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EVALUATION OF THE EFFECT OF HISTONE ACETYLATION REGULATORS ON CALLUS PROLIFERATION AND SOMATIC EMBRYO DEVELOPMENT IN *SOLANUM BETACEUM* CAV.

Dissertação no âmbito do Mestrado de Biodiversidade e Biotecnologia Vegetal,
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List of abbreviations

2,4-D – 2,4-Dichlorophenoxyacetic Acid

AMC – Amino-methylcoumarine

Ala-AMC - L-Alanine-4-methylcoumaryl-7-amide

Arg-AMC – L-Arginine-4-methylcoumaryl-7-amide

BSA – Bovine Serum Albumin

EF1-α - ELONGATION FACTOR1-α

Gly-Pro-AMC - Glycyl-L-Proline-7-amide-4-methylcoumaryl

HDA14 – HISTONE DEACETYLASE 14

HDCA – HISTONE DEACETYLASES

Met-AMC - L-Methionine-4-methylcoumaryl-7-amide

MU – Methylumbelliferyl

MU-C - 4-methylumbelliferyl-b-D-cellobiosid

MU-G – 4-Methylumbelliferyl-β-D-glucopyranoside

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

MU-P - 4-Methylumbelliferyl-acettyl-β-D-phosphate

ncRNA – Non-coding RNA

NEP-TC – NON-EMBRYOGENIC PROTEIN – TAMARILLO CALLUS

LEA14 – LATE EMBRYOGENIC ABUNDANT

Leu-AMC - L-Leucine-4-methylcoumaryl-7-amide

Lys-AMC - L-Lysine-4-methylcoumaryl-7-amide

Phe-AMC - L-Phenylalanine-4-methylcoumaryl-7-amide

qPCR - Quantitative real-time PCR

SE – Somatic embryogenesis

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 embriogénese somática está bem estabelecida e ocorre através de um protocolo em duas etapas. A primeira etapa passa pela formação de um calo embriogénico através da indução em segmentos de folhas jovens ou embriões zigóticos num meio rico em auxina (2,4-D ou Picloram) e numa elevada concentração de sacarose (9% m/v). Este calo pode ser mantido nesse mesmo meio, sem perda da sua capacidade embriogénica. Após transferência para um meio sem auxina e com uma quantidade de sacarose mais reduzida (3% m/v), o calo embriogénico desenvolve-se em embriões somáticos que se convertem em plantas. Algumas limitações deste processo são ainda a frequência de ocorrência de anomalias nos embriões somáticos, com consequentes restrições no seu desenvolvimento e conversão. O objetivo principal deste trabalho foi otimizar o desenvolvimento de embriões e regeneração de plantas diversos através da alteração de estados de cromatina promovida pelo uso de um composto inibidor de desacetilases de histonas, a tricostatina A (TSA).

Calos embriogénicos foram sujeitos ao tratamento com TSA (1 µM), antes de serem transferidos para meio de desenvolvimento. Através deste tratamento, observou-se maior formação de embriões somáticos (738) do que em calos sem tratamento com TSA (213), mas também maior conversão de embriões em plântulas (39%).

Após a análise molecular, em relação à expressão dos genes *LEA14*, NEP-TC e *HDA14*, verificou-se pequenas diferenças entre calos do controlo e calos do tratamento com TSA. Em que *LEA14* tinha maior expressão em controlo e *NEP-TC* era maior no tratamento com TSA. *HDA14* após o tratamento com TSA era mais expressa nos calos tratados com TSA, mas após uma semana à luz era mais expressa no controlo.

Na proliferação de calos não-embriogénicos, a utilização de TSA não melhorou a produção de novas células. A produção de proteínas foi baixa, não correspondendo aos resultados esperados.

Palavras-chave: conversão embrião/planta; desenvolvimento embrionário; embriogénese somática; TSA;

Abstract

Somatic embryogenesis (SE) is an effective tool for plant 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 two-stage protocol. In the first stage, 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 (9% w/v). These *calli* can be kept in the same medium. Following transfer to an auxin-free medium containing lower sucrose levels (3% w/v), 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. The main objective of this work was to optimize embryo development and regeneration of diverse plants by altering chromatin states promoted by the use of a histone deacetylase inhibitor compound, trichostatin A (TSA).

Embryogenic *calli* were treated with TSA (1 µM), before being transferred to embryo development medium. The number of somatic embryos formed was higher for TSA-treated callus (738 embryos) to the 213 embryos from control callus, also the conversion rate was higher in callus treated with TSA, with 39%.

Through molecular analyses, *LEA14*, *NEP-TC* and *HDA14* were tested, obtaining slightly differences between control and TSA-treated callus. In which, *LEA14* had higher expression in control callus and *NEP-TC* had in TSA-treated callus. As for *HDA14*, was expressed higher after TSA treatment but after one week in light, the control callus had the highest expression for this gene.

In non-embryogenic callus mass proliferation, TSA treatment did not result in a higher production of new cells. The protein production was low relative to expectation.

Key words: embryo development; plant conversion; somatic embryogenesis; TSA;

I. Introduction

1. Contextualization of the work

Tamarillo (*Solanum betaceum* Cav. syn. *Cyphomandra betacea* Sendtn.), is a small solanaceous tree that produces edible and high nutritious fruits from which other products might be produced, such as smoothies and jams. Nowadays, the search for new alternative products gives tamarillo an advantage in the market since it is a healthy and appealing option. The fact that Portugal possesses adequate climate conditions for fruit production and tamarillo's fruits can reach a high market value (10-13€/Kg) are aspects that somehow generated an increase of interest from the producers in recent years.

Tamarillo is also used as a model to study the process of somatic embryogenesis (SE). SE has several biological and scientific advantages, such as, the potential for improvement of plants of commercial relevance, as well for the study of physiological changes and molecular mechanisms during embryo development (Méndez-Hernández et al. 2019). The protocol for SE in tamarillo is well-established in the Laboratory of Plant Biotechnology of the University of Coimbra yet, a better understanding of the somatic embryogenesis process is still required (Guimarães et al. 1988; Canhoto et al. 2005). In this context, this work was carried out to obtain new data about SE regulation, using the SE experimental system in tamarillo. The information obtained can then be used in the optimization of tamarillo SE propagation method or extrapolated to other relevant woody species.

2. *Solanum betaceum* Cav.

2.1. Characterization and distribution

Tamarillo, also known as tree tomato, or "tomate de la paz" in Spanish, is a small tree belonging to one of the most economically relevant families - Solanaceae (Correia and Canhoto 2012).

The tree tomato is native to the Andean regions of South America, in Bolivia and Argentina, where it has been cultivated for a long time. From there, the production spread to other subtropical and tropical parts of South and Central

America. Later, it reached the British colonies and Portugal (Bakshi et al. 2016). Colombia, Peru, and Ecuador are the countries with most production for commercialization (Ramírez and Kallarackal 2019).

