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DNA methylation dynamics throughout indirect somatic embryogenesis of tamarillo

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DNA methylation dynamics throughout indirect somatic embryogenesis of tamarillo

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Front cover:

Tamarillo regenerated by somatic embryogenesis

Table of contents

Table of figures viii

List of abbreviations x

Agradecimentos xiii

Abstract xiv

Resumo xvi

Introduction 1

1. CONTEXT OF THE WORK 2

2. SOMATIC EMBRYOGENESIS 3

2.1. General considerations and applications 3

2.2. In vitro procedures for somatic embryogenesis: approaches and advances 4

3. TAMARILLO 12

3.1. Characterization and distribution 12

3.2. Somatic embryogenesis in tamarillo – previous work 15

4. EPIGENETICS 17

4.1. Chromatin structure 18

4.2. DNA methylation 19

4.3. Post-translational histone modifications: H3K9 methylation 23

5. EPIGENETICS ON THE CONTEXT OF IN VITRO CULTURE, DEVELOPMENTAL AND SOMATIC EMBRYOGENESIS PATHWAYS 24

6. OBJECTIVES 27

Material and Methods 28

1. PLANT MICROPROPAGATION AND INDIRECT SOMATIC EMBRYOGENESIS PROCEDURES 29

1.1. In vitro micropropagation of the tamarillo lineages used as starting point for somatic embryogenesis induction 29

1.2. Callus induction from leaf explants of seedling-derived shoots of tamarillo 29

1.3. Proliferation and maintenance of embryogenic and non-embryogenic material 30

1.4. Previously-established cultures of embryogenic masses and non-embryogenic calli 30

1.5. From embryogenic masses to embryo conversion and differentiation 32

1.6. Plant acclimatization 33

2. ANALYSING THE BEHAVIOR OF DIFFERENT EMBRYOGENIC MASSES 34

2.1. Measuring proliferation rates 34

2.2. Embryogenic potential and observation of embryo morphology 35

3. CELLULAR CHARACTERIZATION OF IN VITRO PROCESS OF SOMATIC EMBRYOGENESIS 36

3.1. Material fixation and processing for semi-thin sectioning and light microscopy analysis 36

3.2. Cytochemical techniques 37

4. 5-METHYL-DEOXY-CYTIDINE IMMUNOFLUORESCENCE 37

4.1. Denaturation of DNA of sections 38

4.2. Immunofluorescence of 5-deoxy-methyl-cytidine 38

4.3. Negative controls of immunofluorescence 38

4.4. Confocal analysis 39

5. QUANTIFICATION OF GLOBAL DNA METHYLATION 40

5.1. Extraction of total DNA from samples 40

5.2. Quantification of global DNA methylation 40

6. GLOBAL HISTONE H3K9 METHYLATION 41

6.1. Preparation of the samples for histone extraction 41

6.2. Extraction of histones 41

6.3. Quantification of total histones extracted 41

6.4. Quantification of H3K9 methylation levels.. 42

Results 44

1. OBTENTION AND CHARACTERIZATION OF NEW EMBRYOGENIC LINES OF TAMARILLO 45

1.1. Somatic embryogenesis induction and different yields among genotypes 45

1.2. Different behavior of embryogenic lines of different age during maturation phase 46

2. COMPARATIVE ANALYSIS OF EMBRYOGENIC VS NON-EMBRYOGENIC LINES 52

2.1. Cellular organization and 5-methyl-deoxy-cytidine immunolocalization 52

2.2. Global DNA methylation levels of embryogenic and non-embryogenic lines 57

2.3. H3K9 methylation within an embryogenic / non-embryogenic pair... 60

3. DYNAMICS OF DNA METHYLATION DURING SOMATIC EMBRYOGENESIS: CHANGES IN GLOBAL METHYLATION LEVELS AND DISTRIBUTION PATTERNS DURING INDUCTION AND CONVERSION PHASES 61

3.1. Changes in global DNA methylation levels at induction phase 61

3.2. Changes in cellular organization and 5-methyl-deoxy-cytidine immunofluorescence at induction phase 62

3.3. Changes in global DNA methylation levels during embryo conversion phase65

3.4. Cellular organization and 5-methyl-deoxy-cytidine immunofluorescence during embryo conversion phase 67

Discussion 72

1. DIFFERENCES IN ACQUISITION OF EMBRYOGENIC COMPETENCE, PROLIFERATION RATES AND EMBRYO CONVERSION YELDS IN CULTURES OF DIFFERENT GENOTYPE AND AGE 73

1.1. Acquisition of embryogenic competence by somatic cells is affected by explant genotype 73

1.2. Embryo conversion competence strongly decays with long-term culture 73

1.3. Proliferation rates are higher in non-embryogenic lines and habituated long-term embryogenic lines 74

2. DNA METHYLATION LEVELS IN EMBRYOGENIC AND NON-EMBRYOGENIC CULTURES: DIFFERENCES WITHIN PAIRS AND OVER CULTURE TIME 75

2.1. In recently induced lines, embryogenic masses have lower levels of methylated cytosines and H3K9 than non-embryogenic calli 76

2.2. Culture time modifies DNA methylation state of originally embryogenic cells, what may account for loss of the embryogenic competence 77

3. EMBRYOGENIC VS NON-EMBRYOGENIC CALLI AT A GLANCE: A CYTOLOGICAL AND EPIGENETIC PERSPECTIVE 78

3.1. Embryogenic calli show a very heterogeneous cellular structure and differential 5-methyl-cytidine distribution 79

3.2. Non-embryogenic calli are constituted by sparse cells with large vacuoli and condensed chromatin 80

4. FROM PLANT EXPLANTS TO SOMATIC EMBRYOS: UNDERSTANDING DNA METHYLATION DYNAMICS THROUGHOUT INDIRECT SOMATIC EMBRYOGENESIS OF TAMARILLO 81

4.1. Induction treatment and dedifferentiation process 81

4.2. Embryo conversion and differentiation 85

Conclusions 89

References 92

Table of figures

Figure 1 – Schematic representation of the regeneration of plantlets by SE. (p. 5)

Figure 2 - The various ways of triggering somatic embryogenesis. (p. 9)

Figure 3 – Tamarillo tree growing on the Botanical Garden of the University of Coimbra. (p. 13)

Figure 4 – Schematic representation of the methylation reaction of cytosines in C5. (p. 21)

Figure 5 – The hierarchy of events and conditions intervening in the change of cell fate. (p. 26)

Figure 6 - Schematic representation of SE induction and plant regeneration in tamarillo. (p. 33)

Figure 7 – Morphological aspects of SE induction process starting with young leaves of micropropagated shoots of tamarillo. (p. 48)

Figure 8 – Morphological aspects of embryo conversion and germination of tamarillo from recent embryogenic masses. (p. 49)

Figure 9 – Proliferation of embryogenic cell lines during the maturation phase. (p. 50)

Figure 10 – Morphology of old embryogenic cell lines after three weeks in MS medium without auxin. (p. 52)

Figure 11 – Cellular organization and 5mdC immunofluorescence of old embryogenic cell lines. (p. 54)

Figure 12 - Cellular organization and 5mdC immunofluorescence of recent embryogenic lines. (p. 55)

Figure 13 – Embryogenic cell masses exhibiting protusions. (p. 56)

Figure 14 - Cellular organization and 5mdC immunofluorescence of embryogenic cell lines. (p. 58)

Figure 15 – Cellular organization and 5mdC immunofluorescence of non-embryogenic cell lines. (p. 59)

Figure 16 – Global DNA methylation levels among embryogenic / non-embryogenic cell lines. (p. 59)

Figure 17 – Global H3K9 and DNA methylation levels in embryogenic and non-embryogenic lines. (p. 61)

Figure 18 – Global DNA methylation levels of young fresh leaves and during induction phase. (p. 62)

Figure 19 – Cellular organization and 5mdC immunofluorescence of dedifferentiating tissues of a TSM10 leaf after 6 months on induction medium. (p. 63)

Figure 20 – Cellular organization and 5mdC immunofluorescence of young leaves before induction phase. (p. 64)

Figure 21 – Global DNA methylation levels throughout maturation phase of YL2016a2 embryogenic line. (p. 65)

Figure 22 – Global DNA methylation levels throughout maturation phase of YL2014 embryogenic line. (p. 66)

Figure 23 – Embryogenesis initiation stage. (p. 68)

Figure 24 – Developing embryo stage. (p. 69)

Figure 25 – Developing embryo stage. (p. 70)

Figure 26 – Developing embryo stage, protoderm differentiation. (p. 71)

List of abbreviations

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

5mdC: 5-methyl-deoxy-cytidine

ABA: abscisic acid

APTES: aminopropiltriethoxi silane

ATPases: adenosinetriphosphatases

AzaC: 5-azacytidine

BAP: 6-benzylaminopurine

BER: base-excision repair

BSA: bovine serum albumin

CMT3: chromomethylase 3

DAPI: 4’,6-diamidino-2-phenylindole

dC: (unmethylated) deoxicytosine

DDM1: DEFICIENT IN DNA METHYLATION 1

DME: DEMETER

DML(2/3): DEMETER-LIKE (2/3)

DNA: desoxirribonucleic acid

DRD1: DOPAMINE RECEPTOR D 1

DRM2: DOMAINS REARRANGED METHYLTRANSFERASE 2

dsDNA: double-stranded DNA

DSE: Direct Somatic Embryogenesis

GA_3: gibberellic acid

H1: histone protein 1

H2A, H2B, H3 and H4: octameric histone proteins

H3K9me(2,3): (di-, tri-) methylated histone-3-lysine-9

ISE: Indirect Somatic Embryogenesis

KYP/SUVH4: *KRYPTONITE*

LEC1/2: *LEAFYCOTYLEDON*

MBD: methyl-CpG-binding domain

mdC: methylated deoxicytosine

MET1: METHYLTRANSFERASE 1

MS: Murashige and Skoog basal medium (1962)

NAA: 1-naphthaleneacetic acid

OD: optical density

PBS: phosphate buffered saline

PEM: proembryogenic masses

PGRs: plant growth regulators

PHD: plant homeo domain

PKL: *PICKLE*

RdDM: RNA-directed de novo methylation

RH: relative humidity

RNA: ribonucleic acid

RNase A: ribonuclease A

ROS1/DML1: DNA N-glycosylase protein/DEMETER-LIKE 1

SAM: S-adenosylmethionine

sd: standard deviation

SE: Somatic Embryogenesis

siRNA: small interfering ribonucleic acid

TF: transcription factor

TP: induction medium for young expanded leaves of tamarillo

UV: ultraviolet

VIM1-3: VIMENTIN 1-3

WUS: *WUSCHEL*

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// Abstract

Plants are endowed with a developmental plasticity which allows them to adapt to changes in environmental conditions that surround them. At a cellular level, the balance between stability and plasticity is accomplished through temporal and spatial control of gene expression, chromatin organization and adequate response to external stimuli, which might induce reversion and switch of the fate of a cell population. Epigenetic mechanisms are hallmarks of the regulation of development and acquisition or loss of embryogenic competence both *in vivo* and *in vitro* plant systems.

Somatic embryogenesis (SE) is a powerful biotechnological tool, with practical applications in agriculture, such as crop improvement and large-scale production. Moreover, SE has great utility for research on plant's development, since it provides a reliable *in vitro* system in which almost any physiologic, morphologic and biochemical aspect of embryogenesis can be studied.

However, woody species are frequently recalcitrant to this technique, impairing scientific advances and profitable solutions for agriculture and forestry, particularly the possibility of selecting and propagating interesting traits. Despite all the difficulties, successful SE in some tree species has already been achieved. *Solanum betaceum* Cav. (Sendt.), the tamarillo tree of the solanaceous family, is among those promising cases.

In this work it was analysed how one of the most relevant epigenetic marks, cytosine methylation, varies on tamarillo indirect somatic embryogenesis (ISE) *in vitro* system. Both global DNA methylation levels and immunofluorescence of 5-methyl-deoxy-cytidine (5mdC) were assayed, among different cell lines and throughout the first stages of somatic embryo development, enabling a comprehensive characterization of the process.

The 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. Non-embryogenic *calli*, even when recently induced, show high methylation levels. A

similar relationship was found when histone methylation was compared between embryogenic and non-embryogenic *calli* recently induced from the same line.

Embryogenic masses are histologically very heterogeneous, often exhibiting protrusions where 5mdC immunofluorescence signal is generally low. Non-embryogenic cells present large vacuoles and small nucleus with condensed chromatin.

Throughout dedifferentiation of somatic tissues towards the formation of non-embryogenic and embryogenic *calli*, achieved with application of stress and auxin-containing induction medium, a global decrease on DNA methylation levels was verified. Otherwise, embryo differentiation was accompanied by a progressive increase of DNA methylation levels. An initial hypomethylation moment, upon auxin removal, might be essential for triggering embryo conversion, though.

The present work is a contribution to the understanding of the epigenetic events, particularly the changes on DNA methylation, that occur on cells and particular structures throughout the ISE of tamarillo system.

Key words: *callus* proliferation; embryogenic competence; global DNA methylation; histone-3-lysine-9 methylation (H3K9); immunofluorescence; *in vitro* culture; somatic embryogenesis (SE); tamarillo tree.

Resumo

Os organismos vegetais são dotados de uma plasticidade no desenvolvimento que lhes permite adaptarem-se às alterações do ambiente circundante. A nível celular, o equilíbrio entre estabilidade e plasticidade depende do controle temporal e espacial da expressão genética, organização da cromatina e resposta adequada aos estímulos externos, que podem induzir a reversão e mudança do programa de desenvolvimento das células. Os mecanismos epigenéticos são pedras basilares do desenvolvimento e da aquisição ou perda da capacidade embriogénica, tanto *in vivo* como *in vitro*.

A embriogénese somática é uma poderosa ferramenta biotecnológica com aplicações práticas na agricultura, como sendo o melhoramento de plantas de cultivo e a sua produção em larga escala. Além disso, a embriogénese somática tem grande utilidade para a investigação na área do desenvolvimento vegetal, uma vez que representa um sistema fiável de embriogénese *in vitro* no qual praticamente qualquer aspeto fisiológico, morfológico e bioquímico pode ser estudado.

Contudo, as espécies arbóreas são frequentemente recalcitrantes à aplicação desta técnica, o que condiciona o progresso científico e o desenvolvimento de soluções proveitosas para a agricultura e silvicultura, nomeadamente a possibilidade de selecionar e propagar características interessantes. Não obstante todas as dificuldades, em algumas árvores a embriogénese somática foi já realizada com sucesso. Um desses casos promissores é a árvore do tamarilho, *Solanum betaceum* Cav. (Sendt.).

No presente trabalho analisou-se o modo como uma das mais relevantes marcas epigenéticas, a metilação de citosinas do DNA, varia ao longo do sistema *in vitro* de embriogénese somática indireta do tamarilho. Levaram-se a cabo técnicas de quantificação dos níveis totais de metilação do DNA e imunofluorescência da 5-metil-deoxi-citidina (5mdC) em diversas linhas celulares e ao longo dos primeiros estádios de desenvolvimento dos embriões somáticos, permitindo uma caracterização bastante completa do processo.

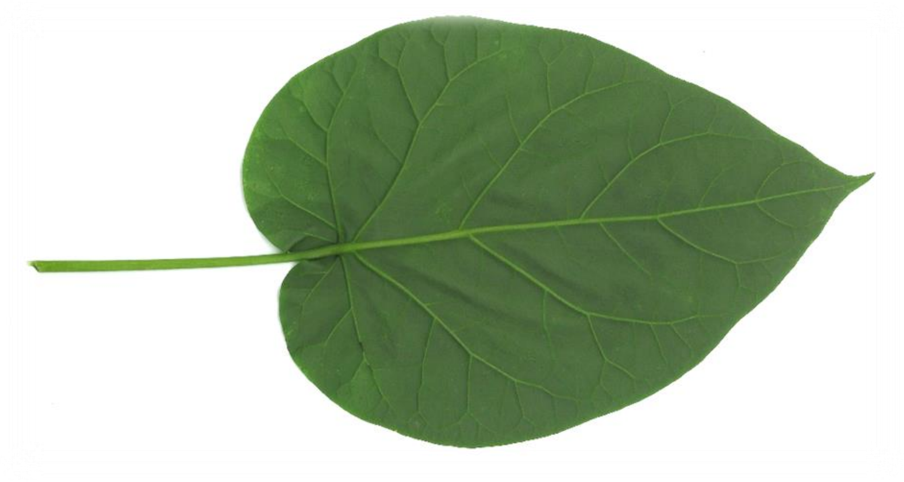
Os resultados revelaram uma tendência geral das linhas de células embriogénicas para proliferar mais e perder capacidade embriogénica quando mantidas em cultura durante um alargado período de tempo. Tais mudanças são acompanhadas de uma progressiva acumulação de metilação de DNA. Os calos não embriogénicos, por sua vez, apresentam elevados níveis de metilação, mesmo quando recentemente induzidos. Quando se comparou o nível de metilação de histonas entre calos embriogénicos e não embriogénicos, a relação encontrada foi semelhante.

As massas embriogénicas são histologicamente muito heterogéneas, sendo comum o surgimento de protusões celulares onde a intensidade do sinal de fluorescência da 5mdC é geralmente baixa. As células não embriogénicas possuem grandes vacúolos e núcleos pequenos com cromatina geralmente condensada.

À medida que os tecidos somáticos desdiferenciam para formar calo não embriogénico e embriogénico, processo que é conseguido através da aplicação de stress e auxinas no meio de indução, foi observada uma diminuição nos níveis globais de metilação do DNA. Por outro lado, a diferenciação dos embriões somáticos foi acompanhada por um progressivo aumento destes níveis. Contudo, um breve momento de hipometilação após a remoção da auxina do meio poderá ser fulcral para o sucesso da conversão de embriões.

O presente trabalho é uma contribuição para a compreensão dos eventos epigenéticos, em particular das oscilações na metilação do DNA, que ocorrem nas células e estruturas específicas da embriogénese somática indireta do tamarilho.

Palavras-chave: árvore do tamarilho; capacidade embriogénica; cultura *in vitro*; embriogénese somática; imunofluorescência; metilação da lisina 9 da histone 3 (H3K9); metilação do DNA; proliferação de calo.



Introduction

1. CONTEXT OF THE WORK

Somatic embryogenesis (SE) is a biotechnological tool which consists of obtaining clonal plantlets from a somatic explant (a leaf, hypocotyl, zygotic embryo, etc.) of a plant (Loyola-Vargas et al., 2008; Canhoto, 2010) through the formation of embryos similar to their zygotic counterparts (Leljak-Levanić et al., 2015). It has great relevance, not only as an option for large-scale propagation of crops and a basis for genetic improvement of plants, but also from a research perspective, since it provides an *in vitro* controlled system to study acquisition of totipotency competence as well as embryo formation and development (Fehér, 2015; Testillano and Risueño, 2016).

Many species, particularly woody plants, are recalcitrant to this technique (Correia et al., 2011; Corredoira et al., 2017). Many approaches have been taken in order to better understand the biochemical, physiological and genetic mechanisms regulating and occurring during plant embryogenesis (Yang and Zhang, 2010; Elhiti et al., 2013; Mahdavi-Darvari et al., 2015). The aim is to improve SE protocols, in a species-specific manner, and therefore increase the yields of the technique and making it suitable for large-scale cloning.

One aspect that has been subject of much recent interest in the scientific community is epigenetics. The term 'epigenetics' comprises all the mitotically and meiotically heritable changes in gene expression which do not relate directly with the DNA sequence itself, but can still be reliably inherited from one generation to another (Tsaftaris et al., 2005; Chen et al., 2010b; Munshi et al., 2015). Amongst the most studied epigenetic marks are DNA methylation and histone-3-lysine-9 (H3K9) methylation, which are associated with transcription-repressive functions (Li et al., 2002). Both of them are known to play a central role in developmental processes (Miguel and Marum, 2011; Nic-Can et al., 2013).

Solanum betaceum Cav. (for many years named *Cyphomandra* betacea), commonly known as tamarillo, is a sub-tropical tree whose main commercial interest is its yellow, orange or red, egg-shaped fruits, with a sweet-sour taste (Prohens and Nuez, 2000). In the recent years, a SE protocol has been developed which allows the full regeneration of plantlets from adult individuals (Correia et al., 2011). Ongoing research has been revealing ways to improve the

process through application of plant growth regulators at specific times and concentration and also through manipulation of other medium components and culture conditions (Correia & Canhoto, 2012; Correia et al., 2009, 2012a). Investigation on the regulatory mechanisms and molecular marks of the embryogenic process, metabolic and proteomic characterization of material in various stages, also provides important insights into the SE in tamarillo (Correia et al., 2016, 2012b). In this context, epigenetic marks and DNA methylation patterns have been arising as promising investigation fields.

2. SOMATIC EMBRYOGENESIS

2.1. General considerations and applications

Somatic embryogenesis consists of a developmental program shift that causes somatic cells to embark on a new developmental pathway, ultimately resulting on the formation of structures similar to zygotic embryos, without occurrence of gamete fusion (Jiménez, 2001; Fehér et al., 2003; Queiroz-Figueroa et al., 2006; Karami et al., 2009).

The competence for SE seems to be exclusive to plant organisms and was first reported by two different research groups (Reinert, 1958; Steward et al., 1958) working on carrot. Since then, the number of species in which entire plants can be regenerated from cell cultures through this process did not stop to increase. (Bajaj, 1995; Fehér, 2005; Omar et al., 2016). Not only somatic cells, but also microspores have been extensively used to embryogenesis induction (Bárány et al., 2005; Prem et al., 2012).

Somatic embryogenesis is a form of asexual reproduction that may be equated to some naturally occurring phenomena, such as spontaneous formation of somatic embryos on the leaf tips of *Malaxis* sp. plants (Taylor, 1967), or to any general apomictic process (Koltunow, 1993; Zavattieri et al., 2010).

Somatic embryos are similar to zygotic embryos in what concerns some morphologic aspects such as bipolar organization, progression through the typical stages of development (globular, heart, torpedo and cotyledonary), absence of vascular connections with parental tissues and even the formation a

suspensor-like structure (Correia and Canhoto, 2010). Furthermore, a comparative transcriptome analysis between somatic and zygotic embryos in cotton revealed that more than 50 % of highly expressed genes throughout embryo development are shared by somatic and zygotic embryogenesis(Jin et al., 2014; Leljak-Levanić et al., 2015).

Nowadays SE is an important biotechnological tool with wide applications in plant improvement, genetic engineering, artificial seed production and large-scale propagation of crops (Deverno, 1995; Loyola-Vargas et al., 2008; Canhoto, 2010; Mahdavi-Darvari et al., 2015). The combination of SE with cryopreservation is especially useful in the selection of superior genotypes (von Aderkas and Bonga, 2000), particularly for perennials with long life cycles.

