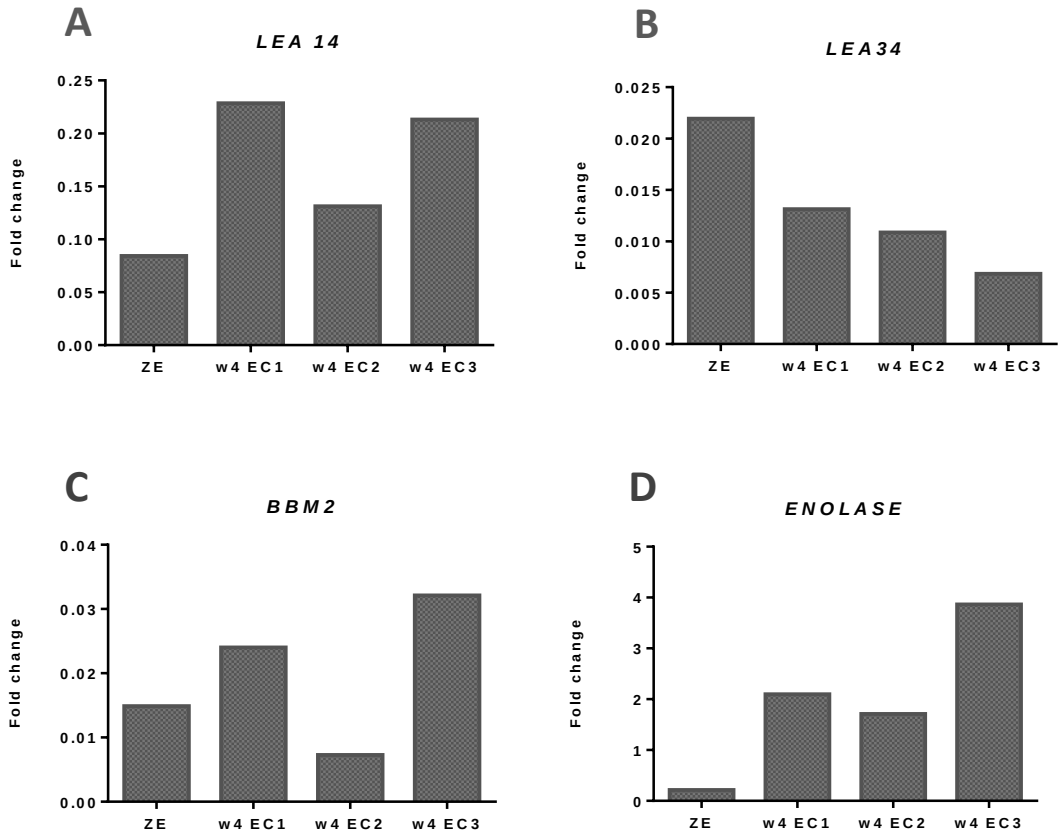

Figure 22 – Comparison of the *LEA14* (A), *LEA34* (B), *BBM2* (C) and *ENOLASE* (D) expression between the zygotic embryo (ZE) and week 4 of somatic embryo development of the lines EC1, EC2, EC3.

Since TSA treatment revealed the best conversion and survival rates among treatments for the line EC1, the levels of expression of the genes were determined for EC1 treated with TSA after two weeks of somatic embryo development - w2, and at the first week of conversion stage - g1. As control, the same line without treatment was used. The expression of *LEA14* was higher in w2 with TSA and in g1 without TSA (Fig. 23A). The results obtained with *LEA34* (Fig. 23B) were very similar to what happened for *LEA14*, the expression was higher in w2 with TSA and in g1 without the treatment. *BBM2* (Fig. 23C) had a similar propensity as the previous gene, with the highest levels of expression in the w2 with the TSA treatment and the g1 without any type of treatment. For *ENOLASE* was noticeable that TSA treatment had an impact and led to lower values of expression in w2 and g1 (Fig. 23D).