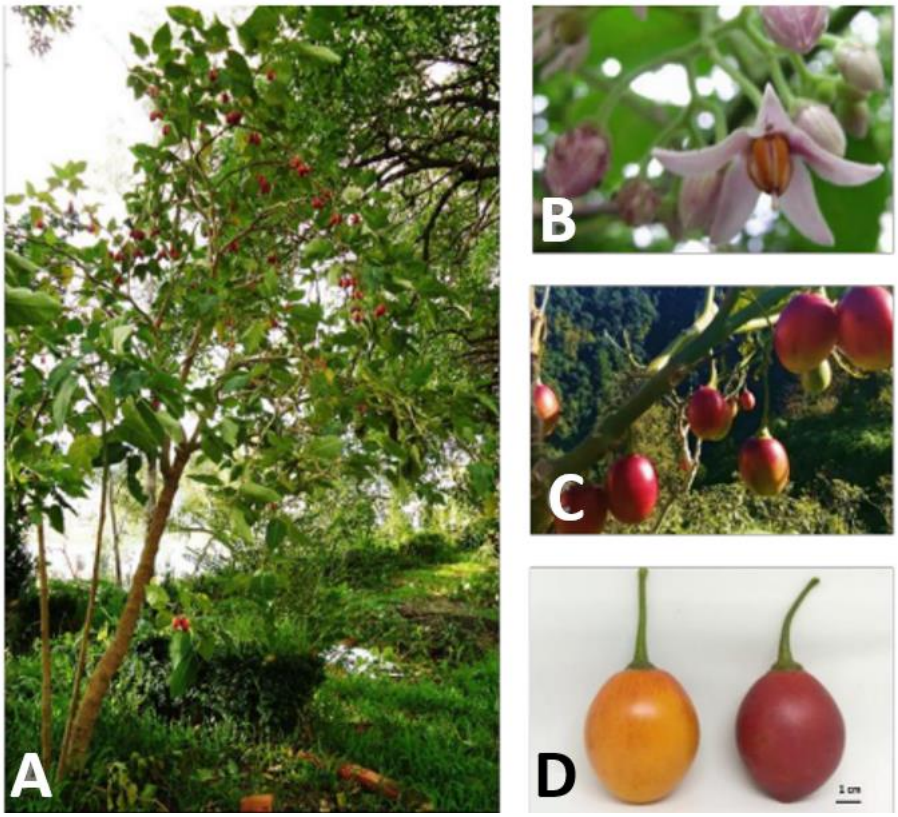

Figura 1 - Tamarillo (*Solanum betaceum* Cav.). (A) - Tamarillo tree growing at the Botanical Garden of the University of Coimbra; (B) - Tamarillo tree flowers; (C)- Tamarillo fruits from a red tamarillo tree variety from Madeira Island; (D)- Yellow and red tamarillo fruits (Correia 2019).

Tamarillo is a small perennial tree that can reach 2 to 4 meters (Fig. 2A), have pale-pink lavender and scented flowers, which develop in small groups (Fig. 2B) and typically blossom since from early spring to late summer. 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 is a smooth epicarp and presents a large variety of colours, such as yellowish, orange, and dark red, or even a mixture of these (Fig. 2D) (Ramírez and Kallarackal 2019).

2.2. Economic relevance

In the Andean region, tree tomato is mainly grown on subsistence farms, being that it represents a non-traditional commodity production and marketing, as well an alternative crop for agricultural diversification (Acosta-Quezada et al. 2011; Jaramillo et al. 2011; Insuasti et al. 2016).

Figure 2 - Different applications for tamarillo fruits such as, smoothies (A and B), whole fruit (C), jams (D) and salads (E). Images retrieve from: A – Tamarillo.com; B - Jaja bakes.com; C - gardeningknowhow.com; D - feastfreely.com; E - thedevilwearssalad.com.

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. 2). Currently, the increase in demand for healthy options and foods with beneficial to consumer's health, tree tomato has been seen as a fruit of interest because of its high content of vitamin C, antioxidants and biocompounds such as polyphenols, flavonoids, and anthocyanins (Wang and Zhu 2020; Viera et al. 2022).

3. Tamarillo propagation methods

Propagation through seeds, grafting or cuttings is usually applied to obtain new plants. Seeds germinate well but the obtained plants are of unknown genotype which make them an inadequate material to clone selected genotypes.

Grafting onto *Solanum mauritianum* is also a propagation method used with the aim of improving the tolerance to wet soil conditions and to avoid the root to rotting. Tamarillo cuttings root easily but produce smaller bushy trees compared with seed-derived plants. (Bakshi et al. 2016).

Over the last decades Plant Biotechnology has evolved due to the emergence of several technologies. One of them is micropropagation, a technique that allows in vitro cloning trough plant cell and tissue culture. The micropropagation techniques can be divided into 3 types, according to the initial material used and the type of response obtained (Canhoto 2010): axillary shoot proliferation, organogenesis and somatic embryogenesis.

Axillary shoot proliferation is the simplest technique in micropropagation since it is based on the development of existent meristems and rooting of shoots. The procedure was the first to be used for commercial purposes and continues to be used for high commercial valuable species, such as orchids. Nevertheless, meristem isolation can be challenging and since axillary shoots are mostly in dormant state, only in determined conditions they are able to develop (Canhoto 2010).

Organogenesis is a process by which cells and tissues are induced develop unipolar structures, namely a primordial root or a stem. There are two processes, direct or indirect. For direct organogenesis, an explant develops adventitious meristems, which grow into stems. After rooting, they form a new plant. In indirect organogenesis, there is first a formation of calli from which buds differentiate, followed by rooting as in the direct process (Canhoto 2010; Kahia et al. 2015).

Finally, somatic embryogenesis (SE), in which under certain conditions somatic cells evolve into embryogenic cells capable of developing somatic

embryos and consequently plants (Guimarães et al. 1996; Canhoto 2010). In natural conditions, the zygotic embryo is the result of sexual reproduction. After a series of successive divisions and specialization the zygote develops into an embryo genetically different from the parents (Canhoto 2010). In SE, the fusion of gametes is not required, and somatic embryos are originated from explants (somatic cells) cultured in vitro. 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 (Canhoto 2010; Correia and Canhoto 2012).

Several explants, such as young leaves and zygotic embryos can be used to achieve induction of SE 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 . For several species SE is associated with a few bottlenecks to an effective application, namely a high number of abnormal embryos (Correia et al. 2012), low rates of conversion of embryos into plants (Stasolla and Yeung 2003; Moon et al. 2006) and low capacity of attaining embryogenic material from adult explants (Correia et al. 2011).

3.1. Somatic embryogenesis in tamarillo

In 1988, Guimarães and co-authors described for first time SE in mature zygotic embryos and hypocotyls from tamarillo, in the Laboratory of Plant Biotechnology from the University of Coimbra (Guimarães et al., 1988). Since then, the same group established successful protocols of SE from different explants (Fig. 3). These protocols are used, not only with the purpose of tamarillo clonal propagation, but also as tools to acquire more knowledge on the cytological and molecular mechanisms related with somatic embryos formation and development (Correia et al. 2012, 2019; Cordeiro et al. 2020).