As an *in vitro* system of embryo development, SE can also provide valuable insights into the biochemical, physiological and morphological aspects regulating dedifferentiation, totipotency and higher plant embryogenesis, which would otherwise be difficult to study due to the obstacles on accessing specific cell types inside the very young embryo or endosperm (Zimmerman, 1993; Queiroz-Figueroa et al., 2006; Mahdavi-Darvari et al., 2015; Testillano and Risueño, 2016).

From a cellular perspective, during SE a high number of genes functioning in differentiated cells shall be suppressed at the same time as some genes necessary for embryogenesis are activated (Figueroa et al., 2002; Mahdavi-Darvari et al., 2015). Increasing evidence suggest that that epigenetic mechanisms operate by modulating gene expression throughout these events (Miguel and Marum, 2011; Solís et al., 2012; Fehér, 2015).

2.2. *In vitro* procedures for somatic embryogenesis: approaches and advances

To accomplish the formation of somatic embryos in *in vitro* plant systems, a complex network of interactions among PGRs (Rose, 2004) and stresses (Pasternak et al. 2002; Zavattieri et al. 2010; Fehér 2008, 2015) must be managed. Indeed, stresses and hormones (particularly auxins) are recurrently

indicated as the most important factors for controlling differentiation state and inducing embryogenic competence in cells (Thomas and Jiménez, 2005).

Somatic embryogenesis can be performed *in vitro* following either a direct (DSE) or indirect (ISE) process (Rose, 2004). In DSE, somatic embryos form directly on an organized tissue such as a leaf or stem segment explant, whereas ISE is achieved via an intermediary step of callus or suspension culture (Dudits et al., 1991; Fehér et al., 2003). It was suggested that for DSE induction and differentiation, only minimal gene reprogramming is required, once that proembryogenic competent cells might already be present and the simple change of external conditions will trigger its embryogenic expression (Willemsen and Scheres, 2004).

Generally, one considers that SE comprises two main stages: induction of embryogenic potential and expression of the embryogenic program, also known as conversion phase (Jiménez, 2001; Namasivayam, 2007). A schematic representation of the general process of ISE, including induction and conversion phases and an extra stage of embryogenic and non-embryogenic cells proliferation, is presented in Fig. 1.

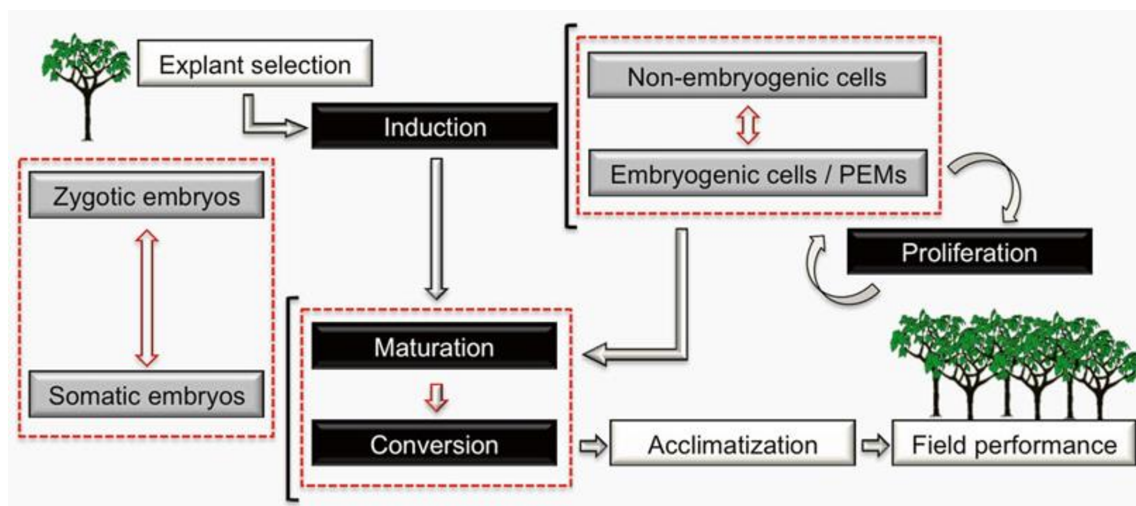


Figure 1 – Schematic representation of the regeneration of plantlets by ISE. Adapted from Correia et al. (2016).

2.2.1. Induction phase, acquisition and maintenance of embryogenic competence

The induction phase is the earliest stage of cellular dedifferentiation (Mahdavi-Darvari et al., 2015), and a step of paramount importance: somatic cells acquire embryogenic competence. It is a multifactorial event (Karami et al., 2009): ranging from the arabinogalactan proteins (AGP) dynamics on the extracellular environment (de Vries et al., 1988; Kreuger and van Holst, 1993; Schmidt et al., 1994; El-Tantawy et al., 2013) to intense enzymatic activity (particularly kinases) (Ramírez et al., 2004), conversion of ATP to ADP, increase of the oxygen uptake and cytoskeleton rearrangement (Mahdavi-Darvari et al., 2015), a lot of changes occur (Testillano and Risueño, 2009; Smertenko and Bozhkov, 2014; Fehér, 2015).

Stress generally causes cell division, alters the differentiation state and inhibits cell elongation (Potters et al., 2007). Cellular functions linked to the stress response can play a role in activation of the embryogenic developmental program (Dudits et al., 1991). However, the exact induction conditions and responsiveness of the cells is highly variable among species, cultivars and tissue employed (Guimarães et al., 1988). Nutritional components of the medium and physical factors can also affect SE induction efficiency in some cases (Rose, 2004).

The role of auxin in embryogenesis induction has been recognized for many years (Schiavone and Cooke, 1985; Liu et al., 1993), since the genetic, epigenetic, metabolic and physiological responses of cultured cells to these regulators will often lead to switching of cell fate, namely the acquisition of embryogenic competence by somatic cells.

In addition, auxins are also known for being related to stress response (Pasternak et al., 2002; Zavattieri et al., 2010). Some interesting correlations between stress, auxins and SE initiation have been extensively reported in the literature (Pasternak et al. 2002; Fehér et al. 2003; Fehér 2005, 2015). It is, indeed, the PGR/stress coupled intervention that is thought to trigger SE induction (Fehér et al., 2003). The fact is, the auxin itself (and other components of the medium) can be seen as stress agents which stimulate cells to adapt and embark on new developmental pathways (Chugh and Khurana, 2002; Karami et al., 2009; Zavattieri et al., 2010; Us-Camas et al., 2014; Fehér, 2015).

The auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is particularly efficient: many *in vitro* systems depend on the use of exogenous 2,4-D as a SE inducer (Pasternak et al., 2002; Fehér et al., 2003; Yamamoto et al., 2005). This synthetic auxin triggers DNA methylation and chromatin structure changes, either directly (De Klerk et al., 1997) or indirectly, by prompting stress-response mechanisms that lead to such epigenetic manifestations (Leljak-Levanić et al., 2004; Fehér, 2015).

The importance and interaction among the various classes of the most well-known inducing factors of plant cell fate reprogramming (namely PGRs and stress) are evidenced in Fig. 2. Notably, this schematic representation of 'competent-differentiated-cell-to-somatic-embryo' pathway highlights the role of changes in gene expression to the switch from the differentiated cell state to a dedifferentiated (and, subsequently, proembryogenic) state.

The induction process often results in the formation of clusters of small cells with dense cytoplasmic content and prominent nuclei (Zimmerman, 1993; Oropeza et al., 2001; Park et al., 2011; Moon et al., 2013). With a friable, opaque macroscopic appearance, the embryogenic clumps usually differentiate after 6-12 weeks on induction medium (a very variable amount of time depending on the species and tissue used as explant), in the case of an ISE protocol (Chakrabarty et al., 2003). These are referred to as proembryogenic masses (PEM) and can either protrude from the somatic explant or grow over it (Halperin, 1966). They have both division and differentiated activity (Pérez et al., 2015).

In ISE these initial embryogenic clumps are often isolated from the remaining cells of the explant of origin, and put onto fresh medium for subculture (Dudits et al., 1991; de Jong et al., 1993). Often new PEM form and a striking heterogeneity on the cellular morphology can happen (Guimarães et al., 1988; Toonen et al., 1994). It is common at this stage to distinguish embryogenic from non-embryogenic *calli*, both of them resulting from the same induction event (Ikeda-Iwai et al. 2002; Queiroz-Figueroa et al. 2002, 2006; Correia 2011).

The competence for embryogenesis is thought to be expressed at the level of the single cell, which usually display an early activation of the division cycle, changes in internal cellular pH (Deslauriers et al., 1991), altered auxin metabolism and non-functional chloroplasts (Pasternak et al., 2002). The endogenous auxin content undoubtedly plays a crucial role during acquisition of embryogenic

competence by cells. However, it is inadequate as a molecular marker, since internal levels of hormones are highly variable and unpredictable among different genotypes and species, and also between competent and non-competent genotypes (Jiménez and Thomas, 2005; Zavattieri et al., 2010). It is crucial then that effective markers for embryogenic competence start to stand out and be characterized. Epigenetic marks arise in this context as potential markers of SE that deserve to be explored (Mahdavi-Darvari et al., 2015).

Embryogenic competence can be retained over successive subcultures of the embryogenic masses (Guimarães et al., 1988; Lopes et al., 2000; Correia and Canhoto, 2012), thus offering great potential for large-scale production and for genetic transformation (Merkle et al., 1995; Thorpe and Stasolla, 2001). Auxins (and sometimes cytokinins) are required also in this 'proliferation phase' and maintenance of PEM (Fehér et al., 2003), generally inhibiting the development of somatic embryos (Nomura and Komamine, 1985; Filonova et al., 2000; Leljak-Levanić et al., 2004).

However, in long-period cultures a substantial loss of embryogenic potential has been reported in many systems (von Arnold et al., 2002; Canhoto, 2010; Correia and Canhoto, 2012; Smertenko and Bozhkov, 2014). *Callus* instability, variations in the chromosome number and DNA amount, somaclonal variation and hormone habituation are some of the phenomena that might account for that loss (Deverno, 1995; Currais et al., 2013; Us-Camas et al., 2014).

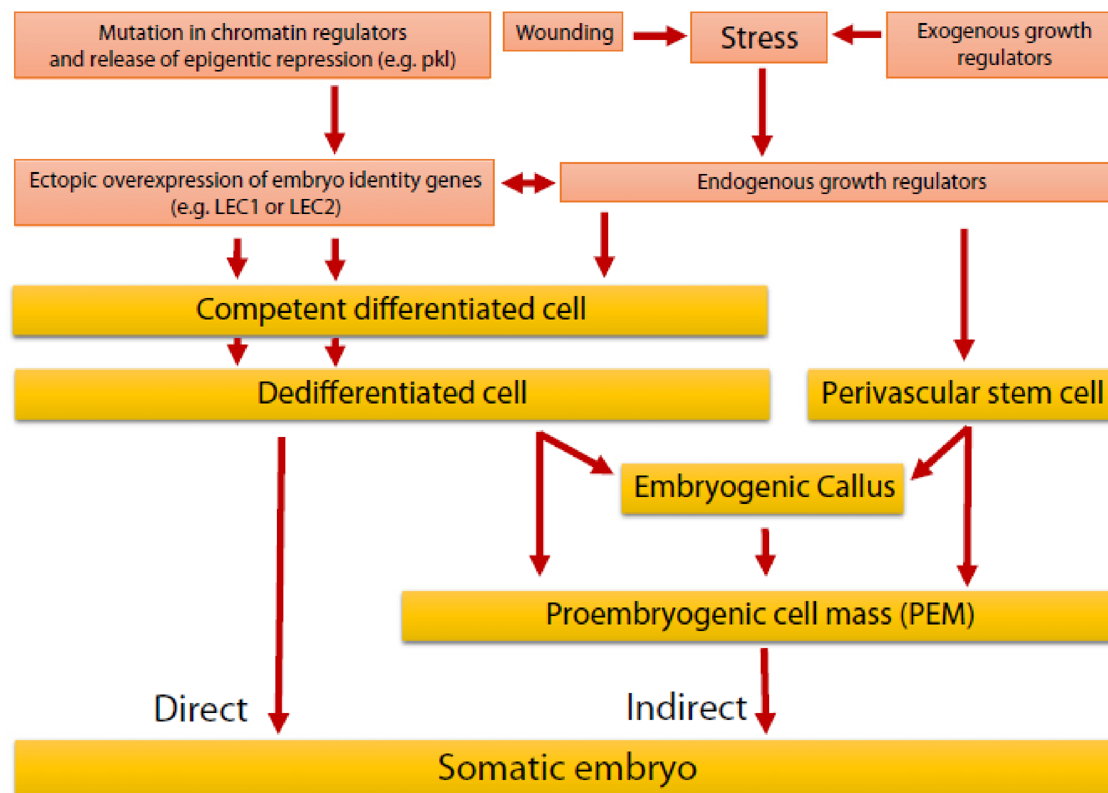


Figure 2 - The various ways of triggering somatic embryogenesis. The embryogenic pathway can be initiated in differentiated cells directly or indirectly or in perivascular cells through *callus*/PEM formation. Endogenous or exogenous signals can result in dedifferentiation in competent cells followed by direct embryogenesis or overproliferation and *callus* tissue differentiation. Under further signals cells of the embryogenic *calli* can proceed to somatic embryo conversion. *PKL*: *PICKLE*; *LEC1* and *LEC2*: *LEAFYCOTYLEDON* (Fehér, 2015).

2.2.2. Conversion phase and somatic embryo formation

Contrarily to the great variability regarding the SE inducing factors effective for each plant species, conversion factors, *i.e.* factors that are required for *in vitro* embryo development, maturation and conversion to plantlets, appear to be quite consistent (Rodríguez-Sanz et al., 2014a) among species: removal of PGRs (auxins) from the medium is indicated in most ISE protocols as triggering the formation of somatic embryos from embryogenic *calli* (Yamada et al., 1967; Thomas and Street, 1972; Matsuoka and Hinata, 1979; Reynolds, 1986; Lopes et al., 2000; El-Tantawy et al., 2014; Testillano et al., 2017). Removing the auxin from the medium has multiple effects. One of them is the lowering of endogenous

antioxidants. The redox status of cells was suggested to play a role on determination of a proliferative *vs* differentiative growth (Earnshaw and Johnson, 1987).

Following that, it becomes evident that auxin concentration plays a central role on the control of SE progression *in vitro*. PGR's other than auxins may be also employed in order to obtain higher embryo formation from PEM in some species, as it is the case of the cytokine 6-benzylaminopurine (BAP) in *Schisandra chinensis* (Chen et al., 2010a).

Manipulation of other culture conditions and media components (such as nitrogen source, chemicals, carbohydrate type and concentration, light conditions and physical stresses) have also showed to be important to the effectiveness of SE, in a system-specific manner (Correia et al., 2012a). For instance, maltose was observed to promote somatic embryo development in *Castanea sativa* Mill. (Corredoira et al., 2003). However, in a phylogenetically close species, *Castanea dentata* (Marsh.) Borkh., this carbohydrate was very poor in supporting conversion of somatic embryos from proembryogenic masses (Carraway and Merkle, 1997), being sucrose most effective in this species (Xing et al., 1999). Higher concentrations of sucrose in the medium were reported to improve somatic embryo's maturation in other species, for example holm oak (Mauri and Manzanera, 2003) and tamarillo (Correia and Canhoto, 2012).

Other examples of compounds that were reported to affect somatic embryo maturation in specific systems were sorbitol, which when added to the culture medium increased the germination and conversion frequency of somatic embryos in soybean (Walker and Parrott, 2001) and the nitrogen source in *Cucurbita pepo* L. embryogenic *calli*: the use of an inorganic nitrogen source (NH4Cl) lead to generation of somatic embryos predominantly in preglobular and globular stage, while use of an organic nitrogen source promoted progression to further stages (Leljak-Levanić et al., 2004).

The development of embryos from PEMs is generally asynchronous and the conversion rates can be very variable depending on species, genotype or *in vitro* conditions (Canhoto et al., 2005). The formation of aberrant embryo-like structures is also common, though this may not always imply impairment of a normal plant regeneration (Correia, 2011).

2.2.3. Effectiveness of somatic embryogenesis protocols

Woody species are often recalcitrant to SE protocols (Pinto et al., 2008; Correia et al., 2011; Corredoira et al., 2017), therefore, the application of SE to tree breeding remains limited. *Acca sellowiana* is considered a reference system for SE of woody plants (Guerra et al., 1997; Fraga et al., 2012; Correia et al., 2016). Other tree species in which SE induction has been experimented are economically relevant, among them various species of the genus *Quercus* (Pinto et al., 2002b; Barra-Jimenez et al., 2014; Correia et al., 2016; Corredoira et al., 2017) and *Eucalyptus* (Pinto et al., 2002a; Corredoira et al., 2015). Concomitantly, some reports have dealt with *in vitro* development of organogenesis and embryogenesis in tree species, as well as with potential molecular markers for early identification of these developmental processes (Bueno et al., 2003; Ramírez et al., 2004; Solís et al., 2008; Germanà et al., 2011; Rodríguez-Sanz et al., 2014a). Tamarillo is a tree species for which SE have been studied not only as a cloning approach but also as an experimental embryology system (Correia and Canhoto, 2012).

We shall take into account that, despite the numerous *in vitro* systems developed in numerous species, these have been frequently based on trial/error results, more than on the knowledge of the regulating mechanisms (El-Tantawy et al., 2013). In fact, the cellular and molecular mechanisms underlying SE induction and progression are just now starting to be understood (Yang and Zhang, 2010). Understanding the cellular processes that operate during induction and progression of SE will help to manipulate the process more efficiently and therefore enhance embryo productivity in plant species of interest (Bárány et al., 2005).

3. TAMARILLO

3.1. Characterization and distribution

Solanum betaceum Cav., commonly known as ‘tree tomato’, ‘tomato de La Paz’ (Bois, 1927) or, since 1967, as tamarillo, is a sub-tropical shrub or small tree of the Solanaceae family which was first described in 1801 by Cavanilles. ”Tama” implies “leadership” in Maori, whereas “rillo” directs to an hispanic relation (New Zealand Tamarillo Growers Association 2008).

The species has its center of origin in the Andean region, more specifically Bolivia, Chile, Ecuador and Peru (Dawes and Pringle, 1983). It spread to Central and Western India, then to the Portuguese atlantic islands of Madeira and Azores (where it is known as ‘pirola’) and to southern Europe and finally, by the end of 19th century, reached Oceania (Hooker, 1899; Slack, 1976; Atkinson and Gardner, 1993).

Currently it is grown on a commercial scale in California, Ecuador, Colombia, Australia and New Zealand (Dawes & Pringle 1983; Prohens & Nuez 2000; New Zealand Tamarillo Growers Association 2008; Correia & Canhoto 2012).

Tamarillo grows fast to 2-5 m high, is a perennial tree and has large (10-30 cm) heart-shaped leaves (Fig.3) with a characteristic musky odor. Pinkish flowers usually blossom between mid-spring and summer time (Prohens and Nuez, 2000; Correia et al., 2009). Fruits can appear isolated or in groups of 3-12 units, reaching maturity from October to April. They are egg-shaped, 5-10 cm long and 3-5 cm wide (Hooker, 1899; Correia and Canhoto, 2012).

Tamarillo cultivars can be grouped in three types according to fruits’ skin color: yellow, red and purple types (Prohens & Nuez 2000; New Zeland Tamarillo Growers Association 2008). Pulp, otherwise, span from orange-red to yellow shades (Correia and Canhoto, 2012). A tree individual can annually produce 15-20 Kg of fruits, during 6-10 years (Duarte and Alvarado, 1977).

Figure 3 – Tamarillo tree growing on the Botanical Garden of the University of Coimbra.

Tamarillo's main commercial interest is precisely the fruits, which can be eaten raw (once peeled, the pulp is consistent and juicy with a sweet-sour taste), canned in syrup or used in processed products such as juices or jams due to their richness in pectin (Bohs, 1991; Duke and du Cellier, 1993). A low carbohydrate content and relatively high content of proteins, vitamins (B_6, C, E and provitamin A) and minerals make these fruits a valuable food resource in nutritional terms (Holland et al., 1992). It has also been indicated as an exploitable source of bioactive components with therapeutic and preventive antioxidant properties (Hurtado et al., 2009; Osorio et al., 2012; Hassan and Bakar, 2013).

Tamarillo has a diploid chromosome complement of 2n=24 (Guimarães et al., 1988). Genetic diversity is usually reduced in natural populations of this tree, what may be partially justified by its mainly autogamic pollination and low success of interspecific hybridization (Pringle and Murray, 1991; Lewis and Considine, 1999).

Tamarillo propagation is traditionally achieved by seeds, cuttings or grafting onto wild *Solanum mauritianum* (Slack, 1976; Prohens and Nuez, 2000). However, these traditional breeding methods are not adequate if one intends to maintain genetic characteristics throughout generations (Pringle and Murray, 1991; Canhoto et al., 2005). Phytosanitary problems such as susceptibility to mildew, nematodes and some viruses like tamarillo mosaic virus (TaMV) (Mossop, 1977; Eagles et al., 1994; Correia and Canhoto, 2012), as well as very low temperatures (under 10ºC) occurring during winter or spring in Mediterranean countries where tamarillo is currently cultivated (Lopes et al., 2000) are other two concerns of large-scale production.

Therefore, biotechnological tools such as *in vitro* cloning and genetic transformation are arising as alternatives to the traditional methods of propagation. The development of new cultivars, more tolerant against biotic and abiotic stress, may play a crucial role in potentiating large-scale production of tamarillo in a near future (Canhoto et al., 2005).

The three methods usually applied for *in vitro* cloning, namely micropropagation from axillary shoots (Cohen and Elliot, 1979), regeneration by organogenesis from leaf explants (Atkinson and Gardner, 1993) and from protoplasts (Obando et al., 1992; Guimarães et al., 1996) and, last but not least, SE (Guimarães et al. 1988, 1996; Lopes et al. 2000; Canhoto et al. 2005; Correia et al. 2009, 2011, 2012a), have been applied to micropropagate tamarillo.

Regeneration of plants from pollen-derived *calli* or anther culture has also been attempted (Cohen and Elliot, 1979; Barghchi, 1986), without success (Correia and Canhoto, 2012).

Thermotherapy (32-36ºC) was proved efficient in virus elimination in contaminated shoot tips (Barghchi, 1986).