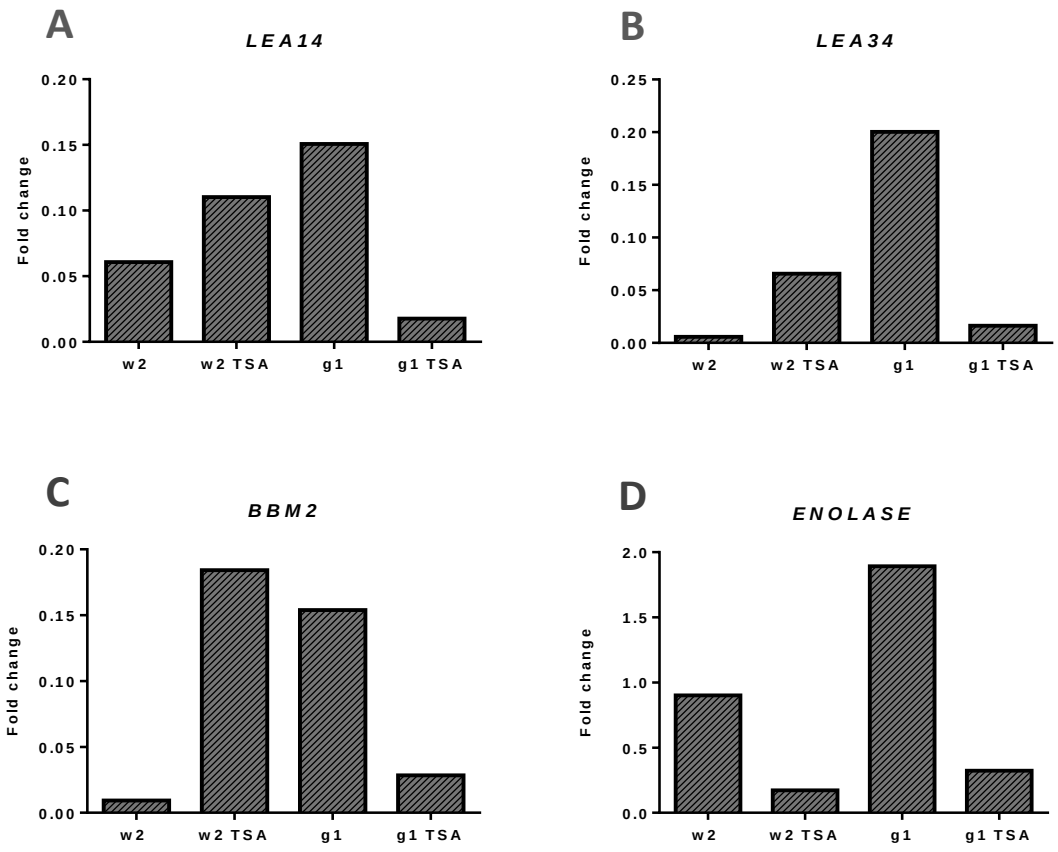

Figure 23 – Comparison of the *LEA14* (A), *LEA34* (B), *BBM2* (C) and *ENOLASE* (D) expression between the week 2 of somatic embryo development and germination stage in line EC1 without treatments and with TSA treatment.

5. Analysis of physiological parameters

Several parameters were analysed to evaluate the physiological state of the plants obtained through somatic embryogenesis. Figure 24 resumes the data obtained for gas exchange parameters and photosynthetic parameters whereas figure 25 shows the data obtained when water potential and growth parameters were assessed.

Regarding the gas exchange parameters intercellular CO_2 concentration, transpiration rate, stomatal conductance and CO_2 assimilation (Fig. 24A-D), the results indicate that the groups G (seed-derived plants) and EC1 (SE derived plant from the line EC1) had a higher intercellular CO_2 concentration and for the remaining parameters the EC1E (SE derived from the line EC1 with the control EtOH from the TSA) and the EC1T (SE derived from the line EC1 with the TSA treatment) showed also higher values.

Concerning the photosynthetic parameters, ϕPSII (quantum yield of PSII) and the Fv/Fm (maximum yield of PSII photochemistry) (Fig. 24E,F) ratios, there were no statistically significant differences between the different groups. Although not statistically significant, there were higher values of ϕPSII for the group EC1T followed by the EC1E group; and for Fv/Fm the group EC2 (SE derived plants from the line EC2) followed by the G group displayed superior maximum quantum yield of PSII photochemistry. Water potential (Ψ_w) is a valuable variable to assess the physiological water status of plants. The water potential (Fig. 25A) showed no statistically significant differences, yet the groups EC1, EC1E and EC1T presented lower values.

For the growth parameters, plant height, dry shoot and root biomass (Fig. 25B-D) means were calculated and analysed. The seed- derived plants (G) were higher and presented more shoot and root biomass than the SE derived plants (EC1, EC2, EC1E, EC1T). The differences were particularly clear for the biomass parameter, where plants from the group G showed higher values to both shoot and root biomass followed by the group EC1, still with a relevant distance, and then the group EC1T. These results are also perceptible in the images of the figure 26. It is important to mention that the groups of plants G and EC1 were acclimatized prior to rest of the groups, as consequence the growth of these plants was higher.