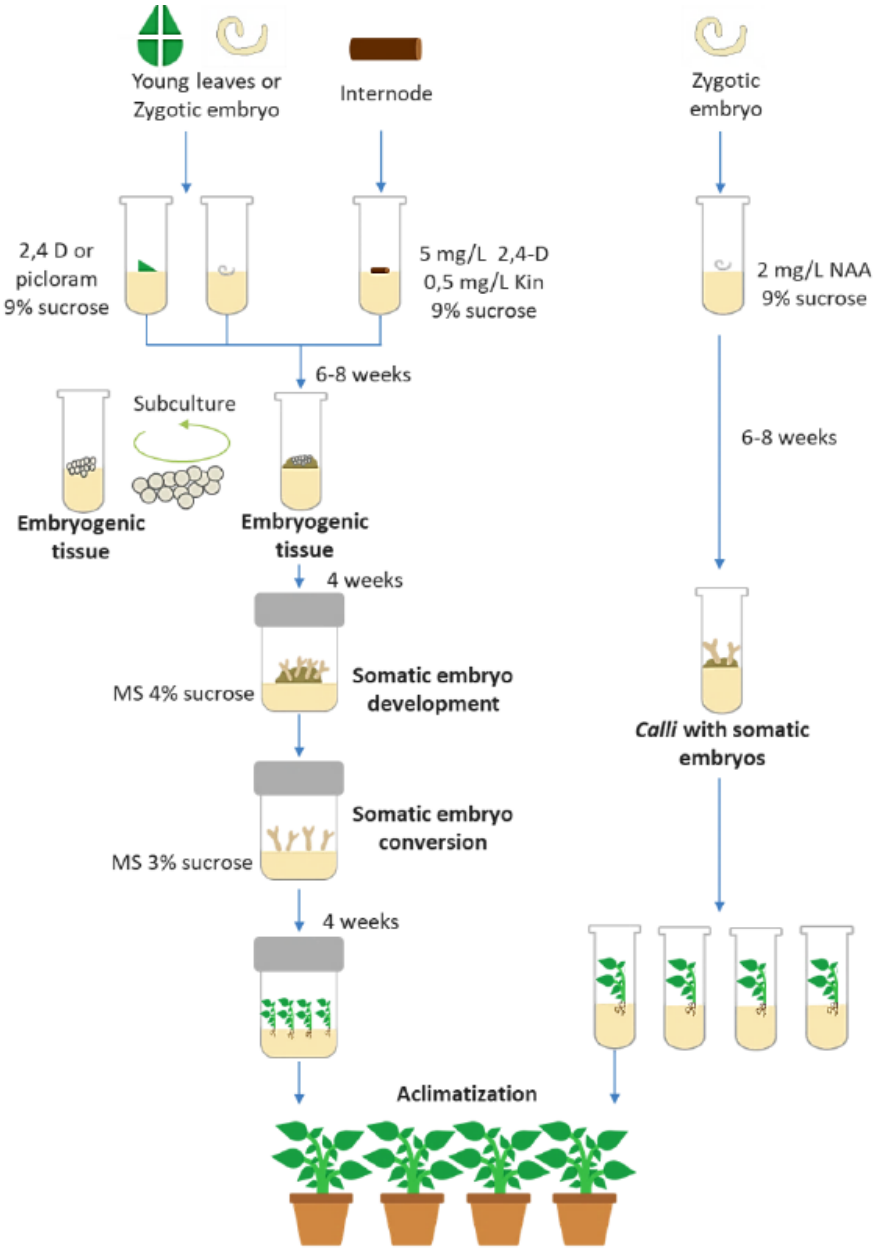

Figure 3 - Somatic embryogenesis in tamarillo (Retrieved from Correia 2019).

There are two pathways leading to SE in tamarillo: “one-step” or a “two-step” process. The “one-step” SE is achieved by exposing explants such as zygotic embryos to MS (Murashige and Skoog 1962) medium with 1-naphthaleneacetic acid (NAA) and high sucrose content. After 4 to 6 weeks of culture, incipient calluses are formed from which somatic embryos develop (Guimarães et al. 1996).

The second process designated as “two-step”, requires two different media to attain full somatic embryo development. The “two- step” SE in tamarillo is achieved by exposing explants such as zygotic embryos (Fig. 4A) or young leaves segments (Fig. 4B) to MS medium with 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram, and high sucrose concentrations (9%). After 4 to 6 weeks of culture a non-embryogenic callus is formed. Then that calli expands and some slow growing whitish clusters of embryogenic calli appear and continue proliferating after the 8th - 10th weeks (Guimarães et al. 1996; Canhoto et al. 2005). Non-embryogenic and embryogenic calli are easily distinguishable (Fig. 4C). Embryogenic callus is formed by globular whitish opaque nodules (proembryogenic masses) which are clusters of small isodiametric cells possessing a large nucleus and a dense cytoplasm (Fig. 4D) while non-embryogenic callus is more friable and presents a darkish color (Fig. 4E).

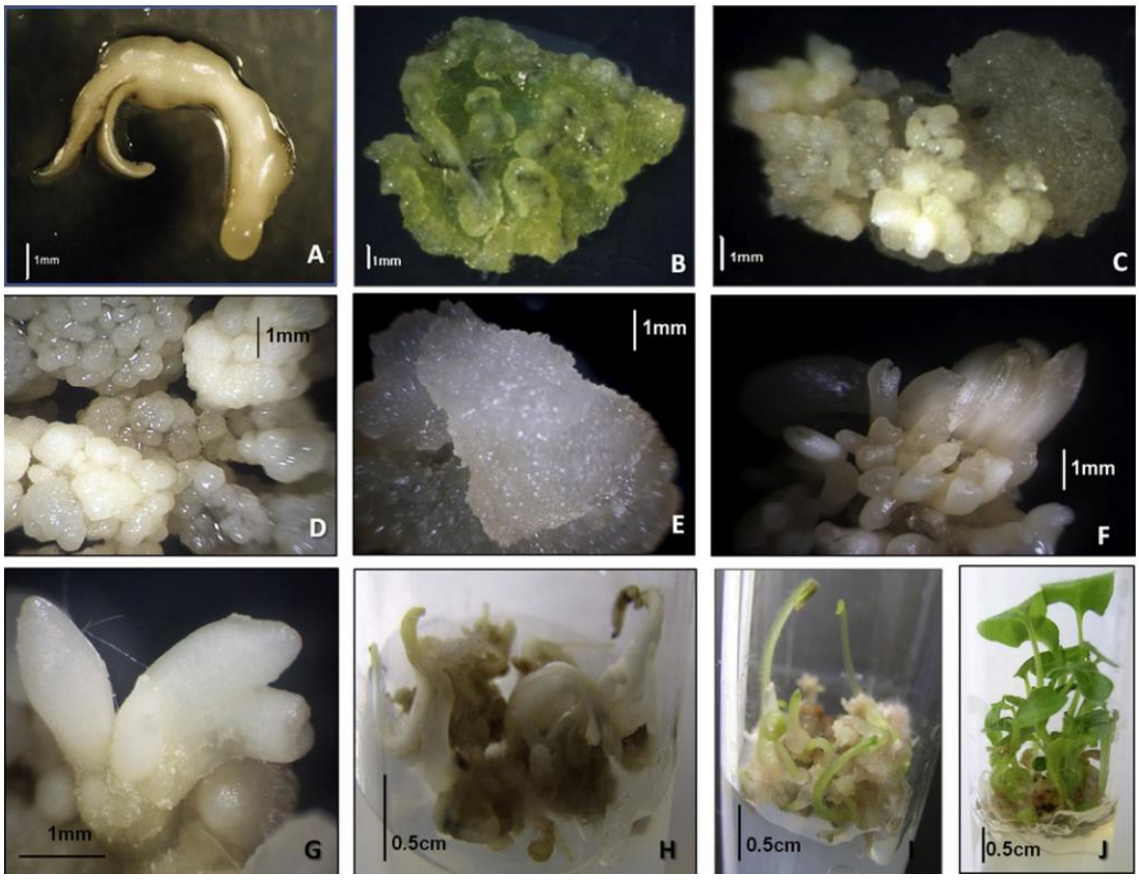

Figure 4 - Somatic embryogenesis in tamarillo. (A) Zygotic embryo after 2 weeks on a 2,4-D containing medium. (B) Leaf explant after 1 month in the induction medium - MS supplemented with picloram. (C) Embryogenic and non-embryogenic (darker) zones formed on a leaf explant after 10–12 weeks of culture.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

In a second stage, the embryogenic tissue is placed in a medium devoid of auxins and with a lower content of sucrose (3%) inducing the development of somatic embryos (Fig. 3F-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. 2005; Canhoto 2010).