Besides its commercial interest, tamarillo has been used in studies of *in vitro* morphogenesis. Thus, the referred biotechnological techniques, and particularly SE, are very useful to explore this plant's potential as a model for embryogenesis in woody plants (Correia and Canhoto, 2012).

Despite the common recalcitrance of adult woody plants material to SE induction (Thorpe and Stasolla, 2001; Bonga et al., 2010; Seguí-Simarro et al., 2011), an

effective system for cloning an adult tamarillo was achieved by Correia et al. (2011). This opens doors not only for the improvement and efficient propagation of selected genotypes of tamarillo, but also for new insights on somatic embryo development in woody species in general.

3.2. Somatic embryogenesis in tamarillo – previous work

Various works regarding SE in tamarillo have been published in the last years, mainly by researchers from the Plant Biotechnology Group in the University of Coimbra (Portugal). Together, they account for making it a suitable model to understand the cytological and molecular mechanisms of somatic embryo formation and development (Guimarães et al. 1988, 1996; Lopes et al. 2000; Canhoto et al. 2005; Correia et al. 2009, 2011, 2012a; Correia & Canhoto 2012)

Guimarães and co-workers (1988) were responsible for the first successful SE induction in tamarillo. They used zygotic embryos and hypocotyls as starting material and induced PEM formation with the synthetic auxin 2,4-D. Somatic embryos developed from PEMs when the auxin was removed from the medium.

In successive protocols, other types of explants such as leaf sections, roots, internode segments, protoplasts and cotyledons have also been experimented for induction of *callus* and somatic embryos, with different success rates (Guimarães et al., 1996; Lopes et al., 2000; Canhoto et al., 2005).

1-naphthaleneacetic acid (NAA) and the synthetic auxin picloram also started to be used as inducing auxins (Guimarães et al., 1996; Canhoto et al., 2005). NAA is used for induction of DES process, because it conduces to formation of a reduced *callus*, unable to maintain the embryogenic potential, and somatic embryos develop instead from a meristematic layer of the explant (Canhoto et al., 2005; Correia and Canhoto, 2012). Picloram, similarly to 2,4-D, induce division of cells leading to the formation of proliferating *callus* after 4-6 weeks of culture and some clusters of embryogenic cells by weeks 8-10 (Lopes et al., 2000; Correia et al., 2011; Correia and Canhoto, 2012), thus representing an ISE process.

Somatic embryo formation is usually asynchronous and aberrant structures (fused embryos and extra cotyledons, for example) are common (Guimarães et al., 1988; Correia and Canhoto, 2012).

Embryogenic *callus* would keep on proliferating for indeterminate time if subcultured on the medium supplemented with auxin, without losing their potential for plantlet regeneration for over a year (Guimarães et al., 1988; Correia and Canhoto, 2012). However, for long-period cultures (more than 2 years), *callus* instability, variations in the chromosome number and DNA amount, as well as lower conversion rates, have been reported (Currais et al., 2013). Plantlets obtained from 5-year-old embryogenic *calli* displayed more abnormalities as well (Canhoto et al., 2005).

Finally, some factors may be managed in order to obtain more efficiency on the process of SE in tamarillo. High sucrose levels in the induction medium, for example, have been reported to improve embryogenesis rate and reduce morphological abnormalities on the somatic embryos (Guimarães et al., 1996; Canhoto et al., 2005). Manipulation of light conditions and the inclusion of abscisic acid (ABA) in the medium before germination may also be beneficial to obtaining more morphologically normal embryos (Correia et al. 2012a). The inclusion of low concentrations (0.1 mg/L) of gibberellic acid (GA_3) in the maturation medium stimulates somatic embryo development and further germination (Canhoto et al., 2005), similarly to what had already been verified in other species (Chakrabarty et al., 2003) and indole-3-butyric acid (IBA) can be used to accomplish shoot rooting, although these are not indispensable conditions (Correia et al., 2011). In fact, even when normal root development does not occur concomitantly with the development of the shoot, adventitious roots often arise and make possible the in regeneration of plants (Canhoto et al. 2005; Correia et al. 2012a).

In addition, it is important to stress that induction, multiplication and conversion rates vary a lot among *callus* lines and cultivars. Explants from yellow cultivars seem to produce embryogenic *calli* more readily than from red cultivars, and are also more stable in culture (Canhoto et al., 2005).

Apart from the works focused on the enhancement of the induction and conversion rates during tamarillo SE, investigation regarding the characterization

of different types of *calli*, induced in different conditions, has been conducted by establishing molecular and biochemical comparisons (Ferreira et al., 1998; Faro et al., 2003; Correia et al., 2012b) and trying to identify molecular markers related with the embryogenic competence acquisition (Correia, 2011; Alhinho, 2016). In parallel, increased metabolism, protein synthesis and stress-related proteins were reported as features of embryogenic cells relative to non-embryogenic *calli* (Correia et al. 2012b).

Other studies invest on developing methods for long-term conservation of tamarillo *calli*, such as cryopreservation (Graça, 2016), aiming to overcome problems arising from culture aging, which can impair cloning (Correia and Canhoto, 2012).

Genetic transformation of tamarillo plants has been attempted too, aiming to obtain cultivars resistant to antibiotics (Atkinson and Gardner, 1993), to TaMV (Cohen et al., 2000), or simply in the context of functional genomics studies (Correia, 2011).

4. EPIGENETICS

Epigenetics was defined as "The study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al., 1996). Epigenetic phenomena thus represent the link between genes and environment, inducing phenotypic variation and 'epiallelic' transmission (Kakutani, 2002). Epigenetics has been subject to a lot of research in the last years, and a hot topic of discussion among the scientific community.

Nowadays, we can assume as a summary definition that epigenetic regulation of gene expression is the set of heritable, but potentially reversible, chemical and enzyme-mediated modifications that act on DNA and associated proteins of chromatin, and do not affect the nucleotide sequence of DNA (Wolffe and Matzke, 1999; Valledor et al., 2007). These changes often lead to the generation of variants ('epigenetic marks') which are not 'coded' by the DNA, but still can be transmitted to the next generation (Boyko et al., 2007; Chen et al., 2010b; Neelakandan and Wang, 2012). The three main mechanisms comprised in

epigenetic regulation are common both to vegetal and animal organisms: DNA methylation, histone modifications and RNA interference (Kouzarides, 2007; Munshi et al., 2015).

Among epigenetic marks, DNA methylation and histone modifications are the best studied. Small non-protein-coding RNAs are also arising as key players of the epigenetic regulation in plants, with particular importance in viral protection and prevention of transposon mobilization (Singer et al., 2001; Neelakandan and Wang, 2012; Munshi et al., 2015).

Epigenetic regulation has endless implications in plant breeding (Tsaftaris and Polidoros, 2000) and evolution (Kalisz and Purugganan, 2004), which are just now starting to be acknowledged.

In context of stress and pathogens' response, for example, the role of epigenetic mechanisms is remarkable and it was comprehensively addressed by Munshi et al. (2015). For example, plants respond to viruses attack by orchestrating, in one hand, chromatin compaction and increased genomic stability and, on the other hand, genetic recombination of resistance genes favoring the emergence of new favorable traits. Then, the fact that this "epigenetically acquired resistance" might be passed on to further generations, reminds us of Lamarck's postulates (Munshi et al., 2015).

Besides, epigenetic mechanisms must not be ignored as key factors regulating the developmental processes in flowering plants, such as gametogenesis, zygotic and somatic embryogenesis (Valledor et al., 2007; Twell, 2011; Neelakandan and Wang, 2012; Solís et al., 2012; De-la-Peña et al., 2015; Testillano and Risueño, 2016).

4.1. Chromatin structure

According to its degree of compaction and therefore its accessibility, we distinguish euchromatin from heterochromatin, the latter described for the first time by Heitz (1928). Heterochromatin is very condensed whilst euchromatin is less compacted, with irregularly spaced nucleosome arrays (Tariq and Paszkowski, 2004; Rosa and Shaw, 2013).

The nucleosomes are under a constant and very complex regulation, its histone suffering changes and modifications that allow the DNA to be accessed for transcription at appropriate times, and by contrast, to be finely packaged within the nucleus (Horn and Peterson, 2002; Jarillo et al., 2009; Rosa and Shaw, 2013).

Efficient modification of chromatin structure is particularly important during cellular dedifferentiation and proliferation in processes such as embryonic development and organogenesis (Grafi et al., 2007a; De-la-Peña et al., 2015). Dynamic changes between chromatin states are relevant in the transcriptional regulation during somatic and microspore embryogenesis (Testillano et al., 2005; Nic-Can et al., 2013). Globally, heterochromatin increases during cell differentiation and organ maturation, while it decreases during cell dedifferentiation, proliferation and reorganization process (Bárány et al., 2005; Tessadori et al., 2007; Exner and Henning, 2008; Solís et al., 2012).

Epigenetic marks like DNA methylation and histone modifications have been revealed as hallmarks that define the functional status of chromatin domains and confer the flexibility of transcriptional regulation necessary for plant development and adaptive responses to the environment (Grant-Downton and Dickinson, 2005; Vaillant and Paszkowski, 2007; Valledor et al., 2007).

4.2. DNA methylation

DNA methylation represents a well-characterized, major epigenetic mark that determines silencing of specific DNA sequences (Finnegan et al., 2000; Neelakandan and Wang, 2012).

According to (Zhang et al., 2006), in the *Arabidopsis* model system, highly expressed genes are usually subject to methylation on its coding region, whilst in genes with low expression activity, methylation occurs in its promoter region.

At its whole, the DNA methylation is species, tissue, organelle and age-specific (Munshi et al., 2015).

Among its many functions, we can highlight: maintenance of the developmental clocks of the organisms (Holliday and Pugh, 1975; Kouzarides, 2007), reduction

of transcriptional “background noise” in organisms with a large genome (Bird, 1995), stabilization of gene silencing initiated by other mechanisms such as genomic imprinting (Bird, 2002) and defense against transposons and parasite mobile elements (Wolffe and Matzke, 1999; Miura et al., 2001; Rosa and Shaw, 2013). Indeed, plants are able to withstand higher demethylation levels than animals, consequently, they cope with higher rates of transposon insertion (Valledor et al., 2007; De-la-Peña et al., 2015).

At a molecular level, DNA methylation consists of a methyl group being added at the 5’position of the pyrimidine ring of cytosine in the DNA, to form the so-called 5-methyl-deoxy-cytidine (5mdC, Finnegan & Kovac 2000). Adenosine methylation only occurs in some bacterial, protozoa and fungi organisms (Vanyushin, 2006). The cytosine shall be adjacent to a guanine (CpG islands) or, in case of plants, that can also happen in CpHpG and CpHpHp contexts, being H any nucleotide other than guanine (Finnegan et al., 2000; Vanyushin, 2006; He et al., 2011). Cytosine methylation occur most often in the first exons or in the promoter of specific genes, thus altering the binding of transcriptional factors and other proteins (Finnegan et al., 2000; Zhang et al., 2006). That way, it plays a key role in gene expression regulation (Valledor et al., 2007). Furthermore, DNA hypermethylation and hypomethylation are associated to heterochromatin and euchromatin regions, respectively (Tariq and Paszkowski, 2004).

DNA methyltransferases are the wide enzymatic family that mediates the methylation reaction and are therefore responsible for either the maintenance (MET1, DRM2 and CMT3 in *Arabidopsis*) of methylation patterns along cell replication, and *de novo* methylation (DRM2 in *Arabidopsis*), which can occur in the context of embryo development, for example (Finnegan et al., 2000; Cao and Jacobsen, 2002). The types and numbers of DNA methyltransferases is highly variable among plant species (De-la-Peña et al., 2015).

Furthermore, a third mechanism of DNA methylation has been recently described: RNA-directed *de novo* methylation (RdDM), which is unique to plants. Small interfering RNAs (siRNAs), which are 24-26 nucleotides long RNAs generally originated from transposable elements and tandem repeats, target specific DNA homologous sequence and trigger methylation at its cytosine

residues in CpG, CpHpG and other sequence contexts (Cao et al., 2003; Chinnusamy and Zhu, 2009; Law and Jacobsen, 2010).

Methyltransferases’ catalytic motifs are highly conserved among species (Munshi et al., 2015). De-la-Peña et al. (2015) have summarized the catalytic mechanism of DNA methyltransferases with the enlightening image that follows (Fig. 4):

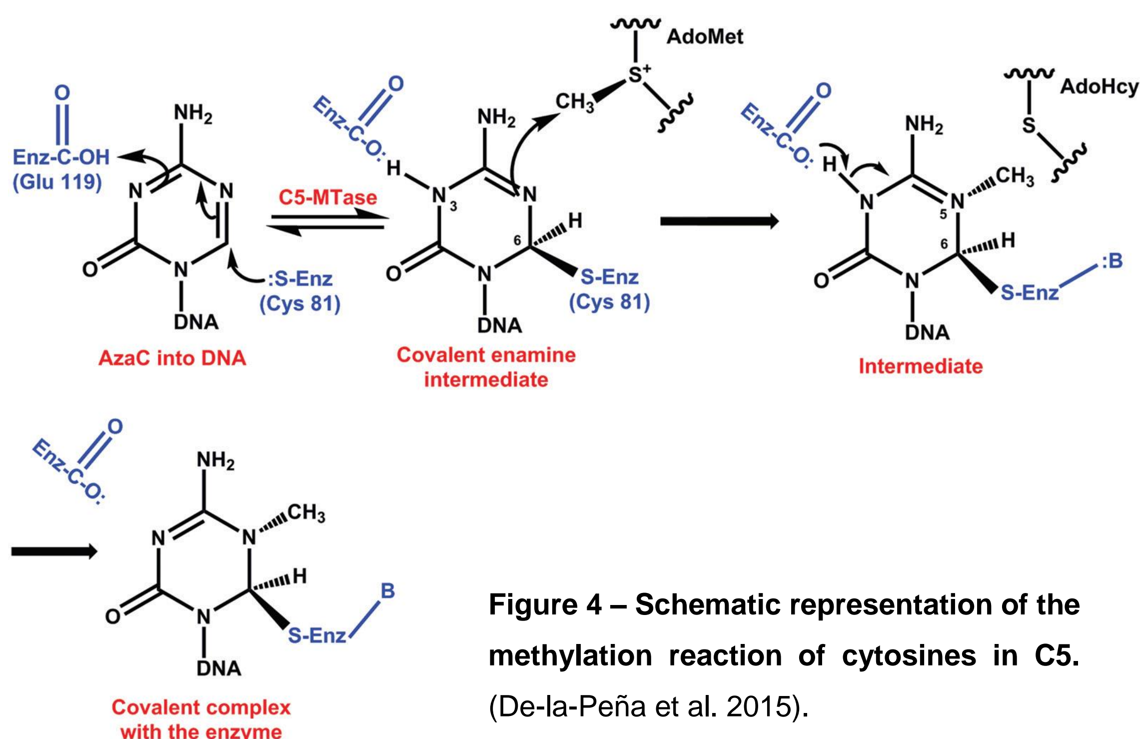


Figure 4 – Schematic representation of the methylation reaction of cytosines in C5. (De-la-Peña et al. 2015).

As one can see, the activation and methylation of the fifth carbon in the pyrimidine ring is primarily triggered by a nucleophilic attack on carbon 6 and transference of a methyl group from a conserved cofactor substrate, S-adenosylmethionine (SAM), to the 5th position (Valledor et al., 2007; De-la-Peña et al., 2015). It was suggested that the effect of 2,4-D on DNA methylation is correlated with SAM levels, in the following way: SAM is not only the methyl group donor for DNA methylation, but also generates precursors for polyamine and ethylene biosynthesis (Miyazaki and Yang, 1987). 2,4-D stimulates ethylene biosynthesis (Herman, 1991), therefore, when it is present, SAM’s consumption in ethylene byosyntesis increases, becoming SAM less available for DNA methylation (Munksgaard et al., 1995). However, this interference is still not fully understood, once that it was observed that in cell suspension cultures of carrot, though 2,4-D was present, SAM pool was high and would drastically decrease upon 2,4-D removal (Munksgaard et al., 1995).

Other than methyltransferases, normal DNA methylation also requires the chromatin remodeling ATPases DDM1 (Jeddeloh et al., 1999) and DRD1 (Kanno et al., 2005), as well as mdC binding proteins VIM1-3 (Woo et al., 2008) to occur.

Although DNA methylation is considered to be a relatively stable epigenetic mark, the demethylation process also occurs, especially during development in plants (Law and Jacobsen, 2010), at its turn, might occur passively (spontaneous substitution of mdC for dC during replication) or actively by enzymatically removal of the methyl group of cytosines (Valledor et al., 2007; Law and Jacobsen, 2010).

Among the various mechanisms of active DNA demethylation that have been proposed in the last years, one appears to unequivocally occur in plants, supported by strong genetic and biochemical evidence (Law and Jacobsen, 2010): a DNA glycosylase directly excises 5meC and is likely associated to base-excision repair (BER) mechanism (Zhu, 2009; Wu and Zhang, 2010).

ROS1/DME (Demeter) family of 5mC DNA glycosylases initiated DNA demethylation is the major active DNA demethylation pathway in plants (Gong and Zhu, 2011; Li et al., 2015). In *Arabidopsis thaliana*, the DME family of DNA glycosylases consists of four members: DME, repressor of silencing 1 (ROS1; also known as DML1), DML2 and DML3 (Zhu, 2009). All members of the DME family have the capacity to recognize and remove 5mdC bases from double stranded DNA (dsDNA) oligonucleotides, irrespective of their sequence context *in vitro. In vivo*, however, mutations in different DME genes lead to hypermethylation at distinct genomic loci (Penterman et al., 2007), indicating that each of these enzymes has a unique *in vivo* function (Wu and Zhang, 2010). Abiotic stresses are known to promote specific demethylation, likely with small RNAs participating in the targeting of DME to specific *loci* (Chinnusamy and Zhu, 2009).

Recently, Li and co-workers (2015) suggested that a histone acetyltransferase complex is essential for the ROS1-mediated active DNA demethylation in *Arabidopsis*, namely for the recruitment of ROS1 glycosylase to specific loci where suppression of gene silencing is necessary. Activation of stress-responsive genes via recruiting ROS1 for active DNA demethylation is one of the aspects in which DNA demethylation might be important in plants (Li et al., 2015). One of the proteins that form that recruitment-complex is a member of the methyl-

CpG-binding domain (MBD), which is also present both in plants and animals and are capable of specifically recognizing and binding methylated DNA (Grafi et al., 2007b). The hypothesis of these enzymes being capable of directly removing the methyl group of 5mdC has been controversial, namely because this would require an enormous catalytic power due to the strength of the carbon-carbon bond that needs to be broken (Wu and Zhang, 2010).

4.3. Post-translational histone modifications: H3K9 methylation

Histones are an important protein family responsible for chromatin structure and packaging of the DNA within the nucleus (Rosa and Shaw, 2013). For instance, the most fundamental unit of chromatin – the nucleosome – is composed by a portion of DNA fiber (approximately 146 base pairs) wrapped around one octamer of core histones (H2A, H2B, H3 and H4, each one twice), and this structure can be further compacted and stabilized by the presence of a fifth histone (H1) (Kornberg, 1974; Kornberg and Lorch, 1999; Munshi et al., 2015).

There are studies reporting that histone modification may play a decisive role in abiotic stress response too (Tsuji et al., 2006; Sokol et al., 2007; Kim et al., 2008).

In order to accomplish the intricate task of letting DNA sequence be readable (or, by contrast, to repress transcription), many non-histone proteins interact with histones and operate the famous but still poorly understood "histone code" (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Pfluger and Wagner, 2007). Among the most common forms of histone modifications there are: acetylation or ubiquitination of lysine, phosphorylation of serine and threonine and methylation of arginine and lysine residues (Munshi et al., 2015). The N-terminal tails of the core histones, which extend outward from the nucleosome core, are especially prone to these post-translational modifications (PTMs) (Neelakandan and Wang, 2012; Rosa and Shaw, 2013).

Among the epigenetic post-translational modifications of histones, histone methylation marks can be correlated both with repression and with transcriptional activation, whereas acetylation is generally an activation mark (Munshi et al., 2015). Also, lysine residues can be mono-, di- or trimethylated and each of these forms can have unique biological functions (Rosa and Shaw, 2013).

H3K9 methylation is a major epigenetic mark for gene silencing, with major functions in many developmental processes: HP1 protein is recruited to H3K9me sites and brings with it deacetylase activity, which in turn prevents the accessibility of the underlying DNA sequence to the transcription machinery (Stewart et al., 2005). It is frequently found in telomeric and centromeric heterochromatin (Munshi et al., 2015). Chromatin associated with transposable elements is prone to H3K9 methylation as well as to DDM1 activity, the first being responsible for transcriptional inactivation and the second for transposon stabilizing (Miura et al., 2001; Neelakandan and Wang, 2012).

In plant genomes, H3K9 methylation is associated with both DNA methylation and small RNAs, as essential drivers for the heterochromatin formation (Saze et al., 2012).

Some data have been published that links H3K9 and DNA methylation (see Tariq & Paszkowski 2004; Munshi et al. 2015). Indeed, the angiosperm-specific enzyme CMT3 (Henikoff and Comai, 1998) is capable of recognizing dimethylated H3K9 (H3K9me2) and directly bringing off DNA methylation at CpHpH sites.

Histone methylation sites are recognized by Plant Homeo Domain (PHD) fingers (Rosa and Shaw, 2013). Histone methyltransferases are extremely specific (Rosa and Shaw, 2013). The kryptonite (*KYP*)/*SUVH4* gene codes for the H3K9 methyltransferase enzyme, being also involved in the activation of cell cycle related genes, thereby initiating the meristematic state and cell division (see Neelakandan & Wang, 2012).

5. EPIGENETICS ON THE CONTEXT OF *IN VITRO* CULTURE, DEVELOPMENTAL AND SOMATIC EMBRYOGENESIS PATHWAYS

Nowadays, thanks to scientific and technological progress, one can look into almost any aspect of SE, from total reprogramming of physiology and metabolism to gene expression patterns (Yang and Zhang, 2010).

Though the relevance and effectiveness of the induction factors of SE (typically stress or exogenous hormones) is variable among species, the response to these

inducers surprisingly similar in all plants, in the sense that the activated enzymatic pathways and gene families are more or less common (Mahdavi-Darvari et al., 2015).