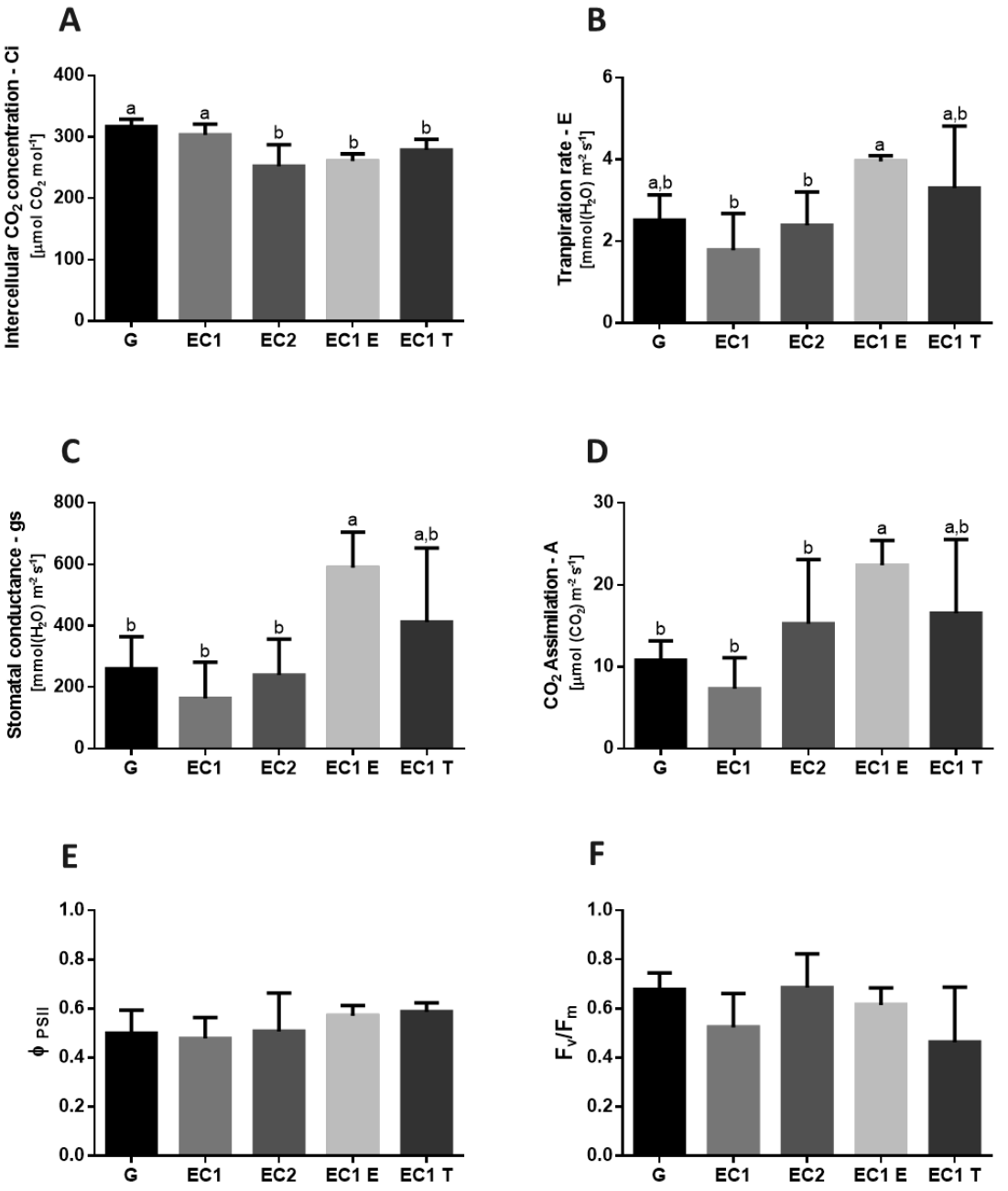

Figure 24 - Gas exchange and photosynthetic parameters in seed-derived plants (G), SE derived plants from the lines EC1 and EC2, and SE derived plants from the line EC1 with treatment with TSA (EC1 T) and its control with EtOH (EC1 E). (A) - Intercellular CO_2 concentration - Ci; (B) - Transpiration rate - E; (C) - Stomatal conductance -gs; (E) - chlorophyll fluorescence parameters quantum yield of photosystem II - ϕ PSII; (F) - Maximum yield of PSII photochemistry – Fv/Fm. Means ± SDs, different letters indicate significant differences between treatments, according to the Tukey's test ($p<0.05$).