The acclimatization stage, in which the plantlets are transferred to the greenhouse, is considered a preparation for the final relocation in the field under ex vitro conditions. The plant survival rate and growth are 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; Canhoto 2010).

3.2. 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; Canhoto et al. 2005).

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 and Canhoto 2012), indicates 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.

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; Reis et al. 2016). Expression of LEA appeared during late embryogenesis and was maintained at a high level in the dehydrated mature embryos. After germination, expression of LEA genes decreases (Park et al. 2011). LEA14 expression provides calli with a variety of stress conditions, mainly to dehydration and salt stress (Park et al. 2011).

NON-EMBRYOGENIC PROTEIN – TAMARILLO CALLUS (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. Furthermore, recent studies showed that this protein is a rRNA methyltransferase, apparently with an inhibitor role during tamarillo's SE (Correia et al. 2019). NEP-TC have a potential role in somatic embryogenesis regulation, namely in embryogenic competence acquisition (Correia et al. 2019).

HISTONE DEACETILASES 14 *(HDA14*, AT4G33470) is a member of the histone deacetylase family proteins that can deacetylate α-tubulin, which play a role in microtubule structure (Eshun-Wilson et al. 2019). Studies indicate that *HDA14* target proteins related to photosynthesis (Alinsug et al. 2012; Hartl et al. 2017), and in somatic embryogenesis, the gene is repressed during the induce of callus (Lee et al. 2016).

4. Epigenetic regulation of SE

Epigenetic mechanisms are important regulators of gene expression that establish potentially heritable changes in gene expression without altering the underlying nucleotide sequence. These mechanisms include DNA methylation, histone remodeling, and regulatory ncRNAs (Aboud et al. 2021).

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 (Fig. 5), specifically its compaction (Pérez et al. 2015; De-la-Peña et al. 2015; Wójcikowska et al. 2020).

This compaction results from two main processes, histone modification and DNA methylation. Both are present in plants and animals; however, DNA methylation in plants is more complex than in animals (De-la-Peña et al. 2015).

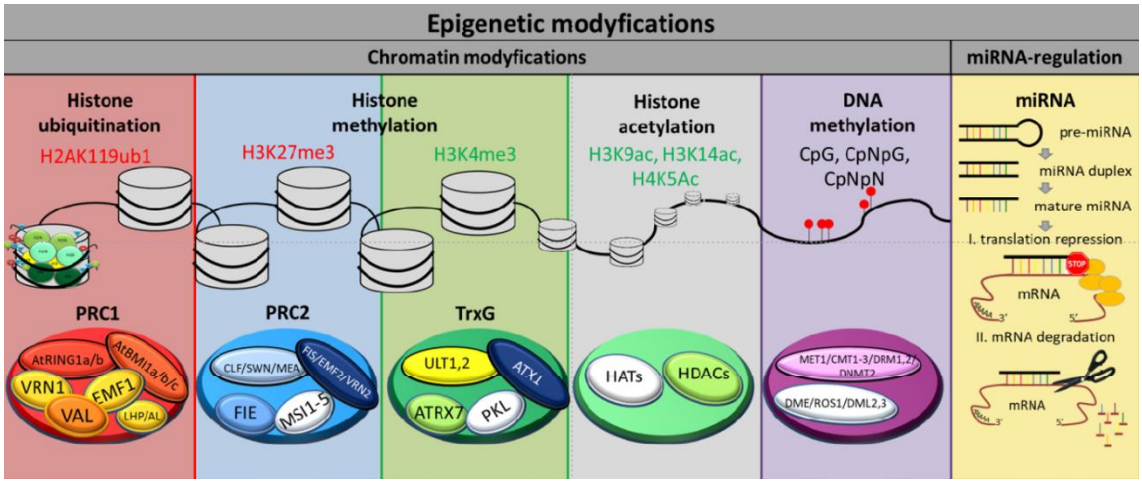

Figure 5 - An overview of the epigenetic processes, including chromatin modifications and miRNA-mediated gene regulation that control embryogenic response of explant cells (Adapted from Wójcikowska *et al.*, 2020).

DNA methylation is an epigenetic regulatory mechanism considered a determining factor in the transcriptional control of gene expression, being essential for correct plant development. During somatic embryogenesis, DNA methylation levels are critical for both morphogenesis and cell proliferation (Pérez et al. 2015).

The acetylation of histone lysine residues produces a relaxation of the chromatin structure, and this phenomenon is associated with increased gene activity. In contrast, the elimination of these acetyl groups leads to a compaction of chromatin, often related to repression and gene silencing (Martínez et al. 2021)

Trichostatin A (TSA) is reversibly and specifically inhibiting histone deacetylases, resulting in hyperacetylation of core histones which modulate chromatin structure. The increase in histone acetylation promotes selective gene transcription (National Center for Biotechnology Information 2022). Since TSA can inhibit histone deacetylases, it can be used to verify the importance of epigenetic mechanisms (Görisch et al. 2005; Li et al. 2014; Wójcikowska et al. 2018, 2020).

Reports have shown that the usage of TSA in somatic embryogenesis can increase SE efficiency and conversion (Wójcikowska et al. 2018), promote totipotency in male gametophyte (Li et al. 2014) and increase of doubled haploidy production in wheat (Jiang et al. 2017)

5. Objectives

There is efficient and well-established SE induction protocols for the solanaceous tree, tamarillo. However, the development of somatic embryos and the germination of the embryos that result from this process still require an optimization to aim the increase of high-quality plant production with a superior field performance. The use of compounds that inhibit histone deacetylases has been described in literature as a way of promoting increased yields in SE.

In this context the main goals of this work were: (1) to analyse the effects of TSA in the development of somatic embryos and their conversion to plants; (2) to observe the effects of TSA on non-embryogenic calli growth and protein expression.

Part of this work was included in the project BP4BP – Tamarillo breeding: better plants for better products (Portugal P2020|COMPETE PTDC/BAA-AGR/32265/2017) developed at the Plant Biotechnology Laboratory of CFE (Centre of Functional Ecology).

II. Material and Methods

1. Effects of histone deacetylases inhibitor (TSA) in the development of somatic embryos

1.1. Embryogenic *calli* maintenance and proliferation

The embryogenic callus line (EC3) used in this work were previously induced from tamarillo zygotic embryos, at the Laboratory of Plant Biotechnology and maintained in subculture for 2 years. This line was subcultured in TD medium, that contains MS basal medium (Murashige and Skoog 1962) supplemented with 9% (w/v) sucrose, 2 mg/L of 2,4-D, 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 4-5 week and maintained in the dark at 25 °C.