As previously stated, DNA methylation variation is a major response of organisms to multiple forms of stress (from mechanical wounding to stress inherent to *in vitro* culturing and PGRs), and one of the possible downstream effects of this is epigenetic instability is the change of the developmental program (Chakrabarty et al., 2003; Neelakandan and Wang, 2012). Likewise, in an *in vitro* culturing context, the ability of an explant tissue to reset its genetic and epigenetic program in response to artificial stimulus and the hormonal environment, will determine its fitness and adaptability to *in vitro* cultures (Neelakandan and Wang, 2012). One can indeed interpret the change of cell fate and developmental program (in SE as well as in organogenesis and other phenomena) as an ultimate response to stressful conditions and *in vitro* conditioning, and for the adaptation to occur, massive genetic and epigenetic reprogramming must occur. This is summarized in Fig. 5.

In the context of plant embryogenesis, there has been an avid demand to characterize DNA methylation patterns in different cultures and *in vitro* systems, epigenetic response to the application of compounds and variation of growth conditions at early developmental stages, because that knowledge might be relevant for plant breeding purposes in the future (LoSchiavo et al., 1989; Leljak-Levanić et al., 2004; Testillano and Risueño, 2016).

For example, in *Arabidopsis calli* and suspension cultures, the undifferentiated state of cells has been reported to be regulated (at least in part) by promoter methylation of specific single-copy genes (Berdasco et al., 2008); also in the same system, *de novo* adventitious shoot initiation is controlled by DNA methylation and histone modification of regulatory sequences of *WUS* gene and auxin signaling components (Li et al., 2011). Recent studies have revealed changes in DNA methylation, histone methylation and acetylation that are associated to the switch of developmental program and *in vitro* embryogenesis initiation in several plant species (Berenguer et al., 2017; El-Tantawy et al., 2014; Rodríguez-Sanz et al., 2014a, 2014b; Solís et al., 2012, 2015).

A better understanding on the topic of epigenetics on the context of SE and *in vitro* systems, can lead to strategies that improve the ability to maintain genomic homeostasis, and that might be an important starting point to overcome undesired somaclonal variation (Kaeppler et al., 2000; Sharma et al., 2007) as well as recalcitrance of some species (specially woody plants) to *in vitro* regeneration and transgene expression (Miguel and Marum, 2011; Neelakandan and Wang, 2012; Testillano and Risueño, 2016).

It is important to emphasize that, although it is clear that DNA methylation and demethylation accompanies differentiation, dedifferentiation and cell division in cell and tissue culture systems, in the available literature there is still no consistent model regarding how DNA methylation behaves on the course of the whole embryogenic development, which applies to all systems studied so far.

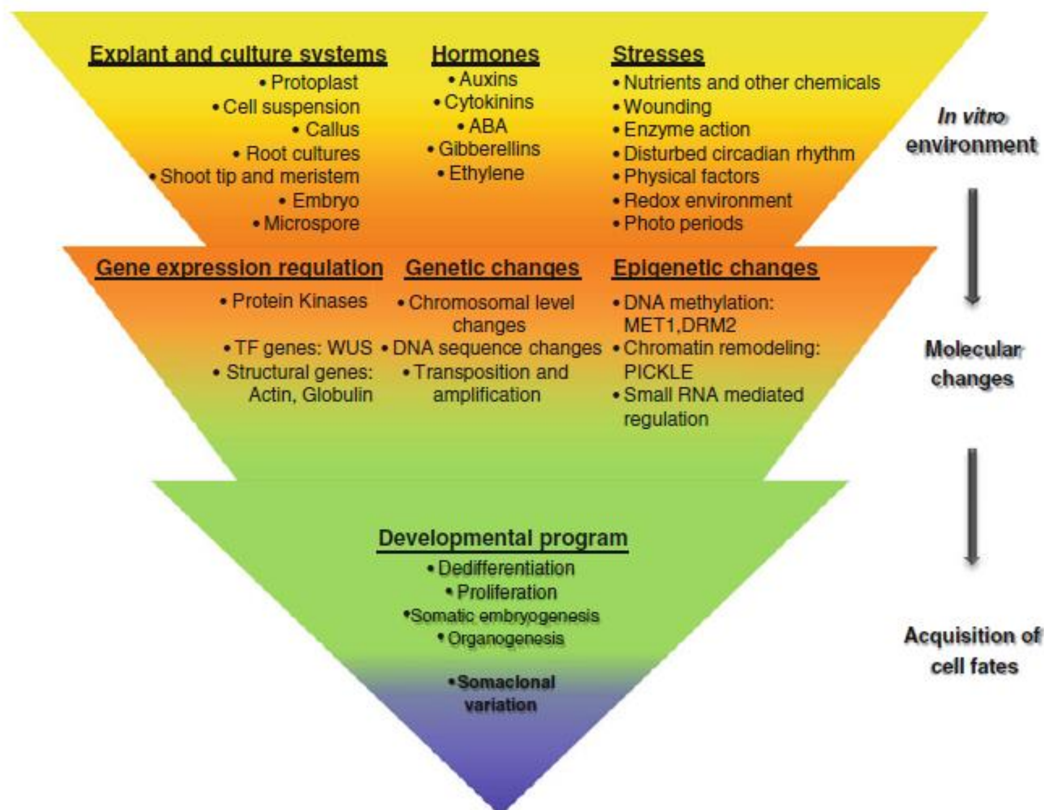


Figure 5 – The hierarchy of events and conditions intervening in the change of cell fate. From *in vitro* conditions to various types of stresses and PGRs, many are the triggering factors to the occurrence of modifications in the way genes are expressed. Epigenetic instability is typical of any cell developmental change. Adapted from Neelakandan & Wang (2012).

Therefore, in the present study we have analyzed the dynamics of DNA methylation, and its relation with H3K9 methylation, during SE of tamarillo, a system in which no information is available on epigenetic marks.

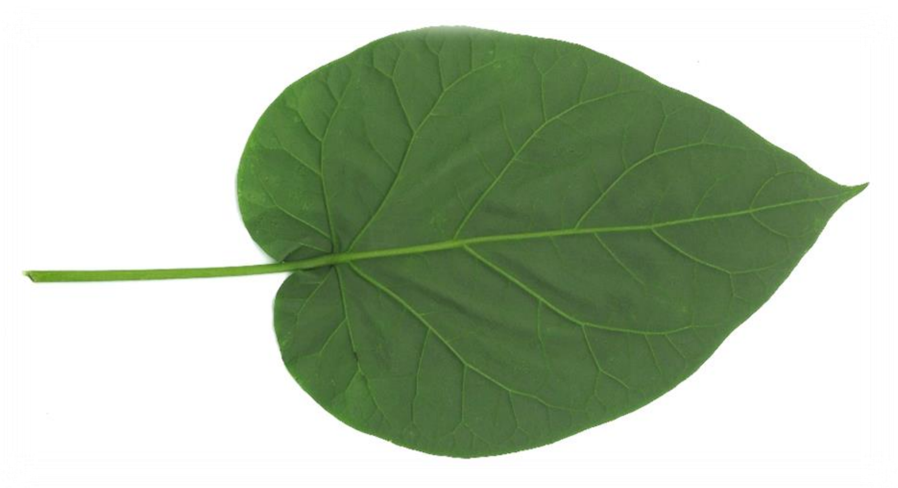
6. OBJECTIVES

The general aim of the present work is the analysis of epigenetic marks during the SE of *Solanum betaceum*, specially focusing on changes in cellular organization, DNA methylation levels and nuclear distribution patterns.

A comparative study between embryogenic and non-embryogenic cell lines, as well as among different stages of SE, like dedifferentiation and early somatic embryo conversion stages are addressed.

Consequently, the specific objectives proposed for this work are:

1. to induce SE from young leaves and generate new embryogenic and non-embryogenic cell lines;
2. to characterize the cellular organization, through histochemical analysis at light microscopy, of embryogenic and non-embryogenic cell lines induced in different years (old and recent lines), and in the main stages of the SE process;
3. to analyze global DNA methylation levels (by quantification assays), and its nuclear distribution (by 5mdC immunofluorescence and confocal analysis), in old and recent embryogenic and non-embryogenic cell lines;
4. to determine changes in global DNA methylation levels (by quantification assays), and nuclear distribution (by 5mdC immunofluorescence and confocal analysis) during the main stages of SE induction (dedifferentiation stage) and embryo development (embryo conversion stages);
5. to determine differences in H3K9 methylation levels between embryogenic and non-embryogenic lines with the same origin and their relationship with DNA methylation levels;
6. to analyze possible correlations between DNA methylation dynamics and embryogenic competence and proliferation rates of different embryogenic cell lines.



Material and Methods

1. PLANT MICROPROPAGATION AND INDIRECT SOMATIC EMBRYOGENESIS PROCEDURES

1.1. *In vitro* micropropagation of the tamarillo lineages used as starting point for somatic embryogenesis induction

In this work, non-embryogenic *calli* and embryogenic masses from three distinct tamarillo genotypes were used. These genotypes (TV310, TSM10 and TDJ3) had been previously established from seed *in vitro* germination, followed by micropropagation through axillary shoot culture, in Murashige and Skoog (1962) (MS) medium containing 3% sucrose, 0.2 mg/L of BAP (Duchefa Biochemie, Netherlands) and 0,6% (w/v) plant agar (Duchefa Biochemie, Netherlands), as described by Correia (2011).

The shoots (1-2 cm long) grew in plastic containers with air filter kept in a growth chamber at 25 ± 1 ºC, in a 16h light / 8h dark photoperiod at 25-35 µmol $m^{-2}s^{-1}$ (cool white fluorescent lamps). They were subcultured into to fresh medium every 4-6 weeks (in the preparation of the culture medium, pH was adjusted (with KOH) to 5.6-5.8 and plant agar (Duchefa Biochemie, Netherlands) was added before autoclaving at 121 °C for 20 min) and excised leafs from developed shoots were used in the induction of SE.

All the following procedures concerning two-step SE induction and somatic embryo maturation and germination were performed as previously described in details by Canhoto et al. (2005).

1.2. *Callus* induction from leaf explants of seedling-derived shoots of tamarillo

Leaf explants from cloned shoots of the three distinct genotypes (TV310, TSM10 and TDJ3) were used for induction of SE.

The most apical expanded leafs were collected, sectioned into 4-6 pieces after removing the main vein and mechanically wounded. Then they were placed with their abaxial sides down onto fresh solid induction medium, which was called ‘TP’ induction medium (Correia, 2011), whose composition is: MS medium with a

concentration of 9% (w/v) sucrose, 5 mg/L of Picloram (© Sigma-Aldrich, Missouri, USA) and 2.5 g/L of Phytagel™ (© Sigma-Aldrich, Missouri, USA). The pH was adjusted between 5.6 and 5.8 before addition of the gelling agent and autoclaved at 121°C for 20 min. The vessel preferably used for SE induction in TP medium were test tubes (15 cm x Ø 22 mm), containing each one approximately 10 mL of medium and one leaf explant per tube.

Leaf explants were kept at 25 ± 1 °C in the dark for 12 weeks of incubation. Samples were also collected at mid-time of the induction phase (6 weeks) for subsequent analysis.

After 12 weeks' time the potentially embryogenic clumps and non-embryogenic *calli* resulting from the induction process were carefully isolated and proliferated in 15 x 2.2 cm test tubes or plastic petri dishes (Ø 90 mm) containing the same TP induction medium.

1.3. Proliferation and maintenance of embryogenic and non-embryogenic material

In vitro maintenance and proliferation of the induced embryogenic clumps and non-embryogenic *calli* is achieved by transferring small portions (80-100 mg) every 4-6 weeks, at a time coincident with the cell's maximum proliferation rate of the masses, to the same culture medium. Embryogenic clumps can also be transferred to liquid medium of the same composition, with subcultures made at 3-4 weeks interval (Canhoto et al., 2005).

1.4. Previously-established cultures of embryogenic masses and non-embryogenic *calli*

Some of the embryogenic/non-embryogenic material used in this work had its origin in SE experiments performed in previous years in our laboratory, having been maintained and subcultured to the present time under the same conditions described above.

Table I – Explant origin and auxin used in the media for induction and proliferation of the embryogenic and non-embryogenic material used in the experiments of the present work.

Line	Embryogenic/ Non-embryogenic	Explant of origin	Year of induction	Hormone in SE induction/ proliferation medium
ZE 2009	Embryogenic	Zygotic embryo	2009	2,4-D
MNE	Non-embryogenic	Zygotic embryo	2009	2,4-D
YL 2009	Embryogenic	Leaf explant from genotype TV310	2009	Picloram
B	Non-embryogenic	Leaf explant from genotype TV310	2009	Picloram
YL 2014	Embryogenic	Leaf explant from genotype TV310	2014	Picloram
CNE	Non-embryogenic	Leaf explant from genotype TV310	2014	Picloram
YL 2016	Embryogenic	Leaf explant from genotype TV310	2016	Picloram
NER	Non-embryogenic	Leaf explant from genotype TV310	2016	Picloram
YL 2016a1	Embryogenic	Leaf explant from genotype TSM10	2016	Picloram
YL 2016a2	Embryogenic	Leaf explant from genotype TSM10	2016	Picloram
YL 2016b	Embryogenic	Leaf explant from genotype TDJ3	2016	Picloram

For instance, the pair ‘YL 2009’ and ‘B’ (of apparently embryogenic and non-embryogenic cell cultures) had its origin in a unique SE induction event, performed in 2009, from leaves of the already established shoot culture of the TV310 genotype. ‘YL 2014’ embryogenic and ‘CNE’ non-embryogenic cell cultures had the exact same origin, except for the starting date of the induction protocol, which was in 2014.

Besides these leave-induced *callus* lineages, another pair has been maintained, subcultured and used for experiments in this work. The so named 'ZE 2009' (presumably embryogenic) and 'MNE' (non-embryogenic) cell cultures, had its origin in an unique induction experiment, performed in 2009, not on leaves but on zygotic embryos excised from seeds, according to a recurrent existent protocol for SE induction in different source of explants (Guimarães et al., 1988; Lopes et al., 2000; Canhoto et al., 2005; Correia, 2011). The auxin used both for induction of embryogenic and non-embryogenic material from zygotic embryos and its subsequent maintenance and proliferation was 2,4-D (© Merck KGaA, Darmstadt, Germany) (Canhoto et al., 2005; Correia, 2011).

1.5. From embryogenic masses to embryo conversion and differentiation

Embryogenic and non-embryogenic material obtained as described in section 1.2., as well as the oldest embryogenic and non-embryogenic lines accounted in section 1.4., have been used, sampled for immunofluorescence experiments, total DNA methylation assays and other purposes and repeatedly referenced throughout the present work.

The protocol for embryo formation from embryogenic masses is based on removal of the auxin from the culture medium (Canhoto et al., 2005) and lowering the level of sucrose: 4% during the first 1-1.5 months and 2.5% on the subsequent subcultures. pH was adjusted between 5.6 and 5.8 and Phytagel™ (© Sigma-Aldrich, Missouri, USA) at a 2.5 g/L concentration was used as gelling gum. This medium with no PGRs is called 'maturation medium'. While at MS with 4% sucrose (*i.e.* the first 1-1.5 months of incubation) the cultures were kept at 25 ± 1 °C in the dark, after which period they were changed to 2.5% sucrose and 16h light / 8h dark photoperiod at 25-35 µmol $m^{-2}s^{-1}$ (cool white fluorescent lamps).

During this embryo conversion and differentiation phase, one can witness, or not, an evolution of the cultured masses undifferentiated state to the arising and development of whitish embryo-like formations, that might later on originate somatic embryos which follow the normal embryogenic development (Correia, 2011)

1.6. Plant acclimatization

When the somatic embryos formed develop into juvenile clone plantlets and their roots reach minimum 3 cm length, they are carefully transferred to pots containing substrate (soil, sand and vermiculite 1:1:1) and taken to a greenhouse at 18 ± 1 °C, 16h light / 8h obscurity photoperiod and 18% relative humidity (RH). Water was supplied once a week.

Fig. 6 summarizes the SE protocol followed in the present work.

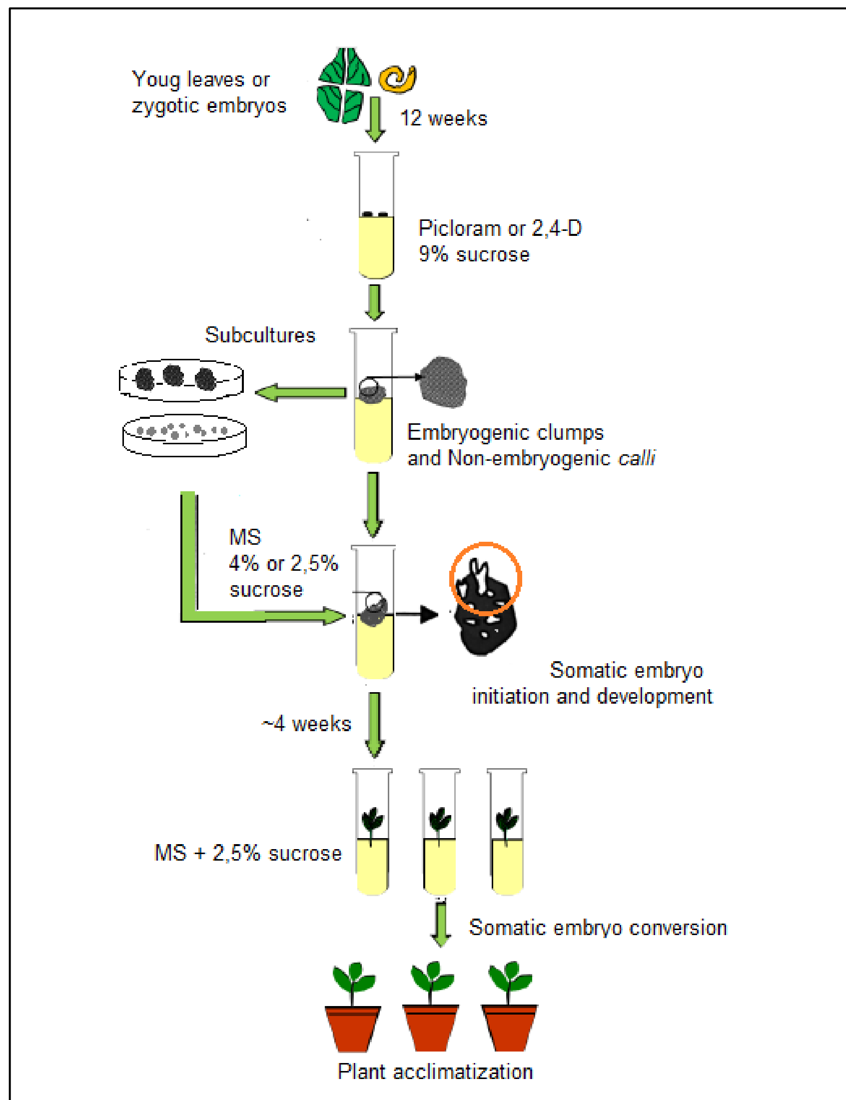


Figure 6 - Schematic representation of SE induction and plant regeneration in tamarillo. Adapted from Canhoto et al. (2005).

2. ANALYSING THE BEHAVIOR OF DIFFERENT EMBRYOGENIC MASSES

Embryogenic (or formerly embryogenic) masses with different ages and genotypes behave differently in what concerns proliferation rates, embryogenic potential, robustness to culture conditions and contamination, etc. (Correia, 2011).

In order to characterize the various lineages used in this work, and also to clarify in which extent the embryogenic callus lineages kept in culture since year 2009 (ZE 2009 and YL 2009), year 2014 (YL 2014) and some from year 2016 (YL 2016a1, YL 2016a2 and YL 2016b) maintained their capacity to regenerate somatic embryos (eventually correlating this behavior observations with methylation further on), a comprehensive assay was performed as described below.

Using 2-5 biological replicas from each subcultured embryogenic line, a known initial mass of 0.1 – 0.2 g fresh weight was transferred to maturation medium. After 3 and 5 weeks of proliferation and embryo conversion, each replica was weighted again (excluding somatic embryos eventually formed) and 'proliferation rate' was calculated as the weight gain divided by the initial weight. The mean proliferation rate of replicas was calculated for each embryogenic line.

Besides, representative samples of material on key stages (early embryos, proliferative masses, etc.) were collected for further global DNA methylation, histone methylation and 5mdC immunofluorescence assays.

All material handling and sampling was performed under aseptic conditions in flow chamber.

2.1. Measuring proliferation rates

Each replica of embryogenic material was weighed on transferring to maturation medium using a precision scale (±0.001 g). New weighing was performed at week 3 and week 5, these measurements being conservative (*i.e.* masses were kept proliferating).

Replicas from which a substantial amount of material had been subtracted at some point (many embryo-like structures or/and embryogenic clumps) were excluded from further weighing, as these would not accurately describe weight increment relative to the initial weight.

Proliferation rates were calculated by dividing fresh weight increment (Week3 - Initial), (Week5 - initial) by initial weight.

One-way ANOVA and Tukey test were employed for statistical analysis of the results.

2.2. Embryogenic potential and observation of embryo morphology

The methodology for identifying and counting the embryo-like structures different time points after auxin withdrawn was based on descriptions and procedures adopted by (Correia, 2011).

As so, at week 1 we took note of the existence (or absence) of whitish structures that would eventually develop into somatic embryos; at week 3 both early embryos (2-4 mm) and new presumptive embryo-like structures were counted and collected, because embryo formation in these cultures is asynchronous (Correia and Canhoto, 2012; Correia et al., 2012b).

Based on previously described morphologically features (Correia, 2011), 'normal' somatic embryos developed as erect structures, with 4-6 mm hypocotyls and sometimes with already evident cotyledon *primordia*, whilst 'anomalous' structures could show fused embryos, display an enlarged/round morphology or no visible cotyledon development. For the purpose of the present study, both normal and anomalous embryos were considered a manifestation of success in what concerns embryogenic ability of the starting mass, once that, according to previous work (Correia et al., 2012a) and current follow-up observation of the structures, the majority of the accounted abnormalities displayed by the somatic embryos do not interfere with their future development and plant conversion.

For purposes of measuring global DNA methylation levels (see 5.), material in maturation and developing embryos were sampled at the following stages:

1. embryogenic masses before removal of auxin in the culture media;

2. embryogenesis initiation stage: corresponded to the first week of YL2016a2 maturation: 1-2 mm whitish embryo-like structures started to emerge from the embryogenic mass. Due to the difficulty of manually separating the two type of structures, embryogenic masses and very early embryo structures, both were collected and analyzed together;

3. very young embryo stage: when young embryos (2-3 mm) could already be separated from the subjacent embryogenic mass.

4. developing embryo stage: corresponds to larger and more developed embryos (4-5 mm), they could be easily separated from the subjacent embryogenic mass.