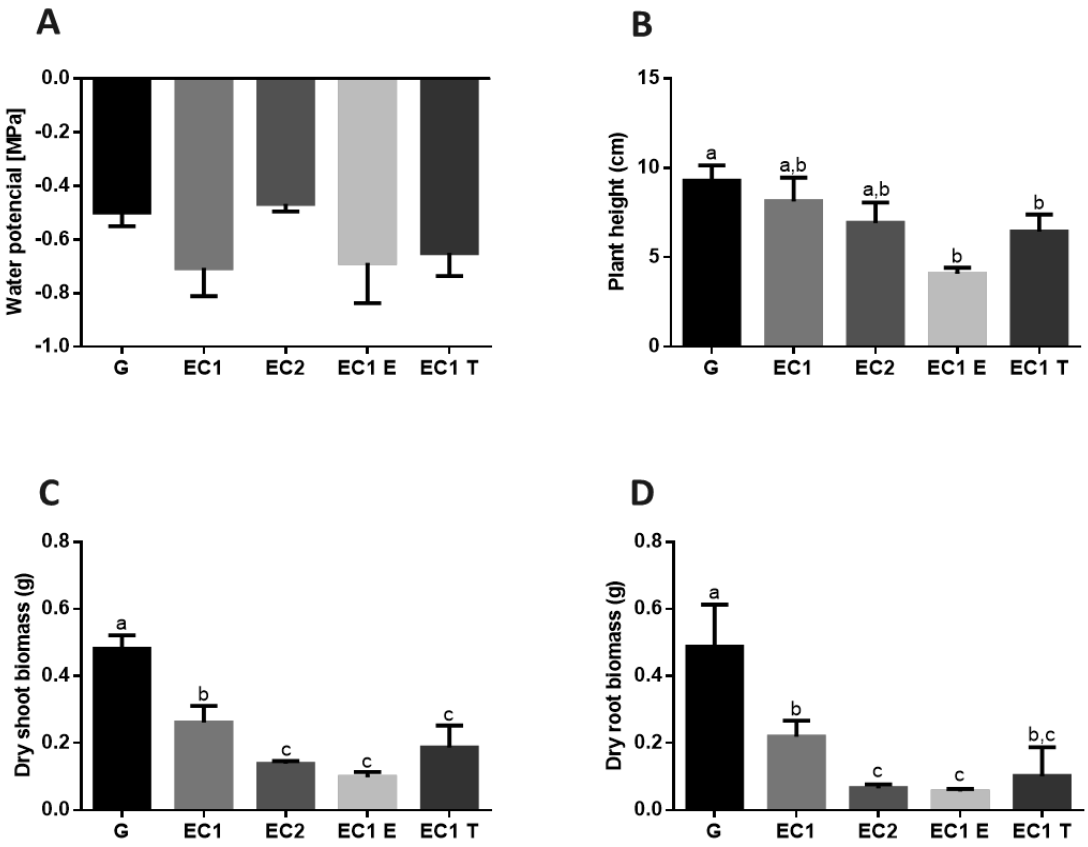

Figure 25 – Growth parameters and water potential in seed-derived plants (G), SE derived plants from the lines EC1 and EC2, and SE derived plants from the line EC1 with treatment with TSA (EC1 T) and its control with EtOH (EC1 E). (A) -Water potential (Ψ); (B) – Plant height; (C) – Dry shoot biomass; (D)- Dry root biomass. Means ± SDs, different letters indicate significant differences between treatments, according to the Tukey's test ($p<0.05$).

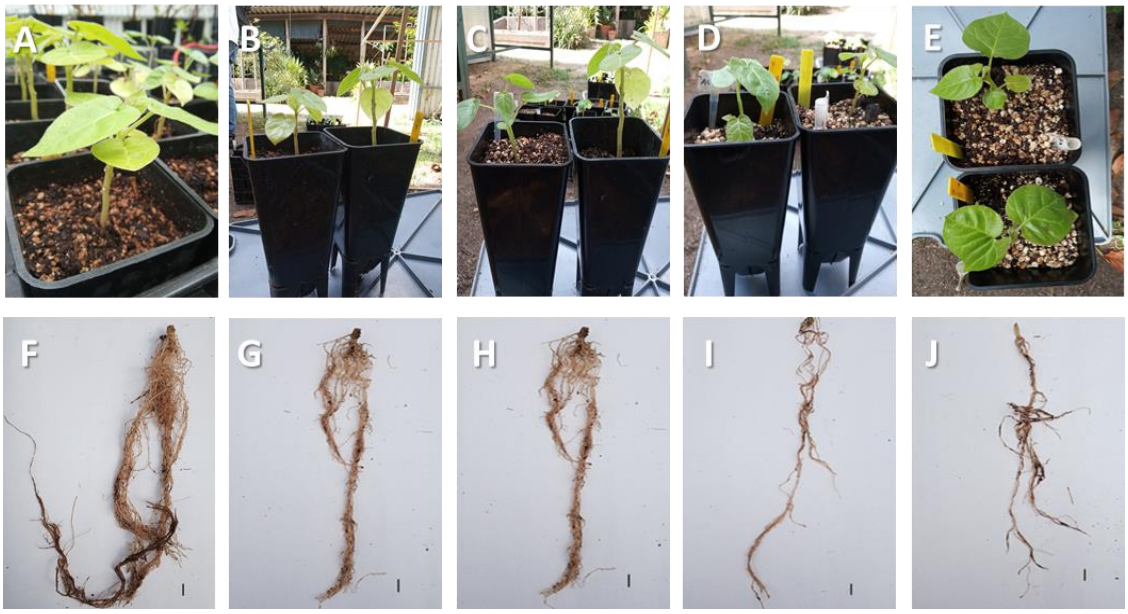

Figure 26 – Photos comparing the morphological differences between groups. (A)- Seed-derived plant- G; (B) – Plant from EC1 group (left) and plant from G group (right); (C) – Plant from EC2 group (left) and plant from EC1 group (right); (D) – Plant from EC1E group (top) and plant from EC1T group (bottom); (E) - Plant from EC1E group (top) and plant from EC1T group (bottom); (F, G, H, I and J) –Roots from G, EC1, EC2, EC1E, EC1T after washing, respectively The bar represents 1 cm**.**

IV. Discussion

1. Assessment of the embryogenic lines

The results showed that the long term established line EC3 had lost embryogenic capacity, meaning that there was no development of somatic embryos when the *calli* were placed in the auxin-free medium; only proliferation. Previous studies have shown that when *calli* are maintained in culture for more than 1 year they become unstable (Lopes *et al.*, 2000) and often display cytogenetic abnormalities and loss of embryogenic potential due to chromosomic variations (Currais *et al*., 2013). Therefore, plant regeneration decreases with the lines' aging and the clonal propagation through SE becomes nonviable. However, the maintenance of these lines in culture is interesting, because they are still able to form pre-embryogenic masses, although further embryo development is compromised. The comparison between these lines and those in which somatic embryos can develop could give important insights about the molecular mechanisms responsible for the differentiation of the pre-embryogenic masses into somatic embryos.