1.2. Effects of TSA in somatic embryo development

For the experiment, 350 mg callus from the embryogenic line EC3 were placed into somatic embryo development medium. The development medium of 40mL consisted of MS medium (Murashige and Skoog 1962) with a content of sucrose of 4% (w/v) and pH adjustment at 5.7 before sterilization for 20 min at 121 °C in the autoclave. The callus was maintained in the development media for 2 days, in an incubator shaker, at 100 rpm, in the dark at 24 °C. Trichostatin A (TSA), a histone deacetylases inhibitor, was added to the mean in a concentration of 1 µM. The callus was exposed to TSA for 2 days.

Due to browning observed during the first treatment, latter treatments follow this method. However, in the first treatment, the embryogenic callus was transferred to a liquid TD medium one month prior to the treatment and then followed the method mentioned.

1.3. Somatic embryo conversion and plant acclimatization

The callus obtained from the control and treatment were then placed in an auxin-free medium that contained the established MS basal medium with sucrose (4% 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 callus was maintained in a dark room for 4 weeks and then transferred for a new development medium, where were exposed to a 16h light / 8h dark photoperiod for 4 weeks, then the somatic embryos were counted. Three months after exposure to TSA, the number of derived plantlets were registered.

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.

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

1.5. Molecular analysis during somatic embryo development

Throughout the development of embryogenic calli to somatic embryos, 3 samples of 80 – 100mg for each treatment (Control and TSA) and for each time (T1 – 2 days; T2 – 7 days, T3 – 21 days or 3 weeks; T4 – 7 days at light or 5 weeks) were collected after the TSA treatment. Samples from the calli in TD medium were also collected to be used as an initial stage (T0).

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 and Quick-RNA™ Mini Prep 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), Late Embryogenesis abundant (*LEA14*), Non-embryogenic protein – tamarillo callus (NEP-TC), Histone deacetylase 14 (*HDA-14*). *LEA14* gene is referred in literature as related with embryo development., *NEP* gene is related to non-embryogenic calli (Correia et al. 2019), as for *HDA-14* gene, it is a gene related with histone deacetylases 14, related to α-tubulin (Eshun-Wilson et al. 2019) and development stage in plants (Alinsug et al. 2012). 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, a qPCR was performed to quantify each gene expression levels. Amplifications reactions were carried out with 1 µL of the previously synthesize cDNA for all the samples, in a final reaction volume of 20 µL, containing NZY Speedy 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, 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 45s at 60°C. The amplification was performed in a BioRad CFX96 Touch™ Real Time- PCR Detection System.

Table 1 - Primer pairs information designed for *LEA14*, *NEP-TC*, *HDA14* and the reference gene *EF1-α*; F stands for Forward and R stands for Reverse

Transcript	Primer pairs (3' – 5')	
LEA14	F	CGCCACTATGAGAAAGCGGA
	R	AGTTGCCGTCAAGAACCCTT
NEP-TC	F	ACATAGCAAAGAGACACAACGTCGGAA
	R	TTGAGGAAGGTTTTAGCATCGGCAA
HDA-14	F	TCCTACTTGCACGAAGAGCC
	R	ACATGATTTACAAACTGCTTATGCC
EF1-α	F	ACAAGCGTGTCATCGAGAGG
	R	TGTGTCCAGGGGCATCAATC

The method used to analyse the qPCR data was the relative quantification method, or $2^{-\Delta\Delta CT}$ method, where the $\Delta\Delta$CT 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.

2. Effects of TSA in non-embryogenic callus proliferation and protein production

2.1. Proliferation of non-embryogenic callus

The non-embryogenic callus was prepared for 27 days, in a medium with MS ½ strength, 3% (w/v) sucrose, 5 mg/L picloran, the pH adjusted to 5,7. Half of the medium was renewed every 9 days. After this time, the cultures were passed through a 300nm sieve, to obtain desegrated cells.

2.2. TSA treatment and sampling

After attaining desegrated cells, they are kept in the same medium as before. The treatment with 1 µM TSA was done on the third and seventh day, being collected samples on day 0, 3, 7, 11.

For each sample, were removed 3mL for analysis. Using this volume of sample, 2mL were put in falcons and centrifuged at 4000rpm for 3 minutes, to determine the volume of cells and through spectrophotometry, for absorbency of 630nm, was calculated the mass of cells for each given time. For the viability test is needed 1mL of sample and a dye, named Evan's Blue in a concentration of 1% (w/v), and this count is done in a counting chamber.

2.3. Extraction and quantification of proteins

The cells for each treatment (Control, TSA-3d, TSA-7d) were grinded into a fine powder in liquid nitrogen, using a mortar and a pestle. Then was added 5mL of phosphate buffer 0,005 nM, pH 7, homogenized and transferred to a falcon. After going to a centrifugation at 14000rpm for 15 minutes, the pellet is discarded, and the supernatant is saved for quantification of proteins.

The quantification of proteins was done following the standard protocol of the QuickStart™ Bradford Protein Assay by BioRad, for microplates and using the protein Bovine Serum Albumin (BSA).

2.4. Enzymatic activities analysis

For the enzymatic activities, were utilized two groups of enzymatic substrates. The substrates of group AMC have a fluorogenic group in the C terminal, amino-methylcoumarine (AMC), and the substrates are L-Arginine-4-methylcoumaryl-7-amide (Arg-AMC), L-Methionine-4-methylcoumaryl-7-amide (Met-AMC), L-Phenylalanine-4-methylcoumaryl-7-amide (Phe-AMC), L-Lysine-4-methylcoumaryl-7-amide (Lys-AMC), L-Alanine-4-methylcoumaryl-7-amide (Ala-AMC), L-Leucine-4-methylcoumaryl-7-amide (Leu-AMC) and a complex substrate • Glycyl-L-Proline-7-amide-4-methylcoumaryl (Gly-Pro-AMC). The substrates of group MU have a flurogenic group methylumbelliferyl (MU), and the

substrates are 4-methylumbelliferyl-b-D-cellobiosid (MU-C), 4-Methylumbelliferyl-β-D-glucopyranoside (MU-G), 4-Methylumbelliferyl-acettyl-β-D-glucosaminide (MU-NAG) and 4-Methylumbelliferyl-acettyl-β-D-phosphate (MU-P).

The protein analysis is done in a fluorimeter, in which the substrate of group AMC has a 380 nm excitation wavelength, and the group MU has a 365 nm excitation wavelength, both have the same emission wavelength 460 nm.

III. Results

1. Effects of TSA in somatic embryo development

Several experiments were done to verify the effects of the histone deacetylases inhibitor TSA in somatic embryo development, however due to contaminations only two experiments were considered valid (Table 2).

There is a slight difference between treatment 1 and 2, that is due to the browning observed during maintenance of callus in liquid medium in treatment 1, which results in the loss of some mass of callus. To overpass this difficulty, in treatment 2 the callus was maintained for a mount in solid medium, and then transferred to liquid medium two days before the treatment with TSA.

Table 2 – Number of embryos and plantlets obtained from the treatments with TSA.