3. CELLULAR CHARACTERIZATION OF *IN VITRO* PROCESS OF SOMATIC EMBRYOGENESIS

3.1. Material fixation and processing for semi-thin sectioning and light microscopy analysis

From leaves to *callus* and embryos, all material was collected at selected *in vitro* culture times and developmental stages in Eppendorf 1.5 mL-2 mL tubes and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C overnight, and then changed to 0.1% paraformaldehyde allowing the samples to be kept in at 4 °C for longer periods before further processing.

After a dehydration in acetone series (30%, 50%, 70%, 90% and 100%), samples were infiltrated in Technovit 8100 resin (Kulzer, Germany) and kept in a rotator 4 °C for 48h. After that, samples were embedded in capsules containing the resin and polymerization was made following Technovit 8100 kit's instructions, at 4 °C overnight.

This processing method may result differently in different samples, depending on its hardness, heterogeneity, tissue compactness, etc. (Testillano and Risueño, 2016). Therefore, fixation of the non-embryogenic *calli* (more vulnerable to unbundling) was sometimes performed with addition of sucrose 10% to the

paraformaldehyde solution, that way minimizing the lysing effects of hypotonic shock.

Good structural preservation of the sampled material will allow a reliable cytochemical characterization as well as antigenicity's preservation (Testillano and Risueño, 2016).

Semi-thin histological sections of 2 μm were obtained from these blocks using an ultramicrotome and disposed over a drop of water on glass slides with, air-dried and stored until use for staining and microscopic analysis.

3.2. Cytochemical techniques

For general structural analysis, sections were placed onto simple glass slides with no coating, stained with toluidine blue for 2-3 min, washed and air-dried. For starch staining, sections were covered by a drop of Lugol's iodine for 10-12 min.

For visualizing and capturing images of these preparations, a Zeiss photomicroscope with a digital camera (Leica DFC 420 C) attached was used, along with its imaging software (LAS V3.6, LeicaMicrosystemsFramework).

4. 5-METHYL-DEOXY-CYTIDINE IMMUNOFLUORESCENCE

Immunofluorescence was performed to localize 5mdC, essentially as previously described by (Testillano et al., 2013).

Semithin sections were obtained and placed over a drop of water on multiwell slides pre-treated with aminopropiltriethoxi silane (APTES) to facilitate adhesion of sections to slides. After air drying, sections were stored until use for immunoflorescence.

4.1. Denaturation of DNA of sections

In order for the 5mdC epitope to be accessible, it is essential to perform a denaturation step previously to the incubation with the anti-5mdC primary antibody. Therefore, sections were incubated with HCl 2N (in distilled water) for 45 min at room temperature.

4.2. Immunofluorescence of 5-deoxy-methyl-cytidine

Incubation of the sections for 10 min with bovine serum albumin (BSA) 5% (w/v) in Phosphate Buffered Saline (PBS) was performed for blocking unspecific targets of anti-5mdC antibody.

The sections were then incubated with the mouse monoclonal anti-5mdC antibody (Eurogentec, Cat. N. BI-MECY-0100, Liège, Belgium) diluted 1:50 in 1% BSA in PBS for 1 hour in humid chamber.

After washing with PBS 1% multiple times, the secondary antibody Anti-Mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, Leiden, the Netherlands) diluted 1:25 in 1% BSA was applied over the sections and incubated in the dark and humid chamber for 45 min.

Finally, and following more rinsing steps with PBS, sections were incubated 10 min with a mixture of 4',6-diamidino-2-phenylindole (DAPI) staining solution 1 µg/mL and Triton™ X-100 detergent (Fluka® Analytical, © Sigma-Aldrich, Missouri, USA) 10 µL/mL in sterile water, for nuclei staining.

Then, the sections were washed in sterile water three times, mounted on Mowiol (© Sigma-Aldrich, Missouri, USA) and stored at -4 °C protected from light until observation on the confocal microscope.

4.3. Negative controls of immunofluorescence

Two negative controls were made, one by lacking the denaturalization step and performing all the protocol of the 5mdC immunofluorescence. Negative results

indicate that the antibody does not cross-react with double-stranded DNA or other nuclear antigens.

The second control was performed avoiding the primary antibody in the incubation. Absence of signal in this case indicate that cross-reactions with other antigens did not occur, since the antibody was completely blocked *in vitro* with the 5mdC (Testillano and Risueño, 2016). Both controls were assured for every immunofluorescence assay carried out.

4.4. Confocal analysis

Immunofluorescence preparations were analyzed in a confocal microscope (Leica TCS-SP5-AOBS, Vienna, Austria) using laser excitation lines of 461 nm wavelength for DAPI and 488 nm for green 5mdC signal. Emission bandwidths were, respectively, 415-482 nm and 500-567 nm.

Observations were performed with 40x and 63x objectives with immersion oil; for each preparation field, fluorescence emissions for the two fluorochromes, DAPI for nuclei (blue) and 5mdC immunofluorescence (green) were collected together with the bright field to visualize the general cellular structure.

Adequate imaging software (Leica software LCS) running simultaneously with the confocal microscope was used for capturing optical sections at 0.8-1 μm z-intervals over a total thickness of approximately 15 μm and images of maximum projections were obtained from z-stacks of 10-15 optical sections, followed by image merging and other functionalities.

Confocal microscopy analysis was performed using the same laser excitation and sample emission capture settings in all immunofluorescence preparations of each set of experiments, allowing an accurate and reliable comparison among intensity of 5mdC signal of different material.

Likewise, quickness in fluorescence image acquisition was essential because the energy of the fluorophore (*i.e.* signal intensity) is rapidly depleted when exposed to the UV excitation light.

5. QUANTIFICATION OF GLOBAL DNA METHYLATION

Global DNA methylation levels were estimated and compared among samples from selected culture times and developmental stages, an ELISA-based colorimetric method was used, following the protocol for plant samples previously described (Testillano et al., 2013; El-Tantawy et al., 2014).

5.1. Extraction of total DNA from samples

Samples of various sources were collected to 1.5 mL sterile Eppendorf tubes, immediately frozen in liquid nitrogen and stored at -80 °C until use.

For homogenization, frozen material was placed in a sterile, cooled mortar and smashed to powder. It was maintained at frozen state by constantly pouring with liquid nitrogen, so that DNA would not degrade, and the resultant powder was put in new sterile Eppendorf tubes (to a maximum of 100 mg per tube) plunged in liquid nitrogen or stored again at -80 °C.

Extraction of total DNA from these disrupted samples was performed using a plant genomic DNA extraction kit (DNeasy Plant Mini, Qiagen) according to the manufacturer's instructions and adding 8μl RNase A (Qiagen).

The DNA extract was diluted in sterile H_2O_d and final concentration was measured in a spectrophotometer (NanoDrop™, Thermo Scientific, Massachusetts, USA), ensuring a symmetric absorbance peak at 260 nm. DNA purity was confirmed with A_{260}/A_{280} ratio between 1.7 and 1.9.

5.2. Quantification of global DNA methylation

Global DNA methylation was determined using the Methyl Flash Methylated DNA Quantification Kit (Colorimetric, Epigentek, Farmingdale, NY, USA) following manufacturer's instructions. Aiming to compare global DNA methylation values among samples further on, a quantity of 100 ng of genomic DNA was set as the sampling amount in all performed assays (Testillano et al., 2013). The mdC content was calculated as a percentage of total DNA.

Each quantification experiment was performed with positive and negative controls and two analytical replicas of each reaction in an ELISA plate, and absorbance was read at 450nm in a spectral scanning multimode reader (Varioskan™, ThermoFisher Scientific, Massachusetts, USA).

Statistical differences among samples were tested using one-way ANOVA and Tukey test for independent samples.

6. GLOBAL HISTONE H3K9 METHYLATION

6.1. Preparation of the samples for histone extraction

Frozen material (previously smashed to powder using liquid nitrogen, similarly to the above described for DNA extraction) was placed in an Eppendorf (approx. 50-100 mg) and, with the addition of a lysis buffer (GC1 solution from the EpiQuik™ Global Histone H3-K9 Methylation Assay Kit, Epigentek, Farmingdale, NY, USA) diluted 1:10 in distilled water, disaggregated by strokes with a sterile Dounce homogener. Homogenized mixture was centrifuged at 10 000 rpm for 1 minute at 4 ^{o}C and the supernatant was discarded.

6.2. Extraction of histones

Extraction of total histones from this collected samples was performed using an adequate histone extraction kit (EpiQuik™ Global Histone H3-K9 Methylation Assay Kit, Epigentek, Farmingdale, NY, USA), according to the manufacturer's instructions.

At the end, distilled water was added to dissolve pellet (10 µl of water per each 40 mg of initial smashed tissue) and stored at -20 °C until use.

6.3. Quantification of total histones extracted

In order to determinate its concentration, an assay was performed following the 'Bradford's method' (Bradford, 1976) histone extracts were added to individual

wells of an ELISA plate, along with Coomassie Brilliant Blue G-250 dye to proteins.

This dye binds primarily to basic and aromatic aminoacid residues (Compton and Jones, 1985). At acidic pH, it is predominantly in the doubly protonated cationic form, presenting a red-brownish colour (maximal absorbance is 470 nm), but when the dye binds to protein, it acquires a blue colour correspondent to its stable unprotonated form (maximal absorbance is 595 nm) (Fazekas de St. Groth et al., 1963; Reisner et al., 1975; Sedmak and Grossberg, 1977).

The amount of protein in the sample is proportional to the optical density (OD) measured at 595 nm. A spectral scanning multimode reader (Varioskan™, ThermoFisher Scientific, Massachusetts, USA) was the spectrophotometer instrument at disposal to do so. A protein standard curve was traced using BSA solutions at seven distinct, known concentration points (diluent was the same as for the samples: distilled water).

The assay was performed using three replicas of each solution; ELISA plate wells were filled with 5 µl of standard BSA solution or diluted sample and 245 µl of dye reagent. Spectrophotometer readings of the plate were made after 5-10 min of incubation at room temperature.

Blanks and negative control OD readings were subtracted to the OD readings of samples.

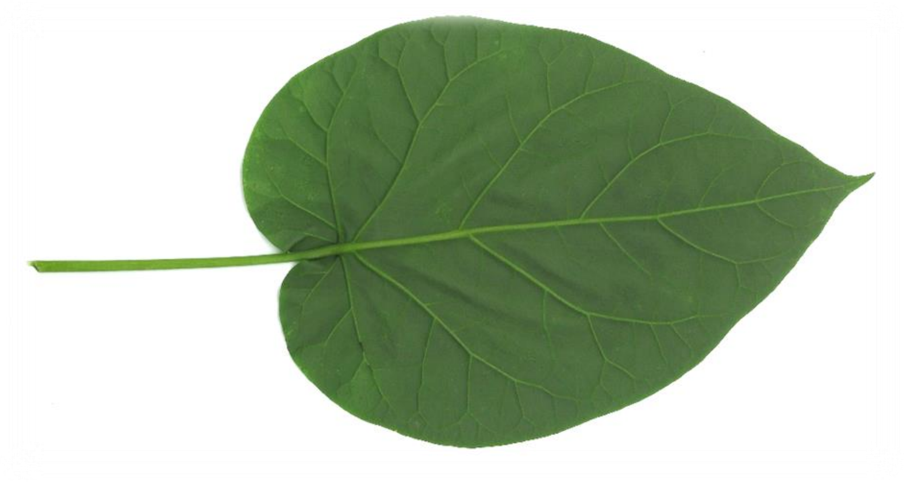
6.4. Quantification of H3K9 methylation levels

To compare relative levels of global H3K9 methylation of samples, the EpiQuik™ Global Histone H3-K9 Methylation Assay Kit (©Epigentek Group Inc., NY, USA) was used, following the method previously described by plant cells (Berenguer et al., 2017).

The protein concentration of histone extracts obtained were adjusted in all samples to 300 ng/µl. Histone extracts were added to individual wells of an ELISA plate which was read at 450 nm in a spectral scanning multimode reader (Varioskan™, ThermoFisher Scientific, Massachusetts, USA).

Blanks and negative control OD readings were subtracted to the OD readings of samples and relative sample OD were calculated in percentages. Assays were performed in duplicate.

Statistical differences among samples were tested using one-way ANOVA and Tukey test for independent samples.



Results

1. OBTENTION AND CHARACTERIZATION OF NEW EMBRYOGENIC LINES OF TAMARILLO

1.1. Somatic embryogenesis induction and different yields among genotypes

Somatic embryogenesis was induced from leaf explants of seedling-derived shoots of tamarillo, as described in Material and Methods section. After 5-6 weeks in multiplication medium, shoots produced several young leaves (Fig. 7A, 7A') that were used for SE induction. Young expanded leaves were excised and cultured in induction medium (Fig. 7B, 7B'), producing two types of structures: non-embryogenic *calli* and embryogenic masses, the former appearing generally by the sixth week of culture and the latter 2-4 weeks later. After completing 12 weeks of incubation, these embryogenic *calli* were easily distinguishable from the surrounding non-embryogenic *callus*, tough they initially grow in mixed aggregates (Fig. 7C). Manual isolation on subsequent subcultures allowed the maintenance and multiplication of the two types of structures separately (Fig. 7D, 7D', 7E, 7E').

Non-embryogenic *calli* are normally yellowish and translucent, with friable texture (Fig. 7E, 7E') and fast growing, whereas embryogenic masses proliferate at slower rates and have a compact, rather granular structure, with more opaque shades ranging from pale yellow to grayish (Fig. 7D, 7D').

The general macroscopic morphology of these two types of cellular masses is maintained in successive subcultures. Beside this, different embryogenic and non-embryogenic lineages have different vulnerability to contamination and resist different time in culture with no signs of oxidation. For instance, YL 2009, YL 2014 and YL 2016 (*i.e.* the three embryogenic lines induced from young leaves of TV310 genotype seedlings) required more attention and more frequent subculturing, in order not to oxidate and darken.

In the present work, SE induction experiments were performed in three different genotypes of tamarillo. In the various induction experiments performed, the yield of the induction process was estimated and different responsiveness to induction among leaves from different tamarillo genotypes was observed.

'Yield of induction' in each experiment was considered as the number of embryogenic clumps per total number of leaf explants plated in induction medium, excluding those subject to contamination. Although there were variations on clump's size, the results, presented on Table II, illustrate differences in the responsiveness to induction among TSM10, TV310 and TDJ3 genotypes.

Statistical analysis (one-way ANOVA and Tukey test) show that for a 95% confidence level, the yield of the induction phase in tamarillo TDJ3 is significantly lower than in TSM10 and TV310 genotypes.

Table II – Yield of the induction phase on young leaves of three clonal genotypes of tamarillo. Values correspond to weighted means of the yield of induction calculated at each induction experiment performed (7-8 experiments with each genotype; 30-100 explants per experiment) and respective standard deviation (sd). Different letters represent significant differences at $p \leq 0.05$, according to Tukey test.

Genotype	Yield of induction (embryogenic clumps / leaf explant)
TSM10	**0.337** ±0.245 a
TV310	**0.260** ±0.187 a
TDJ3	**0.088** ±0.062 b

1.2. Different behavior of embryogenic lines of different age during maturation phase

In the present work, embryogenic lines that were induced 7 and 2 years ago and have been subcultured monthly (old lines), together with new lines induced in this work (recent lines) were analysed. Upon transferring embryogenic clumps to medium without auxin, embryo conversion phase begins. Both recent and old embryogenic lines were subjected to this procedure, having responded very differently. Fig. 8 illustrates the standard behavior of an embryogenic line that

generates embryos and their subsequent *in vitro* development until obtaining a complete plantlet.

1.2.1. Proliferation rates of embryogenic lines of different age during maturation phase

Aside from forming somatic embryos, the embryogenic cell masses in medium without auxin continue to grow and proliferate. We analyzed whether the rate at which this occurs depends on genotype, subculturing age and origin of the explant. Results are shown in Fig. 9 that represents the increase in fresh weight as an estimation of proliferation, of four distinct lines throughout 5 weeks in maturation medium.

The two embryogenic cell lines induced in 2009, YL2009 and ZE2009, were the ones that most proliferated in the maturation medium, respectively with a 7.3 and a 3.7 -fold weight gain (mean values) at the end of third week. Proliferation rate decreased when more recent lines were used. The line induced in 2014, YL 2014, showed a 3.1-fold growth at the end of third week. The trend continued with the prolongation of culture time, with YL 2009, ZE 2009 and YL 2014 having increased their initial fresh weight 10.5, 7.3 and 5.5 –fold, respectively, at the end of fifth week.

The embryogenic line induced in 2016 (YL 2016) was the one that proliferated the least, having just duplicated its initial weight at the end of third week. We did not keep track of the proliferation behavior of this embryogenic line up to the fifth week, due to the fact that when week-3's record was made, there were already so much material converted in somatic embryos (whose weight was excluded), that these were immediately transferred to MS medium with reduced sucrose concentration (2.5%) and led to their own development fate under light conditions.

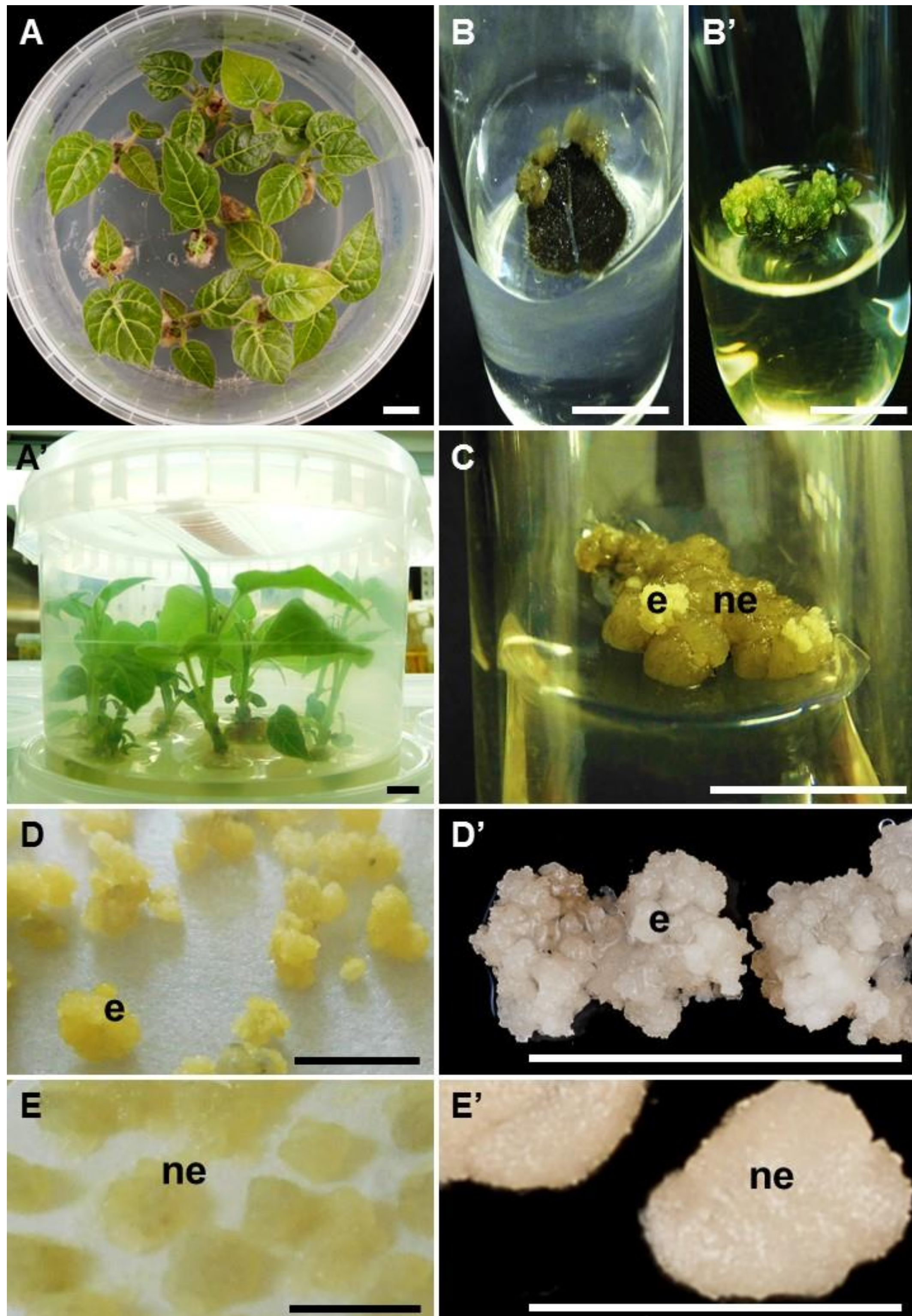


Figure 7 – Morphological aspects of SE induction process starting with young leaves of micropropagated shoots of tamarillo. A, A' – Shoots' micropropagation in plastic container (top and lateral view) with medium with BAP cytokinin. B, B', C – Dedifferentiation of young leaves in induction medium (leaves with distinct aspect), resulting in non-embryogenic *calli* (ne) with embryogenic clumps (e). D, D', E, E' – Separation of embryogenic masses (D, D') from non-embriogenic *calli* (E, E') allowed by subculture in medium with auxin, maintenance and proliferation of tissues. Bars: 1 cm exc D' and E': 0.5 cm.