When lines EC1 and EC2 were tested somatic embryos formation was achieved although a great number of abnormal somatic embryos could be seen. One of the SE limitations is exactly the low quality of somatic embryos developed from the embryogenic *calli* that may lead to difficulties in the stage of conversion into plantlets (Canhoto *et al*., 2005). These anomalies have already been described in tamarillo, as well as their association with the conversion of the embryos into plantlets (Correia *et al.*, 2012). The same study also showed that the abnormal phenotypes of the embryos did not impaired their conversion into plantlets (Correia *et al.*, 2011). Still, previous studies with tamarillo showed that the cells of the somatic embryos are frequently vacuolated, pointing out that the lack of ability to accumulate storage compounds during embryo development, particularly lipids and proteins, is one of the main causes of the poor quality of the embryos and its low potential to convert into viable emblings (Lopes *et al.*, 2000). This may explain the constraints observed in the stage of conversion for the emblings, showing a disturbance from radial to symmetrical differentiation (Canhoto *et al.*, 2005). The results obtained in this work showed that the EC1 line mostly differentiate abnormal somatic embryos and shows a reasonable conversion rate whereas somatic embryos from the line EC2 had major difficulties to convert into plantlets. Since the lines EC1 and EC2 had the same age, this may indicate that tamarillo regeneration through SE is genotype dependent.

Some of the plantlets obtained from the embryogenic lines died during acclimatization. Even so, the survival rate for the SE-plant was still very high, suggesting

that even with irregular morphologies the plantlets may still grow into viable and vigorous plants.

2. Scanning electron microscopy analysis during somatic embryo conversion

Plants have a particular type of development assured by the contiguous formation of organs by the activity of apical meristems (shoot and root). Conversion of somatic embryos into plantlets also implies the organization of shoot and root apical meristems. (Zimmerman *et al.*, 1985). SEM observation of the conversion process in tamarillo were carried out to understand how the somatic embryos develop and further give origin to plantlets. After being placed in auxin-free medium the nodular appearance of embryogenic *calli*, due to the presence of pre-embryogenic masses, is lost and somatic embryo differentiation starts to occur. Most of the embryos did not went through the characteristic morphological stages of embryogenesis and the most developed embryos display a phenotype in which cotyledons are not well differentiated and the whole embryo ends, at the apical pole, by a cup-shaped structure. In some species, like carrot, the formation of somatic embryos with fused cotyledons results from abnormalities in the polar auxin transport (Schiavone and Cooke, 1987) and, in *Arabidopsis thaliana*, cup-shaped embryos are related with the pin mutants (Aida *et al.,* 1999). An unbalance on auxin polar transport may also occurs during somatic embryo differentiation in tamarillo, since to achieve development embryos must be transferred from auxin-rich medium to a medium without auxin. Studies aiming to characterize zygotic embryo development in tamarillo could give important insights into the embryogenic process, by comparison to what has been observed during somatic embryogenesis.

In many plants, the process of shoot growth is preceded by several shoot maturation stages where morphological and anatomical changes occur in which plants may produce distinct vegetative structures, such as trichomes (Telser *et al*., 1997). Trichomes are single or multicellular epidermal appendages with different functions on the aerial part of the plants (Hülskamp, 2019). In SEM analysis the presence of trichomes was noticeable, either in SE-derived emblings or in seedlings shoots, not only in the foliage but also in the stem. Trichomes, usually present in leaves and stems, are present in organs build up by SAM activity, therefore their presence in both emblings and seedlings reveals that SAM behaviour is very similar between them, originating the shoot part and the leaves of the plantlets during the somatic embryo conversion.

Trichomes serve different purposes such as protection against herbivores and pathogens, UV radiation and water loss and in some species, they can even play an important role of detoxication of the plant by accumulating the toxic compound (Hülskamp, 2019).

3. Mass increment

Mass increment results revealed strong differences between the lines that still possessed embryogenic capacity (EC1 and EC2) and the line that lost the ability to produce embryos (EC3). From t0-t1, all the lines remained in proliferation stimulated by the auxin present in the liquid TP medium although EC3 showed higher mass increment values. The distinct proliferation behaviour already noticeable at the end of this period between EC3 and the other two lines may be related to the EC3 line's epigenetic state, embryogenic ability and hormonal habituation among other culture condition factors (Us-camas *et al.*, 2014).

Once the treatments were applied and the *calli* placed in the development medium (t1-t2) differences became evident. The line EC3 showed the highest mass increment values and the *calli* proliferated and remained unaffected with all the treatments. Long term established lines, like EC3, generally have a fast-growing behaviour in comparison with lines with embryogenic competence (Correia *et al.*, 2012). This suggests that line EC3 may have reached a stationary phase, where the lack of auxin is no longer a trigger to promote embryos development. In the lines EC1 and EC2 the effect of the treatments was noticeable on the *calli* growth leading to lower increment values. This may suggest that the lines still react to *stimuli*, thus are still responsive to the lack of auxin in the development medium and can still produce somatic embryos.

4. Somatic embryo development optimization experiment

As stated before, two-step SE is a promising biotechnological tool, but has limitations, such as, the loss of embryogenic ability of lines when subculture for a long period of time and the low conversion rates of embryos into plantlets in woody species. Since SE is such a complex process that relies on different kinds of interactions (Fehér *et al*., 2003), these problems may relate to DNA variations occurring in long-term embryogenic *callus* (Currais *et al*., 2013) or even embryo anomalies, like, defective accumulation of storage compounds (Lopes *et al*., 2000). With that in mind, the three treatments, with TSA, 5-AzaC and AC, were chosen with the main goal of obtaining good-quality embryos able to convert into plantlets without difficulties.