	Treatment 1		Treatment 2	
	Control	TSA	Control	TSA
N. º of Embryos	315	95	213	738
N. º of Plantlets	15	9	47	288

In table 2, it is possible to observe two different results of the effects of TSA in somatic embryo development when comparing treatment 1 and 2. In treatment 1, there are 315 embryos developed from Control callus and a reduced number of 95 embryos obtained from the TSA-treated callus. As for treatment 2, the results are the opposite, in which there are 738 embryos obtained from the callus expose to TSA a lower number of 213 embryos formed in the control treatment.

There is also a noticeable difference in the number of plantlets obtained from those embryos (Fig. 7A, B). From the embryos of Control callus were grown 15 and 47 plantlets of the treatment 1 and 2 respectively. As for callus exposed to TSA, 9 and 288 plants grown from the respectively treatments, from which of the 9 plantlets 4 of them were acclimate in a greenhouse with controlled environment (Fig.7E, F).

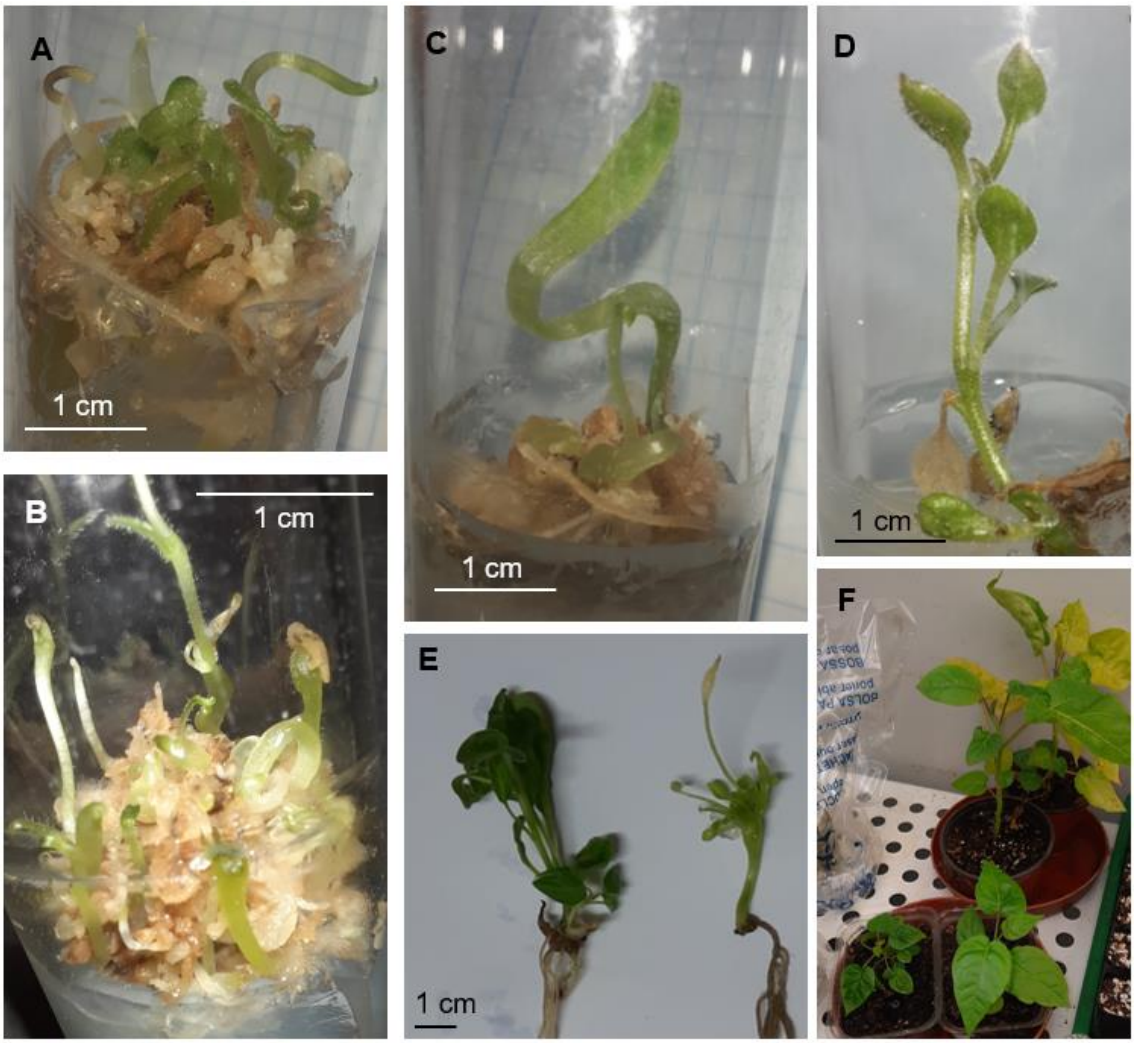

Figure 7 - Plantlets grown from embryos of the treatments (TSA and control). A – Plantlets from control after 1 month; B – Plantlets from TSA treatment after 1 month; C – Plantlet from control after 2 months; D – Plantlet from TSA treatment after 2 months; E – Plantlets with 2 months from Treatment 1 (Left – TSA treated callus; Right – Control callus) transfer to soil for acclimatization; F – Acclimatized plants from Treatment 1 with 6 months.

For treatment 2, plants were not yet acclimatized due to fragility of the plantlets that formed leaves (Fig.7C, D), since from treatment 1, only plantlets like the plantlets from TSA treatment (Fig. 7E) survived acclimatization.

The conversion of embryos/plant for treatment 1 is 9,47% for Control and 4,76% to TSA-treated callus. For treatment 2, the conversion for Control is 22% and 39% for TSA-treated callus.

Since treatment 2 had the most embryos and plantlets, it was chosen for a molecular analysis.

2. Molecular analysis of TSA-treated callus

The gene expression of *LEA14*, NEP-TC and *HDA14* was analyzed through qPCR for both TSA-treated callus and control callus for each time, previously mentioned - T0, T1, T2, T3 and T4. Through the analysis of gene expression, there is a substantial difference between TSA-treated callus and Control callus, but also between the times in both TSA-treated and control callus.

The analysis of *LEA14* (Fig. 6) showed an increase in relative expression from T0 to T1, after transfer of the callus to an auxin-free medium, followed by a decrease in T2 and T3, during embryo development, that then increases after a week in light (T4). *LEA14* expression is slightly higher in control callus than TSA-treated callus.

NEP-TC (Fig. 7) observes n increase from T0 to T1, decreasing in T2, followed by an increase in T3 and decreasing again in T4. The expression of NEP-TC in TSA-treated callus is barely higher than in control callus.

HDA14 (Fig. 8) exhibited a rise in expression from T0 to T1, diminishing in T2. On time T3 for Control callus there is a forward decrease, happening the inverse to TSA-treated callus with an increase in expression. For T4, there is an increase in expression in control callus, with the reverse happening to TSA-treated callus where is a decrease in expression.