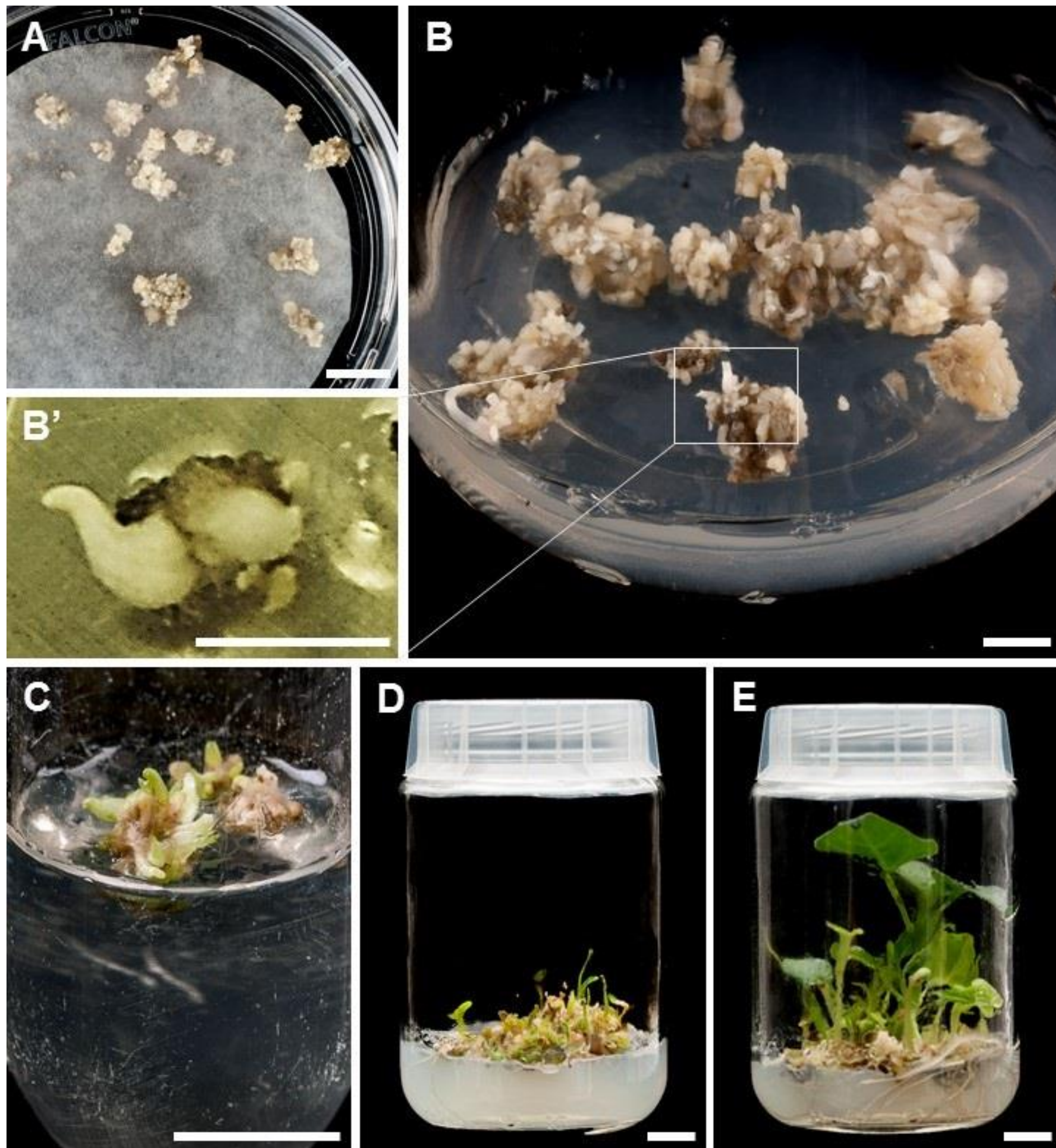


Figure 8 – Morphological aspects of embryo conversion and germination of tamarillo from recent embryogenic masses. A - Embryogenic clumps recently induced (2016) and proliferated in medium with auxin. B, B' – After 1-3 weeks in medium without auxin, whitish somatic embryos start to emerge. C, D – Embryos develop and acquire photosynthetic ability. E – After 5-7 weeks, complete plantlets are ready to acclimatization and transfer to organic substrate. Bars: 1 cm.

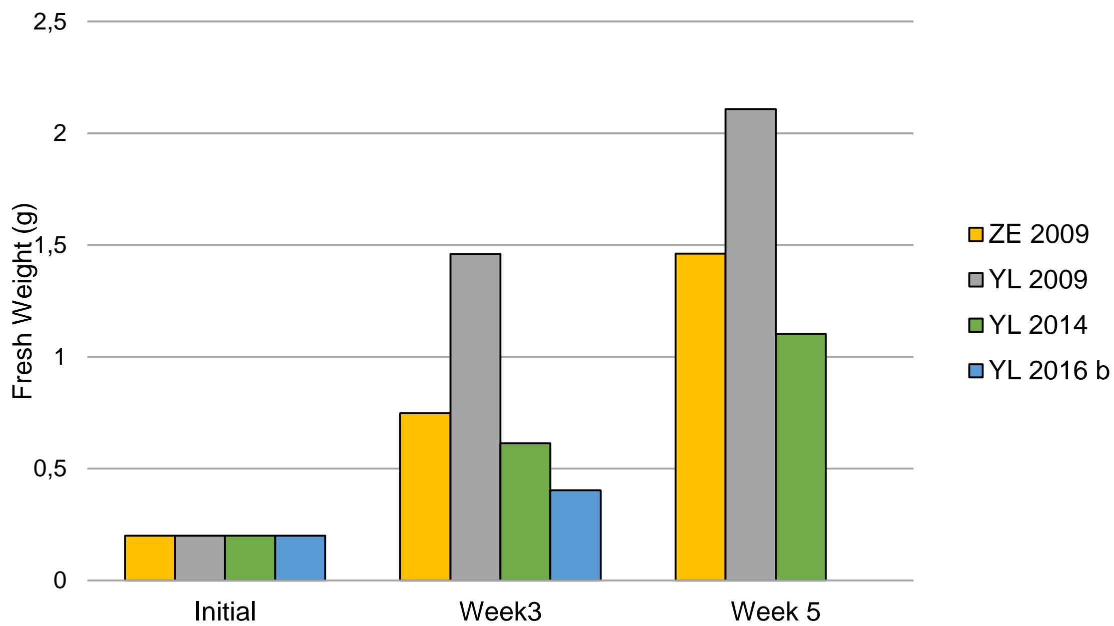


Figure 9 – Proliferation of embryogenic cell lines during the maturation phase. Fresh weight at experiment's beginning was normalized to 0.2 g, aiming for an easier visual interpretation. YL 2009, embryogenic mass induced in 2009 from young leaves of TV310 genetic line; ZE 2009, embryogenic mass induced in 2009 from a zygotic embryo; YL 2014, embryogenic mass induced in 2014 from young leaves of TV310 genetic line; YL 2016b, embryogenic mass induced in 2016 from young leaves of TDJ3 genetic line.

1.2.2. Somatic embryo conversion rates in embryogenic lines of different age

In parallel with weighing, the ability of embryogenic masses to generate somatic embryos was also analyzed. Significant differences in initiation timing, quantity and quality of converted embryos were observed.

Indeed, while 2016's lines usually start exhibiting whitish, early embryo-like structures as soon as one week after auxin's removal, this was not the case for embryogenic lines subcultured since 2009 and 2014. In those, only after three to four weeks some somatic embryos' initiation started to be visible.

Regarding yield of conversion process, presumptive somatic embryos or whitish embryo-like structures discernible to the naked eye were counted at week 3 in maturation medium. Data are shown in Table III.

Fig. 10 shows YL 2009 and YL 2014 embryogenic masses after three weeks in maturation medium, evidencing considerable proliferation but few embryo-like structures.

Table III – Somatic embryo countings during maturation phase of different embryogenic lines.

Embryogenic Mass lineage	Rep	Initial Fresh Weight (g) put onto maturation medium		Developing Embryos (2-3 mm)		Other presumptive embryo-like structures
			Total		Total	
ZE 2009	a	0.235	1.197	0	**0**	0
	b	0.249		0		0
	c	0.264		0		0
	d	0.231		0		0
	e	0.218		0		0
YL 2009	a	0.22	0,824	0	**1**	1
	b	0.233		1		0
	c	0.198		0		0
	d	0.173		0		0
YL 2014	a	0.219	0,939	1	**18**	7
	b	0.177		4		8
	c	0.209		4		6
	d	0.151		3		5
	e	0.183		6		3
YL 2016b	a	0.156	0,292	~30	**>60**	SOME
	b	0.136		~40		SOME
YL 2016a1	a	0.109	0,467	~15	**>90**	~6
	b	0.106		~50		MANY
	c	0.252		~30		SOME
YL 2016a2	a	0.106	0,216	~8	**>40**	~10
	b	0.11		~40		MANY

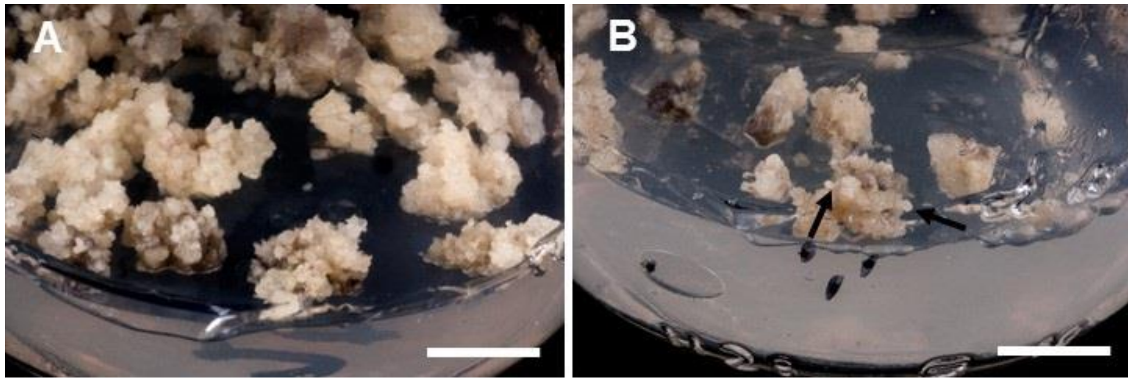


Figure 10 – Morphology of old embryogenic cell lines after three weeks in MS medium without auxin. Black arrows indicate possible embryo initiation events. A - YL 2009; B - YL 2014. Bars: 1 cm.

Besides all the above results, it is important to emphasize that when the few formed embryos from older embryogenic lines were transferred to MS medium with reduced sucrose concentration (2.5%), under light conditions, they atrophied and blackened instead of progressing with their development.

2. COMPARATIVE ANALYSIS OF EMBRYOGENIC VS NON-EMBRYOGENIC LINES

2.1. Cellular organization and 5-methyl-deoxy-cytidine immunolocalization

The differences in structural organization, at cellular level, between embryogenic and non-embryogenic lines were analyzed by bright field and epifluorescence microscop, using several cytochemical methods (toluidine blue staining for general cell structure, iodide staining for starch and DAPI staining for nuclei). Embryogenic potential can be related to DNA methylation epigenetic mark, which is known to play a determinant role on developmental pathways and regulation of the transcriptional activity of cells in each state. Therefore, we performed immunofluorescence with anti-5mdc antibodies to localize methylated cytosines in DNA and compare signals between embryogenic and non-embryogenic material.

2.1.1. Embryogenic cell lines

Embryogenic masses exhibit, under bright field microscopy, an aspect that matches published descriptions of other embryogenic tissues, featuring isodiametric, cohesive cells (Fig. 11A and 11A, 11C) with big nuclei, small vacuoles and frequently starch granules (Fig. 11A, 11A', 11C, 11E), a typical structure of proliferating cells. Toluidine blue staining and high magnification micrographs allow the observation of numerous interphasic nuclei and mitotic phases with chromatin highly condensed in chromosomes; also a prominent nucleolus appears well defined in interphasic nuclei (Fig. 11C, 11E).

5-methyl-deoxy-cytidine immunofluorescence signal appear specifically over nuclei, showing a pattern of labeling related to the chromatin condensation degree, with low-mid intensity in interphasic nuclei and very high fluorescence in mitotic chromosomes (Fig. 11F'). No signal was observed in control experiments with no DNA denaturation or avoiding the first antibody (data not shown).

This general cell structure is similar among embryogenic cells from lines with different ages and origins. The embryogenic masses also exhibit some other particularities. Firstly, budding structures sometimes emerge from the central mass, displaying thicker cell walls, defined boundaries and lower 5mdC signal intensity, typical features of initial proembryo formation (Fig. 13). Depending on the orientation of the section, microscopy images can show these cellular clumps in various positions. They often appear emerging, detaching from the main cellular mass (Fig. 13A, 13D, 13G).

The second particularity to point out is that embryogenic masses are not all-homogeneous: they have zones in which cells show intensely toluidine-stained cytoplasm and low vacuolation, suggesting a cellular activity presumably higher (z1 in Fig. 14), whereas in other zones (z2 in Fig. 14) cells have a less dense, highly vacuolated appearance, which closely resembles non-embryogenic *calli* (addressed in the next section).

This heterogeneity applies to all embryogenic lines observed, regardless of its origin and culture age. However, something interesting stands out: generally, the more recent the line is, the more embryogenic-like cells ('z1') it has. In other

words, zones with presumably low cell activity are the most abundant in embryogenic lines maintained in culture for longer time.

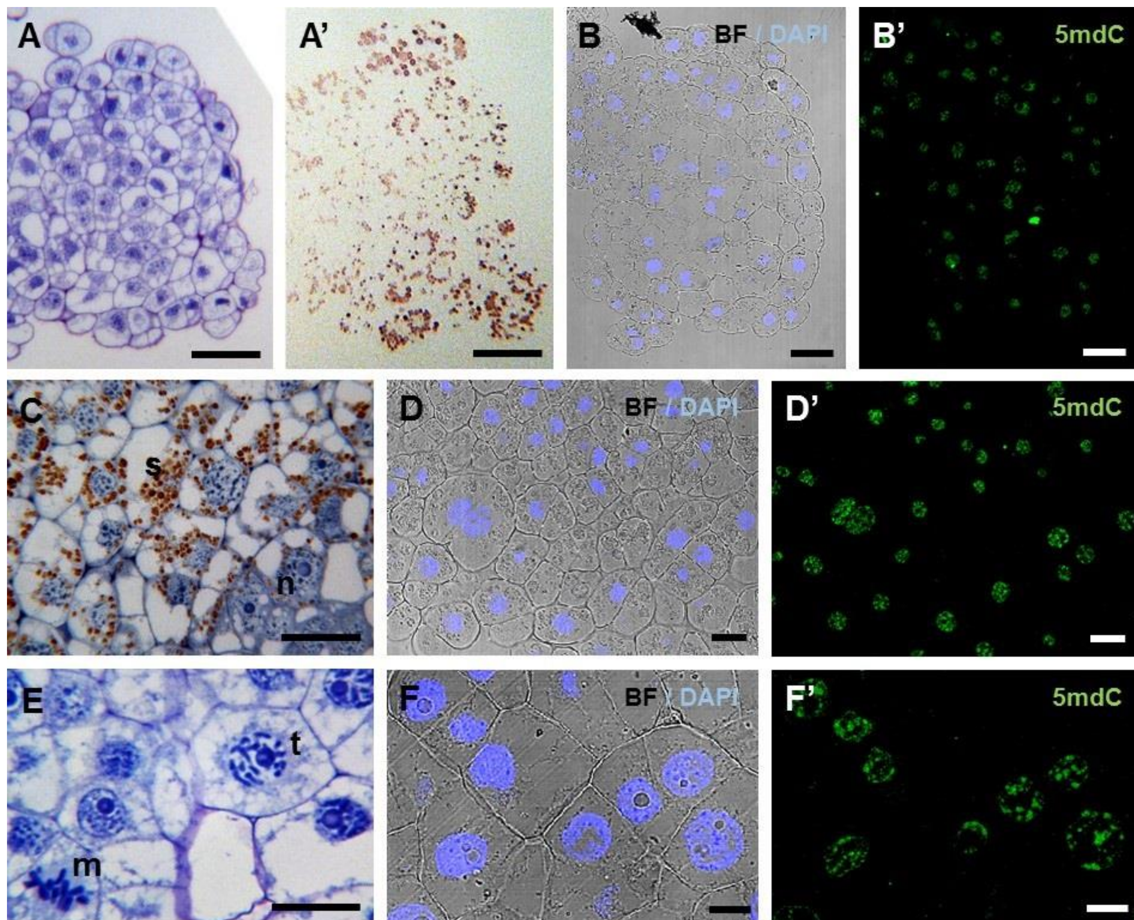


Figure 11 – Cellular organization and 5mdC immunofluorescence of old embryogenic cell lines. Toluidine blue (A, C, E), iodine staining (A', C), bright field / DAPI (B, D, F) and 5mdC signal (B', D', F'). Embryogenic masses induced in 2009 from young leaves of TV310 line (A, B, C, E, F) and zygotic embryos (D). s: starch; n: nucleus; m: metaphase; t: telophase. Bars: A and B: 40 µm ; C and D: 20 µm; E and F: 10 µm.

There does not seem to exist significant differences of the global intensity of the 5mdC immunofluorescence signal among the various embryogenic lines observed, regardless of its origin or age.

There is, however, a noticeable intra-sample variability on the intensity of the signal, particularly when comparing zones more internal to the mass with its periphery, or the protrusion situation already mentioned.

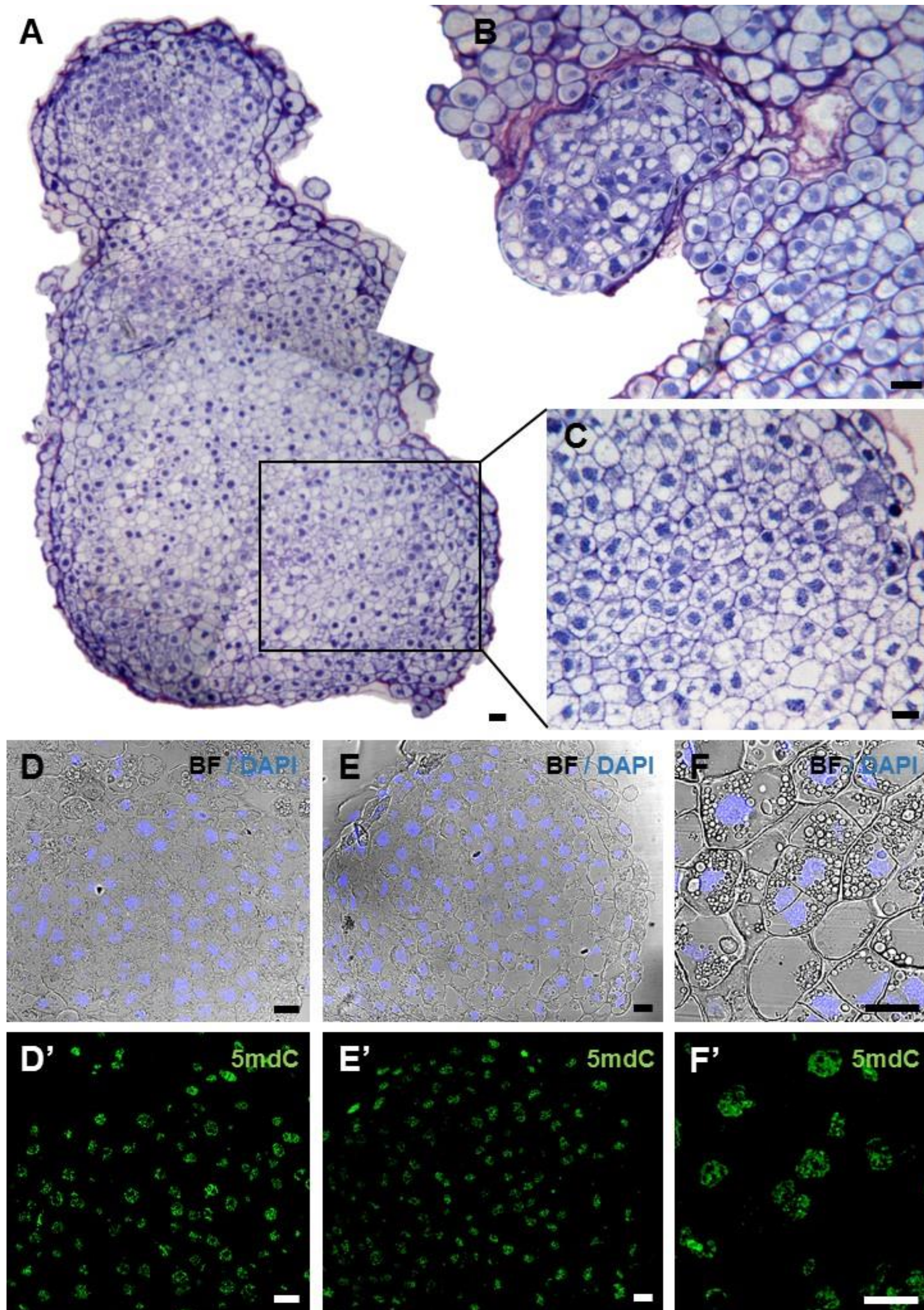


Figure 12 - Cellular organization and 5mdC immunofluorescence of recent embryogenic lines. Toluidine blue (A, B, C), bright field / DAPI (D, E, F) and 5mdC signal (D’, E’, F’). Embryogenic masses induced in 2016 from young leaves of TSM10 (A, E, F) and TDJ3 (B, C, D) lines. A – Complete section of a small embryogenic cell clump, in which one can perceive its granular organization; D, E – similar and equivalent cell areas in the two different embryogenic lines. Bars: 20 µm.

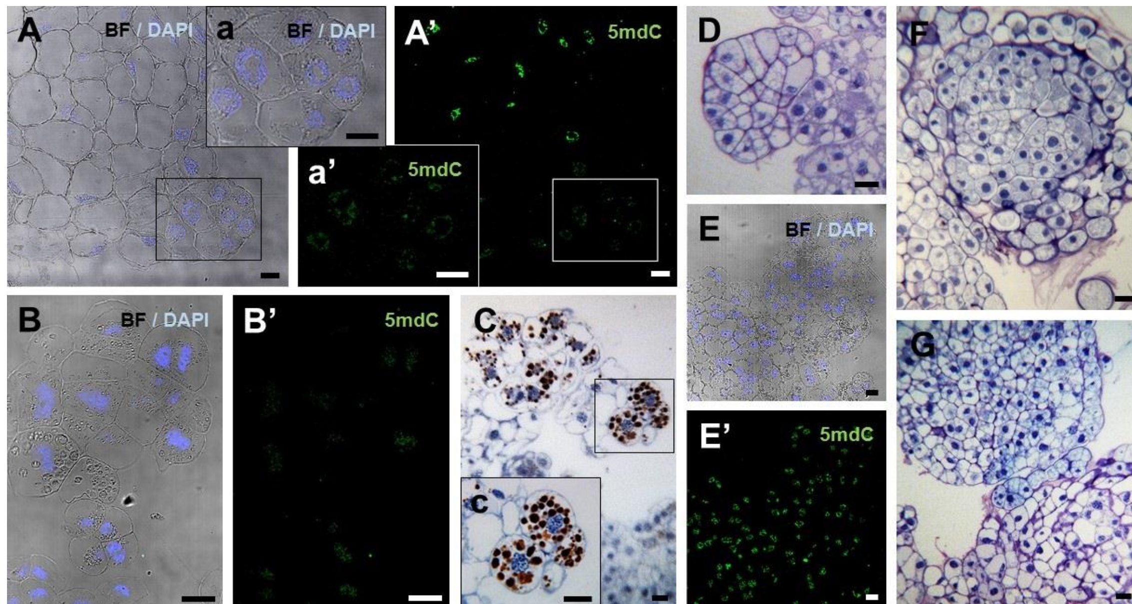


Figure 13 – Embryogenic cell masses exhibiting protrusions. A, a, B, E: bright field / DAPI; A', a', B', E': 5mdC signal; C, c: toluidine blue / starch staining; D, F, G: toluidine blue staining. Lines of different age and origin are presented, namely: ZE 2009 in A, D and G; YL 2009 in B, C and E; YL 2016b in F. Bars: 20 μm.