TSA (trichostatin A) is an inhibitor of histone deacetylases. Since histones modulate DNA transcription due to modifications in the structural organization of DNA, and that epigenetic mechanisms control developmental processes (De-la-Peña *et al.*, 2015), TSA was tested to find whether histone acetylation is involved on somatic embryo development of tamarillo. There a is connection between chromatin structure and expression of somatic-embryogenic related genes (De-la-Peña, 2015). TSA was already used as stress agent to induce the formation of embryogenic *calli* in *Brassica napus* and when applied to *Arabidopsis thaliana* microspores indicated that totipotency relies on a histone deacetylases dependent mechanism (Li *et al.*, 2014).

The results for the TSA treatment revealed a higher number of embryos for the EC2 line whereas the line EC3 remained without response. The somatic embryos resulting from both lines EC1 and EC2, showed good quality in the sense that they presented an opaque white colour, suggesting the accumulation of storage compounds. However, morphological anomalies were often present. In the control (EtOH) translucent embryos were formed that may present low capacity of storage compounds accumulation.

Since the somatic embryos presented decent quality was expected good conversion rates, but they were very low. The control that supposedly had low quality embryos presented a higher percentage of conversion. The embryos developed from line EC1 showed a more efficient conversion compared to the line EC2. This may suggest that, since EC1 is considered the most competent line, TSA had led to a better chance of obtaining good quality embryos. TSA presented better conversion and survival rates than the other treatments. Maybe the adjustment of concentrations or time of exposition to the substance may lead to better results.

5-AzaC is an hypomethylation agent that leads to a decrease of methyltransferase activity and a consequent decrease of DNA methylation and assurance the genetic stability and genome integrity (De-la-Peña *et al.*, 2015). 5-AzaC was already used to study DNA methylation during SE induction in carrot (Shibukawa *et al.*, 2009) and its application led to embryogenic potential recovery of long-term embryogenic lines in *Theobroma cacao* by decreasing DNA methylation (Quinga *et al*., 2017).

Therefore, it was expected that the 5-Azac treatment could had an effect in the line EC3, but the *calli* continued to proliferate normally after being placed in the development media, and after 4 weeks nothing was noticeable but some oxidation. The

EC1 line revealed a higher number of embryos, with very different morphologies: translucent with abnormal shapes or opaque but very small, questioning their quality. The treatment control (DMSO) led to oxidation of *calli*, probably because of high level of toxicity. The conversion rates were very low, confirming that the embryos anomalies resulted in constrains in the conversion stage. The survival rate was also very low. This treatment turned out to be very aggressive to the embryogenic *calli*, this is also noticeable in the mass increment results specially for line EC1 but also for line EC2, leading to the lowest mass increment values when the *calli* was placed in development media.

The exact role of AC in SE needs to be clarified, but this compound is very used in embryo development and germination studies in due to its powerful adsorption capacity (Thomas, 2008). In the two-step SE process is required the transition of the *calli* from an auxin-rich medium to a medium without auxins. The decrease in yield of the development process may related to the fact that the auxin (picloram) remains in the embryogenic *calli* since auxins inhibit the development of embryos and promote *calli* proliferation (Rai *et al*., 2008). In tamarillo, AC was already used to improve somatic embryo development and conversion and it had positive results for embryo quality when added to the development medium (Mano *et al*., 2016).

This treatment led to a high number of developed embryos for the line EC2 and was the only treatment that led to a response of the line EC3. Despise this result, the somatic embryos also showed anomalies. The conversion rate turned out to be very low for the embryos resulting from the line EC1, and for the line EC2 and EC3 there was no conversion at all. This result may suggest that AC may led to adsorption of the auxin but also of other important substances that help the development of the embryos. Thus, the AC concentration should be lower and maybe conversion would have been more successful.

In all treatments tested the embryos from the line EC1 had a more efficient conversion into plantlets then the ones from the lines EC2 and EC3, in accordance with what happened with results of the assessment of the embryogenic lines. Overall, the treatments did not prevent anomalies and there was not a very successful treatment, but TSA treatment resulted in a higher conversion rate when applied in the line EC1 and higher survival rates.

5. Molecular analysis during somatic embryo development

LEA proteins have an important role during the terminal stages of zygotic embryogenesis and some of these proteins are considered SE markers, therefore *LEA14* and *LEA34* genes were chosen to be analysed during the somatic embryo development (Heringer *et al.*, 2018). *BBM2* (also known as AP2 – Like ethylene responsive transcription factor) was selected since it works as key regulator of embryo development in plants (Yang and Zhang, 2010). *ENOLASE* was also selected due to its association with carbohydrate and energy metabolisms which are related with storage compound for an efficient conversion into plantlets (Chugh and Khurana, 2002).

Although, the results did not show a tendency when the genes expression was compared between the three lines throughout the embryo development, there was a current result in gene expression from w0-w1. When the *calli* was transferred from an auxin-rich medium to the development medium (without auxin and low on sucrose content), there was frequently a gene expression augmentation, either subtle or more noticeable. This suggests that the stress from the lack of auxin may influence the gene expression. This was followed by a decrease in expression.