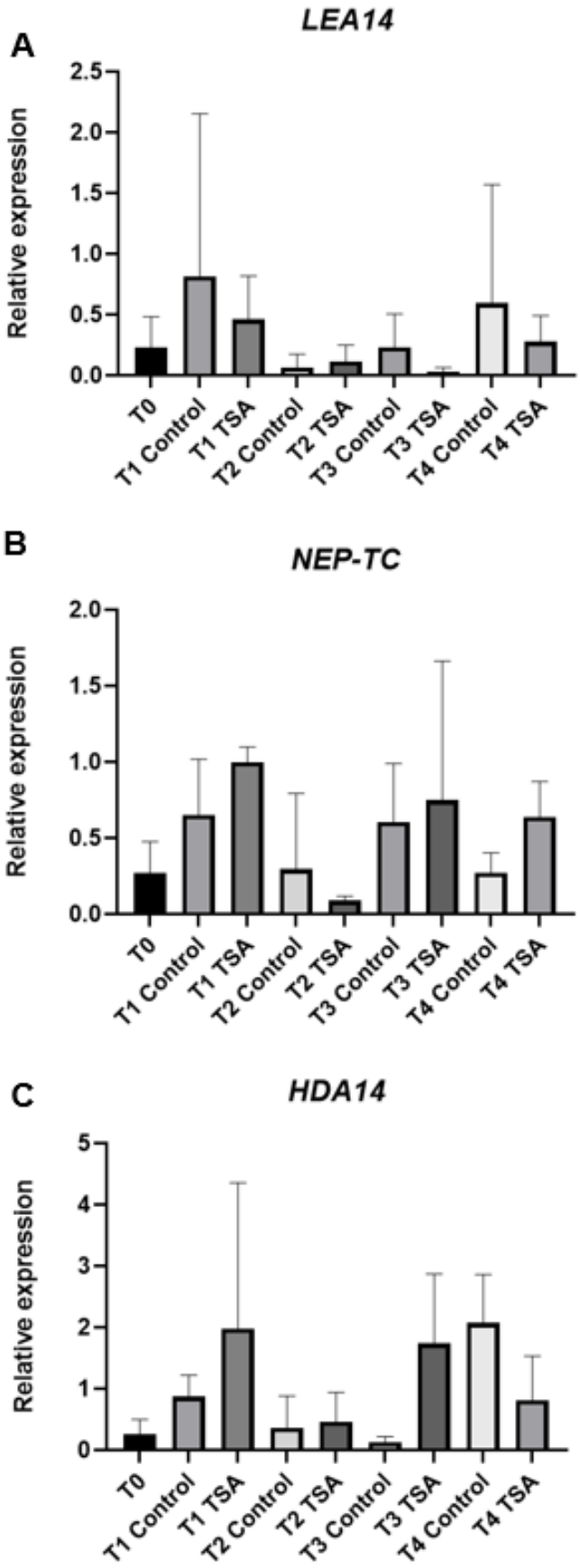

Figure 6 - Relative expression of the *LEA14* (A), NEP-TC (B) and *HDA14* (C). T0 – 0 days; T1 – 2 days; T2 – 7 days; T3 – 21 days (3 weeks); T4 – 7 days at light (5 weeks)

3. Effects of TSA on non-embryogenic callus proliferation

For the proliferation of non-embryogenic callus, it was verified the cell count in different times and those cells viability.

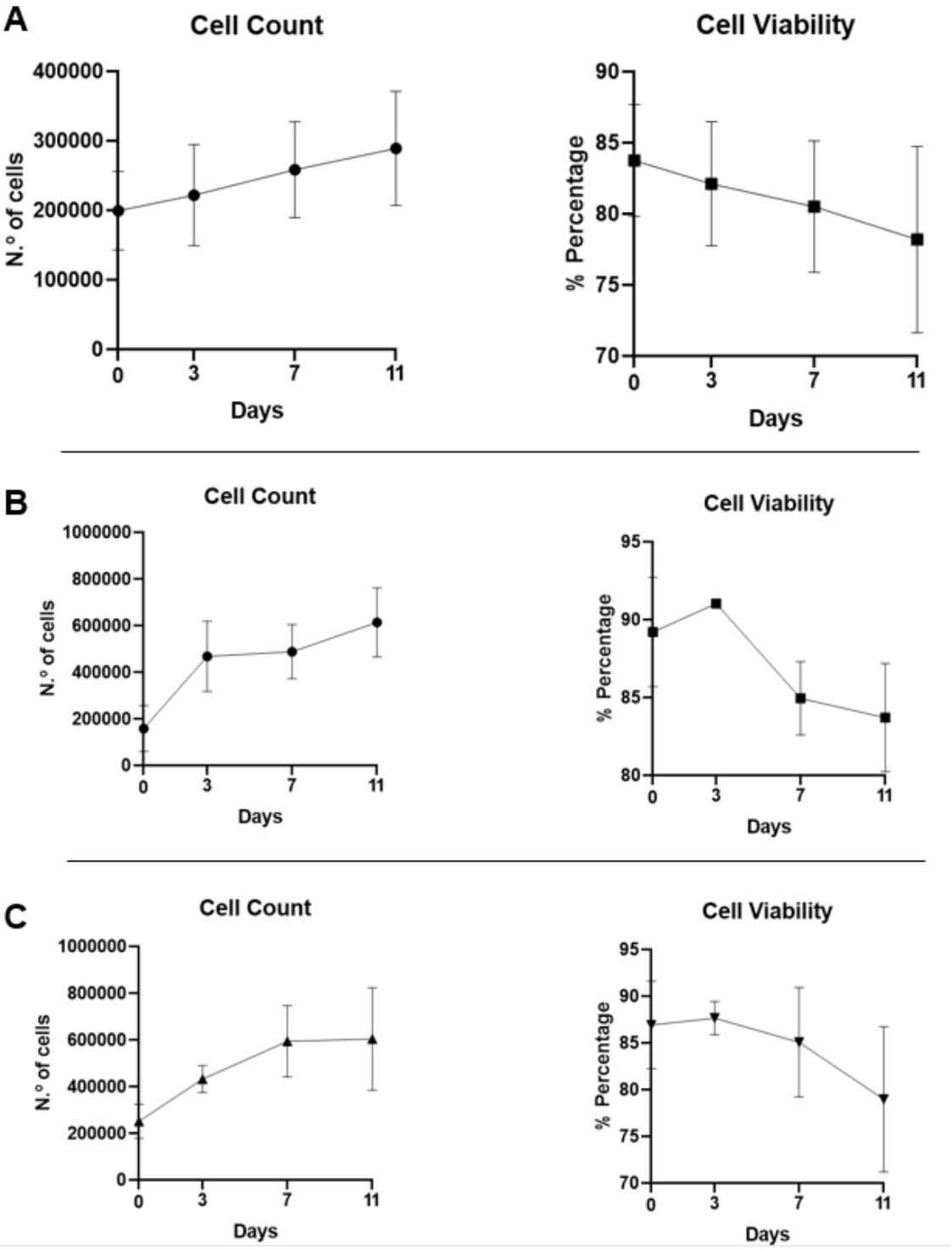

Figure 7 – Non-embryogenic callus proliferation. A – Control; B – TSA Treatment on day 3 (3d TSA); C – TSA treatment on day 7 (7d TSA).

The callus from control (Fig. 7A) grown from -± 200,000 cells to ± 300,000 cells, corresponding to an increase of 145%. These cells started with ± 83% viability and it decline to ± 78% after eleven days.

The callus treated with TSA on third day, increased from ±160,000 cells to ±600,000 cells, which is 389% increase. In figure 7B, after the treatment with TSA, the increase in cells is slow between day 3 and day 7. As for the cell viability, in the beginning was ±89%, increasing a bit to 91% and after the treatment decreasing to ±83% at day 11.