2.1.2. Non-embryogenic cell lines

The non-embryogenic cell lines originated, as explained before, as the first dedifferentiated cellular masses that form from the leaf explants during the induction process. They are soft, translucent aggregates which proliferate fast in an appropriate medium. At a microscopic level, these cells are usually large, not appearing to form any type of organized and compact tissue as the embryogenic cells do. They are characterized by round and irregular shapes, rarely isodiametric, very large vacuoles, small nuclei with condensed chromatin and sometimes with starch granules (Fig. 15).

This general cell organization of non-embryogenic lines is observed in most of the lines analyzed, there seems to be no significant differences in structure among non-embryogenic lines based on their age or explant of origin (Fig. 15A-I), 5mdC immunofluorescence experiments to localize methylated DNA provide an intense labeling that covers the whole nuclear area (except the nucleoli) of the small nuclei of cells (Fig. 15F'-I'). Regarding non-embryogenic lines of different age, direct comparisons of 5mdC signal intensities (accomplished by careful analysis of images captured in the confocal microscope under the same settings

and experimental conditions) lead to the observation that immunofluorescence signals do not vary significantly over subculture time.

Among young leaves' derived lines, the 5mdC signal intensity of non-embryogenic cells looks similar to signals of most of the cells of embryogenic lines. However, some cells, frequently the ones located on the periphery of embryogenic masses (and often corresponding to dense-active cells, characterized above as z1 in Fig. 14), show lower 5mdC singles than non-embryogenic cells.

2.2. Global DNA methylation levels of embryogenic and non-embryogenic lines

Assays of quantification of the global levels of DNA methylation in each embryogenic and non-embryogenic tissue were performed since their results can be related to the general state of transcriptional activity / inactivity of its cells' genome.

For these assays, embryogenic and non-embryogenic masses were homogenized and analyzed, therefore, the obtained percentage of total methylation of cytosines is an average value of the contribution of all cells forming the cellular aggregates which are, (as demonstrated by our previous microscopic study), very heterogeneous (Fig. 14 and 15). Taken this into account, the data that follows provide information of interest for our study.

The results of the global DNA methylation levels in embryogenic and non-embryogenic lines of different years are shown in Fig. 16.

As one can see in the histogram (Fig. 16), regarding embryogenic masses originated in different years, global DNA increased as cultures get older. This behavior does not apply to non-embryogenic cell cultures, in which cytosine methylation is less regular.

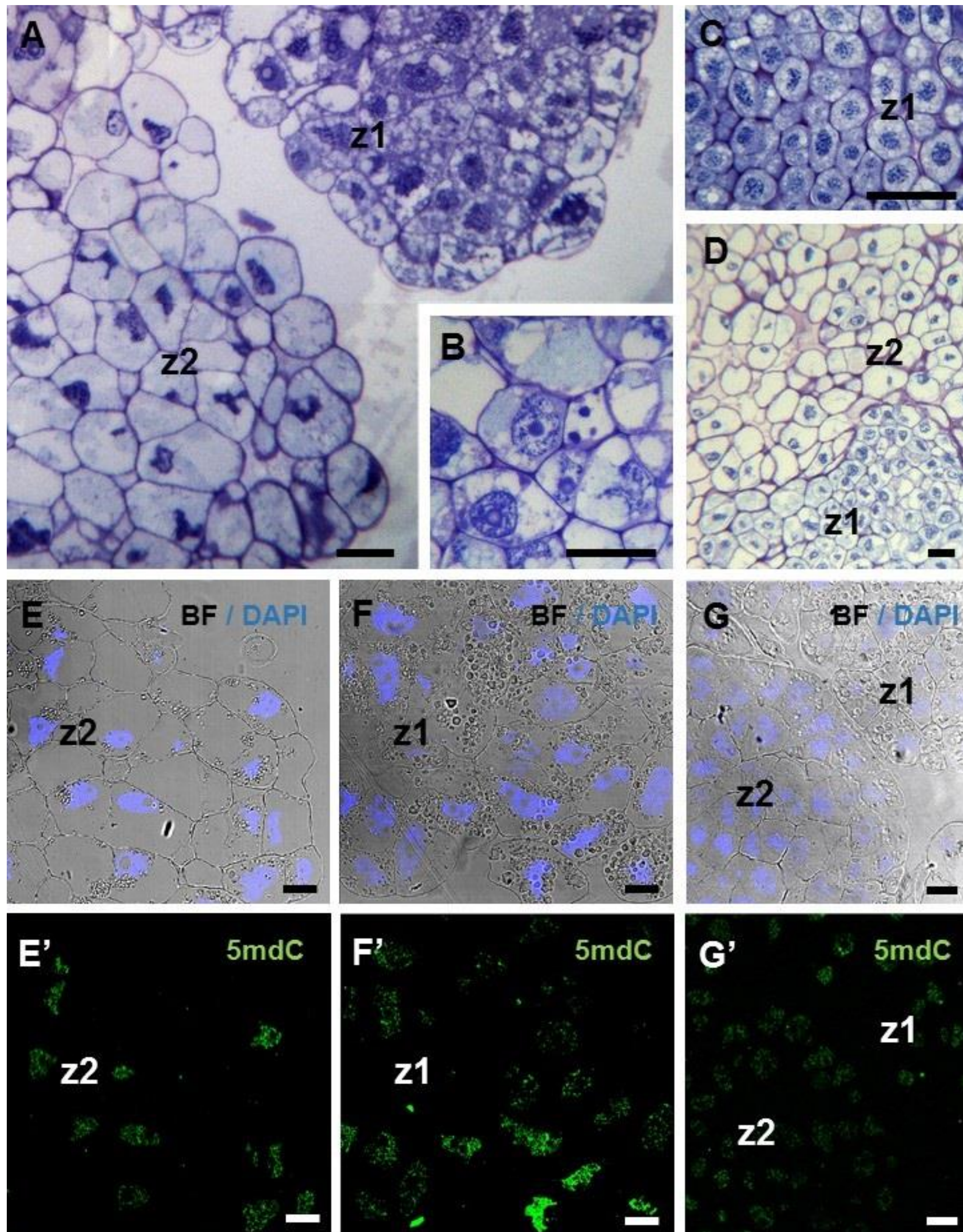


Figure 14 - Cellular organization and 5mdC immunofluorescence of embryogenic cell lines. Toluidine blue (A - D), bright field / DAPI (E - G) and 5mdC signal (E’ – G’) of embryogenic masses evidencing distinct cellular zones. z1, embryogenic zone with high cellular activity; z2, embryogenic zone which resembles non-embryogenic *calli*. Lines YL 2014 (A, B, E, E’, F, F’), ZE 2009 (D, G, G’) and YL 2016 b (C) are represented, proving that these two cell domains are present in embryogenic masses regardless of its age and explant of origin. Bars: 20 µm.

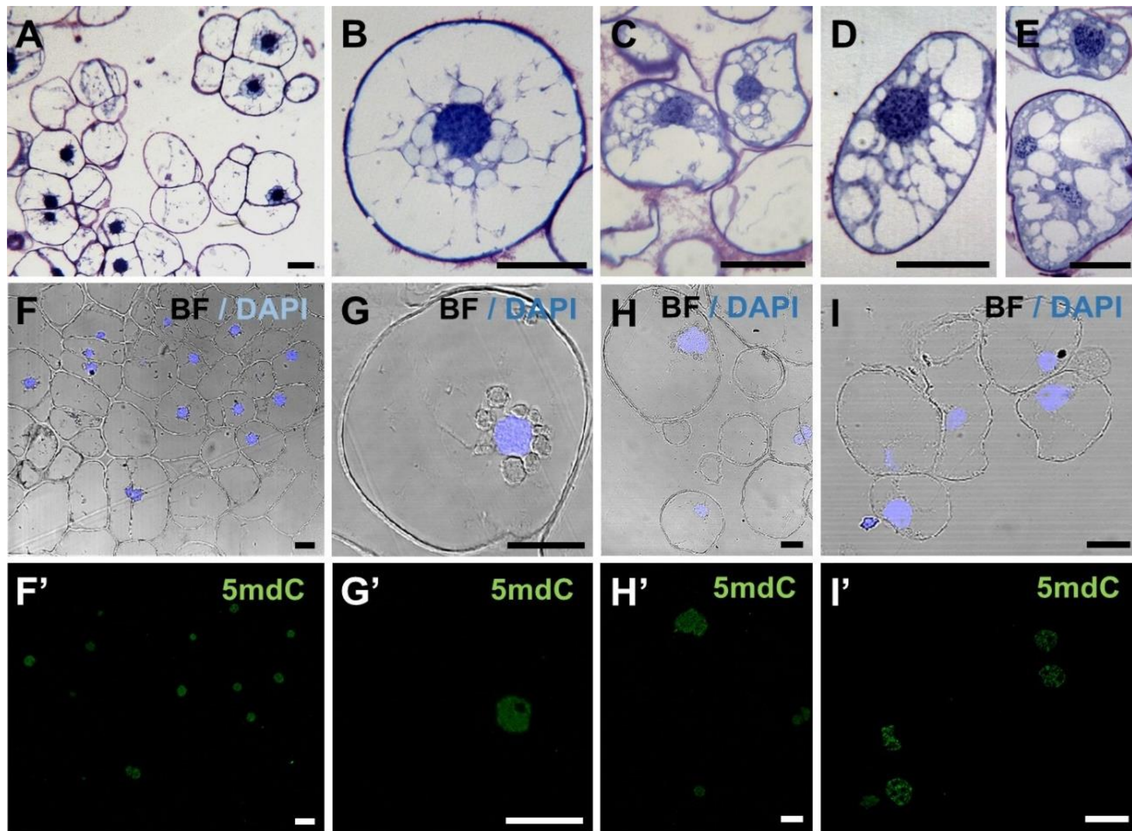


Figure 15 – Cellular organization and 5mdC immunofluorescence of non-embryogenic cell lines. Toluidine blue (A - E), bright field / DAPI (F-I) and 5mdC signal (F'-I') of old (2009) embryogenic masses induced from zygotic embryo (A, B, C, F, F', G, G', H, H') and young leaves of TV310 line (D, E, I, I'). Bars: 20 µm, exc. B and C: 10 µm.

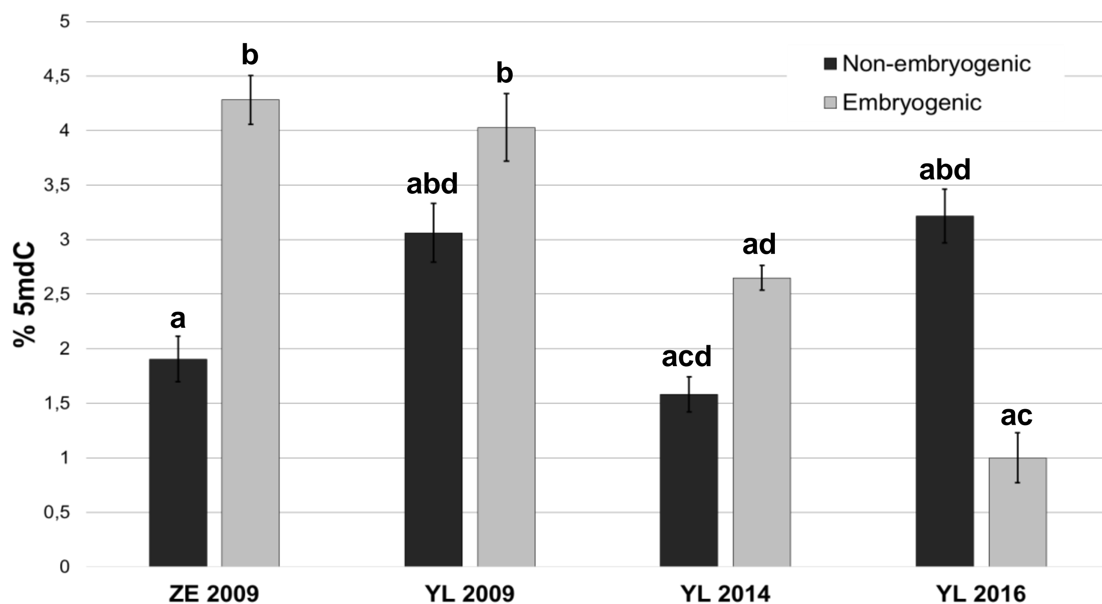


Figure 16 – Global DNA methylation levels among embryogenic / non-embryogenic cell lines. Each pair was originated in a single induction event, from young leaf (YL) or zygotic embryo (ZE) explants, in years 2009, 2014 and 2016. Error bars correspond to sd values. Values indicated by the same letter were not statistically different at $p \leq 0.05$, using Tukey test.

Between the embryogenic and non-embryogenic lines within a pair (lines originated in the same year), differences are only statistically significant in the oldest and newest lines analyzed, ZE 2009 and YL 2016. The older pair shows higher methylation in embryogenic than in non-embryogenic lines, whereas the newest pair shows an opposite relation, *i.e.* non-embryogenic *callus* being more methylated than the embryogenic mass. The rest of lines studied (YL2009, YL2014) do not show significant differences in methylation between embryogenic and non-embryogenic lines. As culture time passes by, this within-pair relation tends to reverse, especially due to the accumulation of DNA methylation in the embryogenic cells.

As previously described, the embryogenic capacity and the abundance of dense-active cell clumps among embryogenic lines decrease in older lines in comparison with newer lines. The data of the global DNA methylation assays also suggest a relationship between levels of methylation and age of the lines. Only in the recent embryogenic lines, with high embryogenic capacity and abundant dense-active cell clumps, the methylation levels are significantly much lower than the corresponding non-embryogenic line of the same year. In contrast, older embryogenic lines, with less embryogenic potential, show higher methylation levels than recent embryogenic lines. The embryogenic line originated from a zygotic embryo in 2009 (ZE 2009), which has almost lost its ability to produce new somatic embryos, exhibits higher methylation level than its corresponding non-embryogenic line.

2.3. H3K9 methylation within an embryogenic / non-embryogenic pair

Some other epigenetic marks, together with DNA methylation, contribute to regulation of gene activity. Among histone methylation marks, the methylation of lysine 9 of histone H3 is one of the modifications with a repressive action of gene expression that correlate with DNA methylation in many developmental processes. Therefore, the more recent pair, YL 2016 embryogenic / non-embryogenic pair was further analyzed regarding its H3K9 methylation levels, by an ELISA-based colorimetric assay which shows the higher absorbance value the higher its percentage of H3K9.

Results show a much higher level of H3K9 methylation in non-embryogenic than in embryogenic masses (Fig. 17). Noteworthy, this result correlates with the above presented DNA methylation result for the same embryogenic / non-embryogenic pair: the former is considerably less methylated than the latter, the quantification of the two epigenetic marks, DNA and H3K9 methylation, showing a similar profile (Fig. 17).

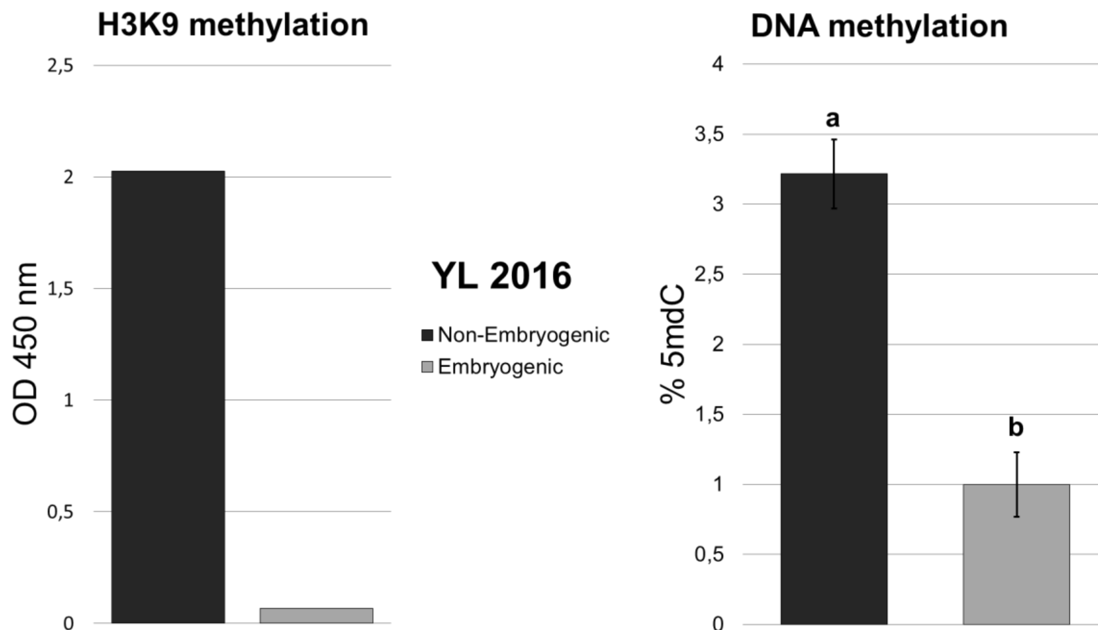


Figure 17 – Global H3K9 and DNA methylation levels in embryogenic and non-embryogenic lines. Mean levels in YL 2016 embryogenic / non-embryogenic pair. Error bars correspond to sd values. Values indicated by the same letter were not statistically different at $p \leq 0.05$, using Tukey test.

3. DYNAMICS OF DNA METHYLATION DURING SOMATIC EMBRYOGENESIS: CHANGES IN GLOBAL METHYLATION LEVELS AND DISTRIBUTION PATTERNS DURING INDUCTION AND CONVERSION PHASES

3.1. Changes in global DNA methylation levels at induction phase

Aiming to clarify how the DNA methylation state of young tamarillo leaves change during the process of induction, we performed a global DNA methylation quantification assay using fresh leaves and dedifferentiating material (6 weeks on induction medium).

The results indicate that, after 6 weeks in induction medium, containing auxin, cells of dedifferentiated masses originated from leaves show a substantial reduction on the global percentage of methylated cytosines on its genome, in comparison with cells of leaves (Fig. 18).

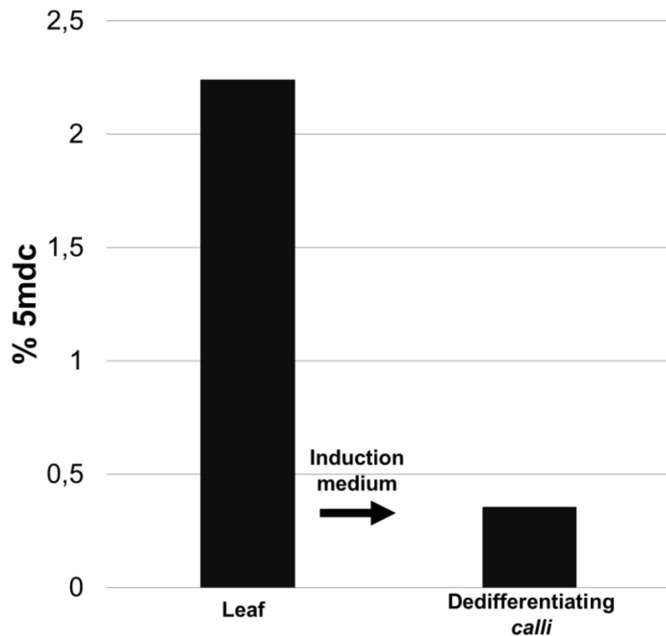


Figure 18 – Global DNA methylation levels of young fresh leaves and during induction phase. TSM10 genotype.

3.2. Changes in cellular organization and 5-methyl-deoxy-cytidine immunofluorescence at induction phase

In order to complement the global DNA methylation assay addressed above, and also to have some insights on the cellular structure and distribution of the 5mdC signal in dedifferentiating tissues, cytochemical and immunofluorescence analysis of the same two stages (fresh leaves and dedifferentiating leaf masses of tamarillo material) was performed.

Microscopic analysis of young leaves reveals a typical, organized tissue structure (Fig. 20): central vein, leaf blade, xylem and phloem veins, stomatic apertures, adaxial and abaxial epidermis, palisade and lacunar mesophyll cells with numerous chloroplast at their peryphery and some trichomas (Fig. 20A, 20B, 20C).

Nuclear signal as revealed by DAPI is very intense, indicating a high chromatin condensation level in most nuclei of the differentiated cells of epidermis and

mesophyll of young leaves (Fig. 20B', 20C'). Immunofluorescence assays provide an equally intense 5mdC labelling to leaf cell nuclei (Fig. 20B'', 20b, 20C''), which seems to homogenously cover the whole nuclear region, except for the small nucleoli which appear dark (Fig. 20B, 20B', 20b).

Regarding the dedifferentiating cell masses, microscopic analysis show that they are heterogeneous and are formed by cells of different sizes and shapes (Fig. 19C), the majority of them have large vacuoles (Fig. 19B, 19b) and look alike non-embryogenic *calli* cells. Others regions are constituted by smaller cells with dense cytoplasms and numerous starch granules (Fig. 19A, 19a). Presumably, these regions are more prone to form, at the end of the induction process, embryogenic clumps that can further originate somatic embryos under specific culture conditions.

In these dedifferentiated cell masses originated in induction phase, both type of nuclei, (from small-dense cells and from larger vacuolated cells) show much lower 5mdC immunofluorescence signal (Fig. 19A, 19A', 19a, 19a', 19B, 19B', 19b, 19b') than leaf cells, before induction.