LEA proteins are usually associated with dissection in seeds during zygotic embryogenesis (Heringer *et al.*, 2018). During SE they may constitute a good indicator of embryos quality. Thus, the fact that *LEA14* and *LEA34* expression increased during late development weeks, w4 and w3, respectively and remained a little higher in g1, was somewhat expectable. Still the expression for these two genes was very low, especially for *LEA34* that showed the lowest values in comparison to all genes.

BBM2 also showed low levels of expression. Since this gene is associated with embryo development and even with shoot development in plants (Yang and Zhang, 2010) it was expected a significant expression in the late stages of embryo development and as well as in the initial conversion stage (g1).

Metabolism-related proteins, such as enolases, are predominant in embryogenic cells (Correia *et al.*, 2012). Therefore, the fact that there was a high expression of *ENOLASE* throughout the embryo development was already assumed, particularly in final stages (w4), when storage compounds are so important to embryos quality and to their conversion into plantlets. In g1 *ENOLASE* expression was only high for EC1 line, coincidentally the line with more success in conversion. *ENOLASE* was also highly expressed in the long-term established line EC3, maybe this is the result of the shock

induced by the lack of auxin and sucrose, resulting in unbridled proliferation as seen in the mass increment values.

When the levels of expression of the genes between the last week of embryo development and the zygotic embryo were compared, *LEA34* showed a tendency, ZE had higher values, followed by EC1 (as stated before the line from which embryos convert more efficiently), then the line EC2 and finally line EC3 (that does not produce embryos). Suggesting that *LEA34* may have a significant role in embryo quality and development. For the *LEA14* expression was higher in EC3, followed by EC1 and EC2 and lastly the ZE, indicating that maybe *LEA14* expression is less important in zygotic embryogenesis then in SE embryogenesis. Expression of *BBM2* was equally very low for ZE similar to what happened with the embryogenic lines, yet, surprisingly EC3 line revealed a higher expression in w4. *BBM2* is related to embryo development and the line EC3 does not even has the competence to produce embryos. *ENOLASE* expression was very low in ZE. This could be related to the fact that zygotic embryos do not have so much need to accumulate storage compound as the somatic embryos, because they are involved in endosperm where storage compound accumulate.

The line EC1 with TSA treatment was also analysed because EC1 was the line with better conversion rates and TSA treatment led to higher conversion rates in that same line. TSA treatment led to higher levels of expression in w2 for all genes except for *ENOLASE*, but when it came to g1, the levels of expression were lower for the TSA treatment. Showing that perhaps in w2 TSA was still present in the *calli* and the DNA was more available for transcription and in g1 stage the TSA was not reactive anymore.

While there is some knowledge for SE induction pathways (Chugh and Khurana., 2002; Fehér *et al.*, 2003; Ikéda *et al.*, 2006; Pais, 2019), the somatic embryos development stage is still not that studied at the molecular level. The type of research is imperative not only because SE is often mentioned as model for the study of morphological, physiological, molecular and biochemical events occurring during the onset and development of embryogenesis in higher plants (Quiroz-Figueroa *et al.*,2006) but also because SE applications as clonal tool would be better understood and thus optimised.

6. Physiological parameters of the plants

Gas exchange parameters: intercellular CO_2 concentration, transpiration rate, stomatal conductance and CO_2 assimilation were measured. Stomatal conductance is related to the interaction between the plant and the global atmospheric environment involving it (Chen *et al.*, 1999). CO_2 diffusion into the mesophyll cells of the leaves and water vapour from the leave to the atmosphere is mainly driven by the stomatal aperture, which is controlled by a complex system of plant physiological processes (Araújo *et al.*, 2011). Therefore, stomatal conductance is closely connected with transpiration rate and CO_2 assimilation.

The group of plants EC1T and EC1E (plants resulting from the line EC1 and treated with TSA and its control EtOH, respectively) noticeably presented higher values for stomatal conductance than the other groups. Thus, similar results occurred for transpiration rate and CO2 assimilation. The intercellular CO_2 concentration is related to the presence of CO_2 in mesophyll cells. The group of seed-derived plants (G) and the group of SE derived plants from the line EC1, revealed higher values of intercellular CO_2 concentration. In comparison with studies performed in well-watered plants of tamarillo (Braga, 2015) some of the groups like G, EC1, and EC2 presented lower values.

The ϕ PSII (chlorophyll fluorescence parameters quantum yield of photosystem II), a parameter measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry (Maxwell and Johnson, 2000), did not show significant differences between groups, with results of approximately 0.8, same as other species like *Eucalyptus globulus* (Jesus *et al.*, 2005). This may suggest that the conditions in the greenhouse were not the best and may led to a state of hydric stress that impairs the photosynthetic capacity (Correia *et al.*, 2014). The Fv/Fm (maximum yield of PSII photochemistry, *i.e*. the quantum efficiency if all PSII centres were open) results also did not revealed any significant difference between groups and has expect were higher than the results ϕ PSII.

The water potential (Ψ_w) results also did not revealed significant differences. According to Braga (2015), well-watered tamarillo plants show water potential values of ≥ - 0.5 MPa. Majority of the groups revealed lower values, suggesting that maybe all the groups, regardless of being SE-derived or seed derived, were a little far from normal concerning water status, possibly again due to conditions in the greenhouse.