The seventh day TSA-treated callus (Fig. 7C), the cells grown from ±250,000 to ±600,000 cells, which is an increase of 240%. The cells viability was at ±87% at the start and it dropped to ±78% after day eleventh.

4. Protein quantification and Enzymatic activities

The determination of protein levels present in the different samples is crucial to verify if there are any effects of TSA in the production of protein in non-embryogenic callus.

As shown in table 3, the concentration of protein in control callus is 58.547 µg per mL, for 3d TSA treatment is 22.09 µg/mL and 24.694 µg/mL for 7d TSA treatment. Control callus was almost double of the concentration of protein to of both of TSA treatments, 3d and 7d.

Table 3 – Protein quantification of TSA treatments. 3d TSA – Treatment with TSA on day 3; 7d TSA - Treatment with TSA on day 7.

	Concentration (µg/ml)	ABS (595nm)
Control	58.547	0.459
3d TSA	22.090	0.445
7d TSA	24.694	0.446

In the enzymatic activities, there was observed negligible activity for all substrates used in the assessment.

IV. Discussion

1. Somatic embryogenesis development after TSA treatment

As stated before, two-step SE is a promising biotechnological tool, but has limitations, such as, the low conversion rates of embryos into plantlets in woody species and the loss of embryogenic ability of lines when subculture for an extended period. Some studies revel the importance of the initial explants use to initiate of embryogenesis, concluding that embryogenesis pathway is confined to a small number of cells that have the potential to activate genes related to somatic embryogenesis (Yang and Zhang 2010; Martínez et al. 2021).

Somatic embryogenesis signaling is an extraordinarily complex process where several molecular mechanisms are involved, however one of the major mechanisms is epigenetic (Yakovlev et al. 2016; Méndez-Hernández et al. 2019). The TSA treatment was chosen with the intention to obtain good quality embryos able to convert into plantlets, already demonstrated in *Arabidopsis* explants (Wójcikowska et al. 2018).

TSA (trichostatin A) is an inhibitor of histone deacetylases. Since histones play an important role in the structural organization of DNA, since it modulates DNA transcription, and those 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.

The results for the TSA treatment showed that a high number of embryos can be induced. The somatic embryos 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.

Since the somatic embryos presented good quality, the conversion rate was also effective. On the contrary, the control had low quality embryos, which almost no plantlets were formed, as referred in a previous study (Correia et al. 2012).

This suggest that TSA had led to a better chance of obtaining good quality embryos. TSA presented better conversion and survival rates than the Control treatment (Jiang et al. 2017). Maybe the adjustment of concentrations or time of

exposition to the substance may lead to better results, since the concentration of TSA was transplanted from a study in *Arabidopsis thaliana* (Wójcikowska et al. 2018).

The process by which the HDAC inhibitors modify morphogenic and embryogenic processes is not clear, although several authors have noted a potential relationship between HDACs and auxins. Wójcikowska et al. (2018)demonstrated that somatic embryogenesis can be induced by TSA in Arabidopsis in the absence of auxin application, suggesting the involvement of histone acetylation in somatic embryogenic responses to auxins. (Li et al. 2014) reported that blocking HDAC activity with trichostatin A (TSA) in cultured male gametophytes of Brassica napus leads to a large increase in the proportion of cells that switch from pollen to embryogenic growth.

2. Molecular analysis of TSA-treated callus

LEA proteins have an important role during late stages of zygotic embryogenesis and some of these proteins are considered SE markers. Therefore, *LEA14* gene has been analyzed during the somatic embryo development (Yang and Zhang 2010; Heringer et al. 2018). NEP-TC gene encodes for a RNA methyltransferase, which is found in non-embryogenic callus and was chosen to verify if the chromatin changes by TSA with affect the expression of this protein in the callus (Correia et al. 2019). HDA14 gene was also selected due to its being a gene that encodes histone deacetylase 14, associated with α-tubulin (Eshun-Wilson et al. 2019).

Although, the results did not show a difference between the control and TSA treatment, can be seen that on T2 (7 days), the expression to the genes tested is the lowest in both TSA-treated and control callus. This can be due the fact that the callus from control and TSA treatment are adapting to the solid medium, which was a lower availability to minerals and vitamins that to the liquid medium.

LEA14 proteins are associated with late development weeks, expecting for an increase in expression through third week and stay high in the conversion faze. However, in the third week (T3), only the control had an increase in

expression and an even higher expression after one week at light (T4), only where is when there is an increase of the expression of LEA14 on TSA treatment. The results obtained are similar to a previous study done by another investigator from my laboratory (Correia 2019).

NEP-TC shows a trend, in which on T1 there is a relatively high expression of protein compared to T0, followed by a decrease and again a rise in expression after another decline (Fig. 7). According to Correia et al., 2019, even though this protein is absent in ECs, it continues to reveal expression, corresponding to the results obtained, were the normal expression of NEP-TC was below 1.

HDA14 should have lower expression overall since it is strongly inhibited by TSA (Hartl et al. 2017). On T1, after the TSA treatment, was a high expression suggesting that due to the presence of TSA, there was a up regulation for the expression of HDACs, and after the removal of the histone deacetylases inhibitor it declines to a similar level to the control callus (Lee et al. 2016).

After a week in light, the increase in expression in both control and TSA treatment shown, it can be explained by being the time where the conversion from embryo to plantlet is happening, since this gene is reported to be expressed during development stages of plants (Alinsug et al. 2012).

There can be errors due to the difference in the NCE/EC ratio, leading to an uneven sampling.

3. Non-embryogenic callus proliferation after TSA treatment

For every treatment, the cell count was similar. The difference is the slight increase of cells between the counts when the TSA treatment was done. For TSA treated callus, was predicted that should have a higher proliferation relative to the control, since HDAC are involved with the processes of proliferation, differentiation and cell death (Xu et al. 2007; Rodríguez-Sanz et al. 2014)

Even though TSA can be cytotoxic in high concentrations, the viability stayed consistent between the treatments and control.

4. Protein quantification and enzymatic activities

The determination of protein quantity and enzymatic activities, permits to decide the role of the TSA on the callus. However, the quantity obtained is extremely low relative to other studies on somatic embryogenesis of tamarillo (Correia et al. 2012).

Due to the low quantity of total protein in the samples, it was not possible to determine the enzymatic activities of those cells.

V. Conclusion remarks

The usage of TSA can give a better efficiency of callus, both in embryo numbers but to with conversion from embryo to plantlet. Although, the presence of abnormal embryos is still high, and some of them can convert however they do not survive acclimatization. However, the conversion rate is low to have any commercial value, so there still a need to determine a TSA concentration suitable for tamarillo.

A molecular analysis throughout the embryo development was also carried out revealing genes' levels of expression during the 5 weeks of the embryogenic in development medium. *LEA14*, NEP-TC and *HDA14* have different roles in embryo development. LEA14 and HDA14 have a higher expression after one week at light and NEP-TC have a regular expression but a lower expression after being at light.

The proliferation of non-embryogenic callus with and without TSA did not show differences between them. As for the protein quantification, it was low than expected for both treatments and the enzymatic activity was not possible to determine due to the low quantity of protein.

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