Overall, even with some differences the seed-derived plants and SE derived plants showed similar behaviours and results. Since all the plants remained in the same

conditions in the greenhouse and watered in the same manner, these results were somewhat expectable.

Regarding plant height, shoot and root biomass the results showed significant differences. This is explained by the fact that G group plants and EC1 are older than the remaining groups, meaning that their biomass was a little higher because they had more time in the greenhouse growing and that tamarillo presents a fast development in the first months of development significant shoot and foliar growth (Acosta- Quezada *et al*., 2016). Thus, between G group and EC1 there was still a difference, with G group plants having higher values for plant height, shoot and root biomass. Between the remaining groups, the SE derived plants from the line EC1 with TSA treatment revealed higher values for plant height, shoot and root biomass. Indicating, that the somatic embryos, resulting from the EC1 line treated with TSA, that converted successfully resulted in vigorous plants.

Studies performed with conifers show superior characteristics of SE emblings compared to zygotic seedlings, with higher height and greater dry mass of new roots (Egertsdotter, 2018). In *Coffea arabica* was also observed that SE emblings were more vigorous than seedlings and the observed vigour in the nursery was carried over to field performance as these plants were more precocious than seedlings and yielded coffee beans 1 year earlier than seedlings (Menéndez-Yuffá *et al.*, 2010). This study performed in tamarillo did not reveal big differences between seed derived plants and SE derived plats. Therefore, it can be said that tamarillo SE-derived plants and seed-derived plants are very alike physiologically.

V. Concluding remarks

Plant *in vitro* propagation techniques are valuable tools that allow genetic improvement and large scale-production. Somatic embryogenesis is one of them but the fact that some of its basic mechanisms are still unknown makes an optimization difficult and the outrun of its limitations hard.

From the tamarillo embryogenic lines (EC1, EC2, EC3) already established, EC3 already lost its ability to produce embryos. In agreement with other works, long-term culture *calli* lost its embryogenic competence. The embryogenic lines developed embryos with abnormal phenotypes frequently cup-shaped and sometimes formed clusters. The conversion rates were low, and some embryos developed roots, suffered an enlargement and constrained, still EC1 had the highest conversion rate. The plantlets that resulted from the somatic embryos revealed some anomalies such as twisted and bulky stems, but still had a high survival rate. Suggesting that, in acclimatization, SE derived plantlets can still develop into vigorous plants.

Scanning electron analyses results showed that the tamarillo somatic embryos usually do not present cotyledons, and are often cup-shaped, but once they reach the embling stage they are very similar to the seedlings.

One of the limitations of SE is the development of somatic embryo, since low quality embryos do not convert into emblings. Therefore, an optimization experiment was carried out with the application of TSA (trichostatin A) or 5-azacytidine in the embryogenic *calli* and a rinse of the *calli* with activated charcoal. Between the three treatments, TSA (an agent that leads to better DNA accessibility) revealed better results when applied to the line EC1. Still, the experimental conditions can be optimized, in order to attain better results.

A molecular analysis throughout the embryo development was also carried out revealing genes' levels of expression during the 4 weeks of the embryogenic *calli* lines in development medium. *LEA14, LEA34*, *BBM2 and ENOLASE* were chosen due to their involvement in different embryogenic stages. *LEA14 and LEA34* related to LEA proteins showed the highest values in late weeks of embryo development. *BBM2* showed low values during the whole development. *ENOLASE* associated with storage compounds accumulation, as expected, showed the highest expression values of all genes, with an increase in week 4 of embryo development. When week 4 expression levels were compared between the embryogenic *calli* lines and the zygotic embryo, was noticeable that *ENOLASE* had the lowest values for the zygotic embryo, which is expected since the zygotic embryo do not have so much need to accumulate storage compound as the

somatic embryos, because they are involved in the endosperm. *LEA34* showed a tendency, the zygotic embryo had higher values, followed by EC1 (the line from which embryos convert more efficiently), then EC2 and finally line EC3 (that does not produce embryos), suggesting that *LEA34* may have a vital importance in embryo quality and later conversion. The line EC1 with TSA treatment was also analysed revealing that TSA led to higher levels of expressions for genes except for *ENOLASE* in week 2 of development. The somatic embryo development is a stage still very unknown at the molecular level, so research in this particular and decisive phase of SE to enlighten some of what happens would be very interesting.

In plant performance analysis gas exchange parameters measured: intercellular CO_2 concentration, transpiration rate, stomatal conductance and CO_2 assimilation revealed significant differences. With EC1T and EC1E (plants resulting from the line EC1 and treated with TSA and its control EtOH, respectively) having higher values for transpiration rate, stomatal conductance and CO_2 assimilation. The photosynthetic parameters (ϕ PSII and Fv/Fm), as well as, water potential (Ψ) results did not showed any significant difference. Significant differences arose between groups when plant height, shoot and root biomass was analysed because the seed-derived plants group and the SE derived from line EC1 plants group were older than the others. Between these two seed-derived plants group showed higher plant height, shoot and root biomass, but still with not a big difference from the SE derived plants. From the remaining groups the SE derived plants from the line EC1 with TSA treatment revealed higher values for plant height, shoot and root biomass. Overall, SE-derived plants performance was very similar to seed-derived plants. In the future, would be interesting to follow these plants in testing fields to see their development and behaviour as adult trees.

VI. References

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