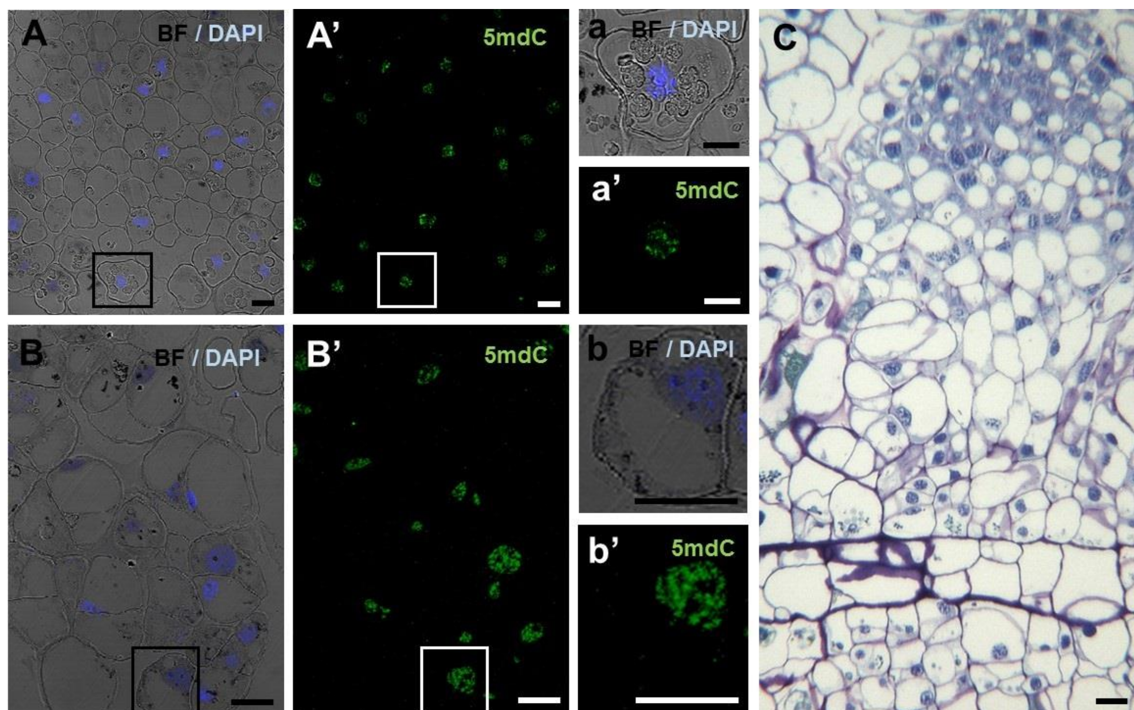


Figure 19 – Cellular organization and 5mdC immunofluorescence of dedifferentiating tissues of a TSM10 leaf after 6 months on induction medium. A, B, a, b – bright field / DAPI; A', B', a', b' – 5mdC immunofluorescence signal; C – toluidine blue staining of a highly heterogeneous region. Bars: 20 μm.

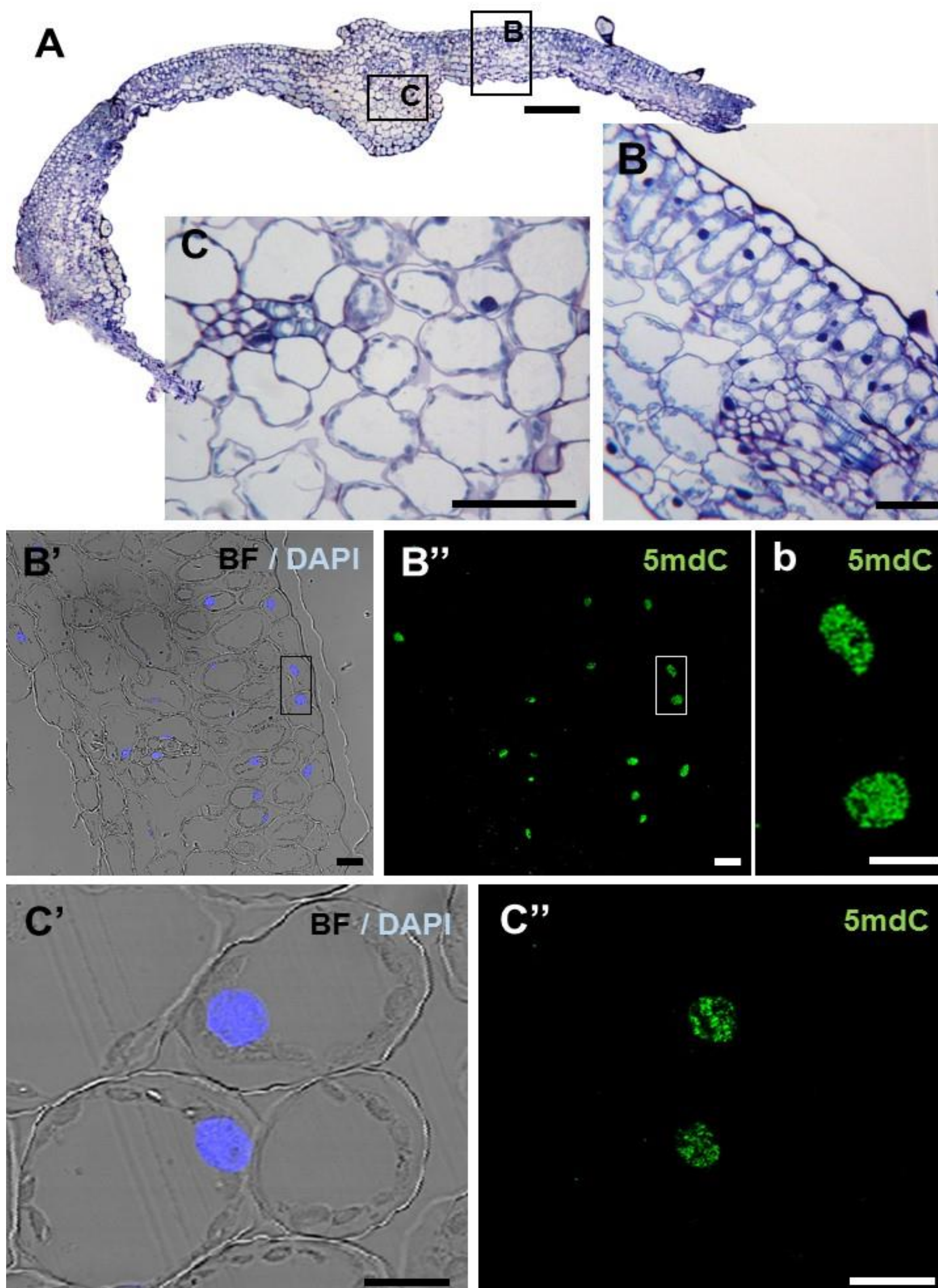


Figure 20 – Cellular organization and 5mdC immunofluorescence of young leaves before induction phase. Fresh young leaves of TDJ3 (A, B, C) and TSM10 (B'-C'') tamarillo on cytochemical and immunofluorescence analysis. A – Leaf's overview, assembly of toluidine blue staining images; B, B', B'' – leaf blade on transversal section, respectively toluidine blue staining, bright field / DAPI and 5mdC immunofluorescence signal; b – detail of two upper epidermis cell nuclei, 5mdC signal; C – mesophyll and vascular cells on central vein region, transversal section, toluidine blue staining; C', C'' – detail of three mesophyll cells, respectively bright field / DAPI and 5mdC signal. Bars: 20 µm exc. A: 100 µm and b: 10 µm.

3.3. Changes in global DNA methylation levels during embryo conversion phase

When embryogenic masses are cultured in maturation media, embryogenesis initiate and somatic embryos develop. To analyze the changes in DNA methylation during this phase, we selected three stages of the embryo development, based essentially on the macroscopic appearance of the maturating material, and we carried out a global DNA methylation quantification assay on them.

Two distinct embryogenic lines were used as the starting material. One of the lines assayed was YL2016a2 which, as previously stated, came from an induction event performed in 2016 with young leaves of tamarillo TSM10 used as somatic explant. The other embryogenic line tested for global methylation levels throughout embryo conversion was YL2014, an older mass also induced from young leaves (TV310 genotype) that had been multiplied and monthly subcultured since 2014.

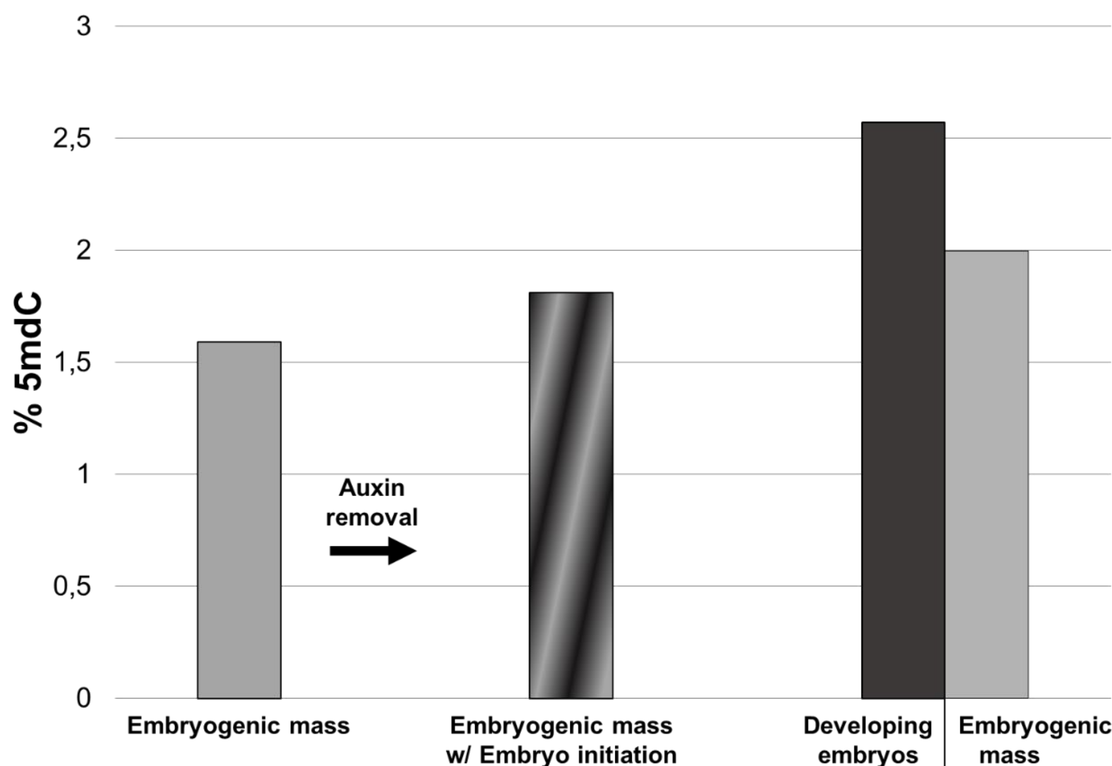


Figure 21 – Global DNA methylation levels throughout maturation phase of YL2016a2 embryogenic line.

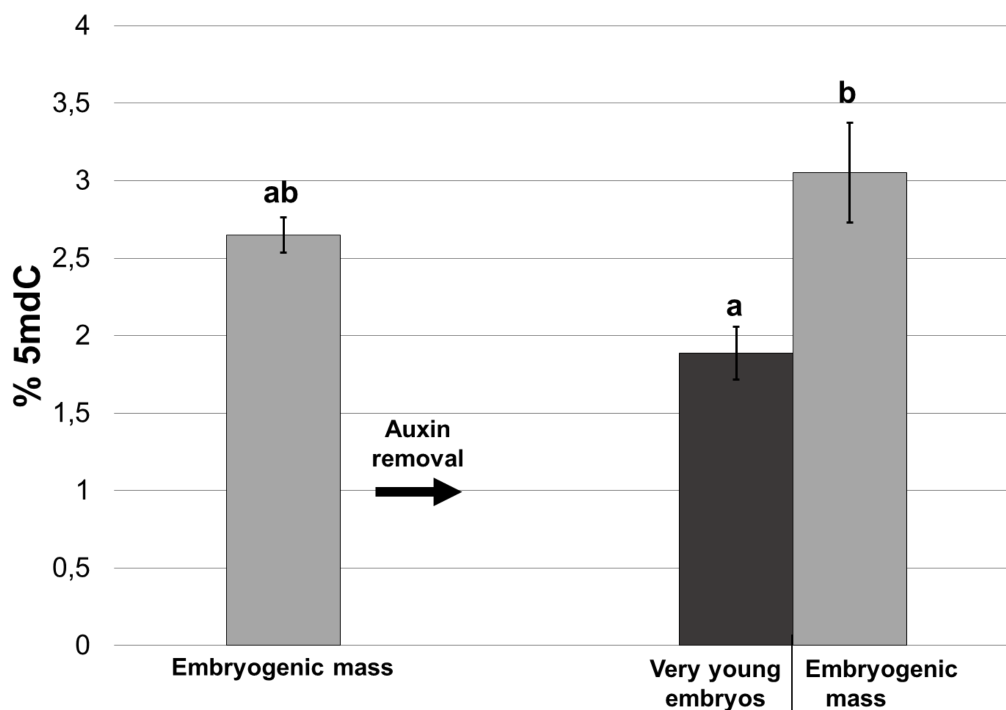


Figure 22 – Global DNA methylation levels throughout maturation phase of YL2014 embryogenic line. Error bars correspond to sd values.

Taken together, the data from both assays showed that early embryos formed after auxin removal are less methylated than the embryogenic masses (analyzed before auxin removal and also during early embryo maturation, subjacent to them) (Fig. 22). In more advanced stages of embryo development, DNA methylation is higher, comparing with the embryogenic mass from which originated (Fig. 21, left and right bars). These results indicate that embryogenesis initiation from embryogenic masses involves a decrease in DNA methylation, whereas embryogenesis progression and embryo development lead to DNA methylation increase, correlating with embryo differentiation and maturation.

Concerning the result of very early stage in which the embryogenic mass and the initiating embryos were analyzed together (Fig. 21, second column), we may justify the absence of significant differences in %5mdC with the original masses before auxin removal (Fig. 21) with the fact that this material contained few and little embryos, and therefore, their possible contribution to lowering the overall methylation level will have been insignificant.

3.4. Cellular organization and 5-methyl-deoxy-cytidine immunofluorescence during embryo conversion phase

In order to better interpret global DNA methylation results and to analyze the patterns of distribution of methylated DNA in nuclei of different cell types in embryos, we found appropriate to perform a microscopic study, using toluidine blue staining and also immunofluorescence images of 5mdC, during the embryo conversion phase, specifically in early embryos and embryogenic material cultured on maturation medium.

Material used for such study came from different lines and maturation events; figures were selected as the most illustrative of the various types of structure found during embryo conversion.

At early stages, after auxin removal, embryogenic masses with very early embryos show a general structure in which we see that, while some regions maintain an organization of apparently inactive embryogenic tissue with highly vacuolated and large cells (Fig. 23B), other regions are formed by more dense cells, showing numerous mitotic images (Fig. 23C). These cells are organized in lines or layers, forming a more organized structure than regions with vacuolated cells. These dense cells may be the ones that will effectively convert somatic embryos.

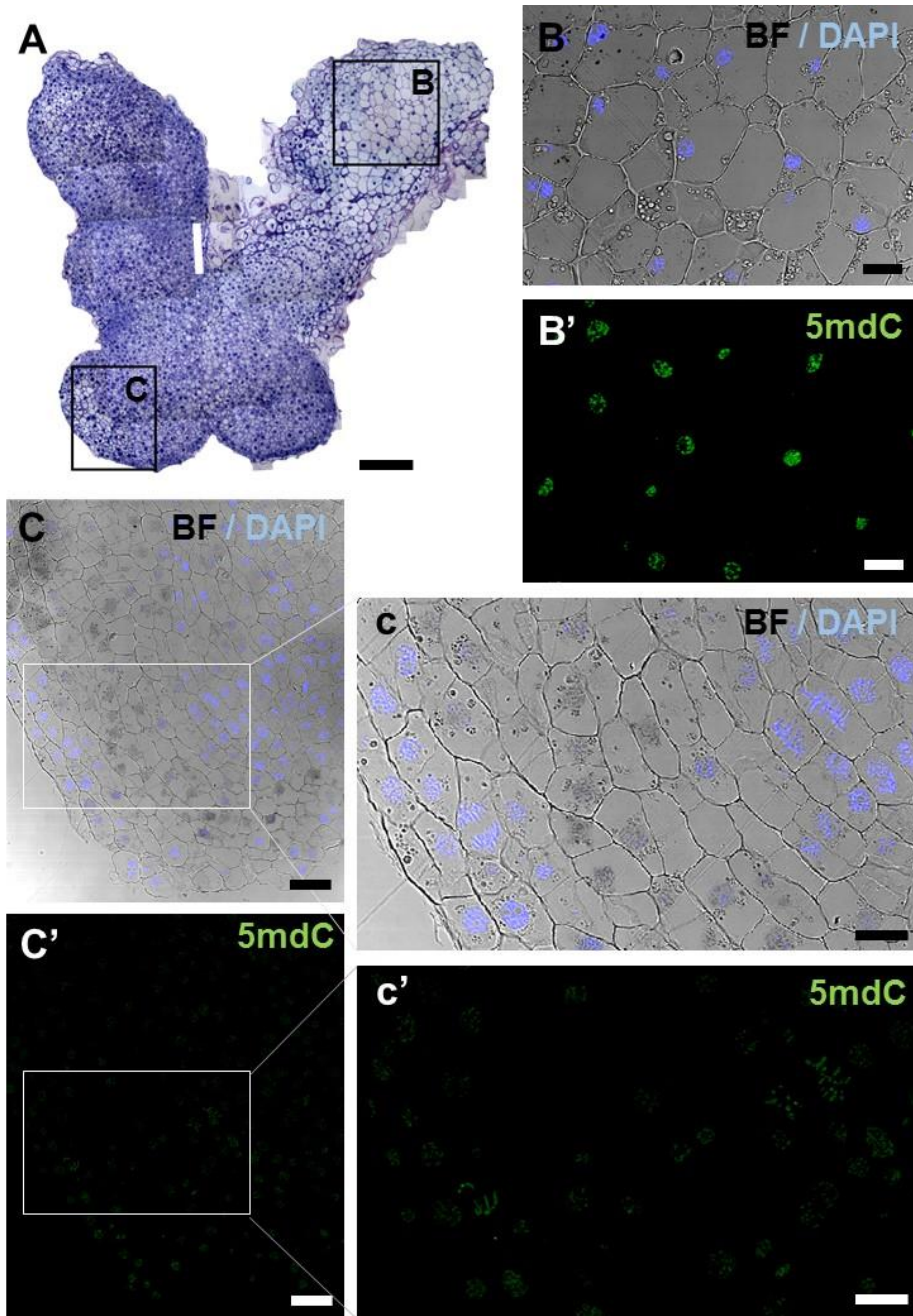


Figure 23 – Embryogenesis initiation stage. YL2016a2 line on maturation medium. A – assembly of images for mass general overview, toluidine blue staining; B, C, c – bright field / DAPI images showing distinct regions of the mass; B', C', c' - 5mdC immunofluorescence of the same regions. Bars: A: 100 µm; B, B', c and c': 20 µm; C and C': 40 µm.

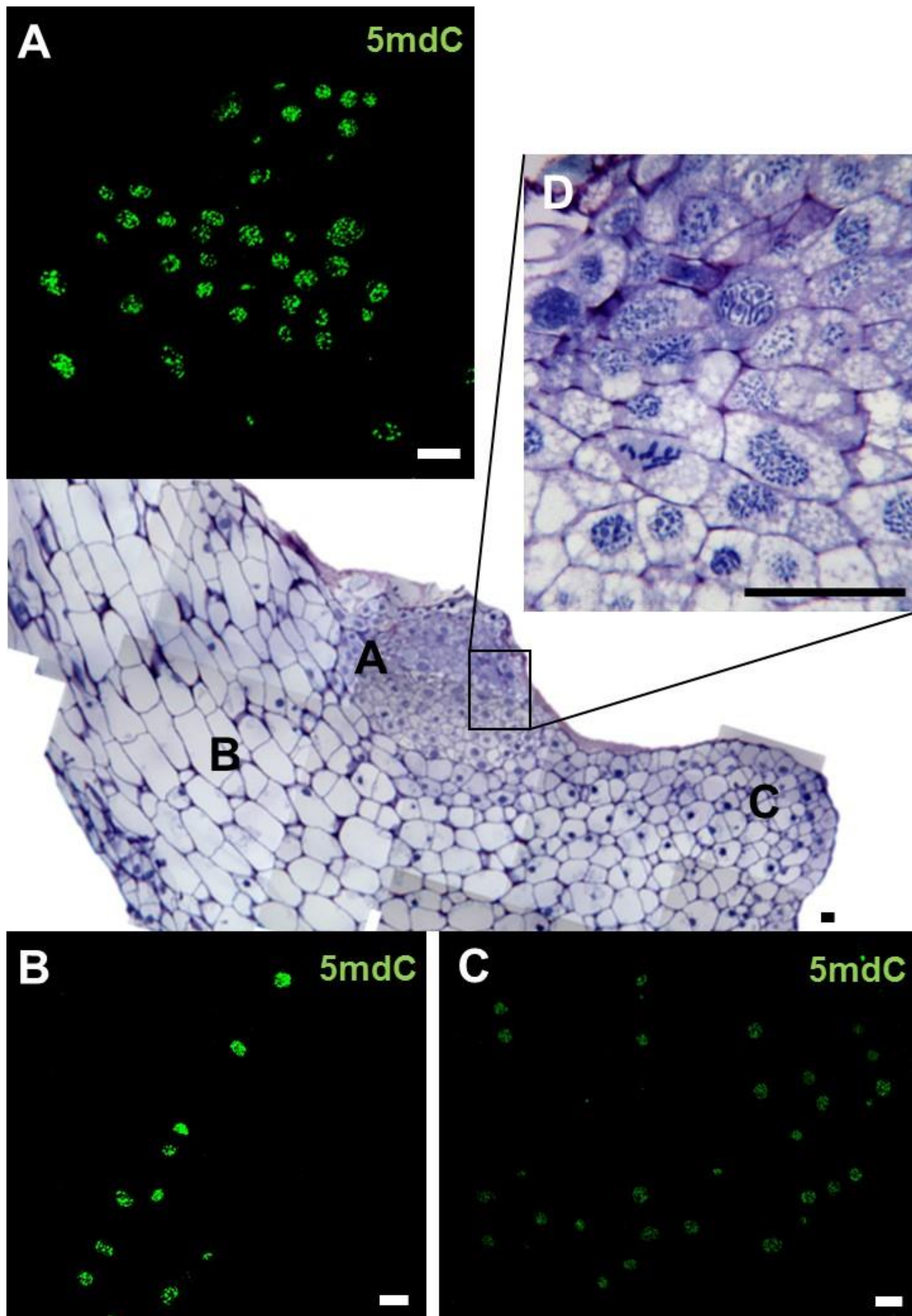


Figure 24 – Developing embryo stage. Somatic embryo converted from an embryogenic mass from YL2016a2. In the center, an assembly of images of toluidine blue staining provide a general overview of the embryo. A, B, C - 5mdC immunofluorescence of selected regions of the embryo, being A described as a presumably meristematic zone, B as ground cells and C a region close to the embryo's tip. Bars: 20 µm.

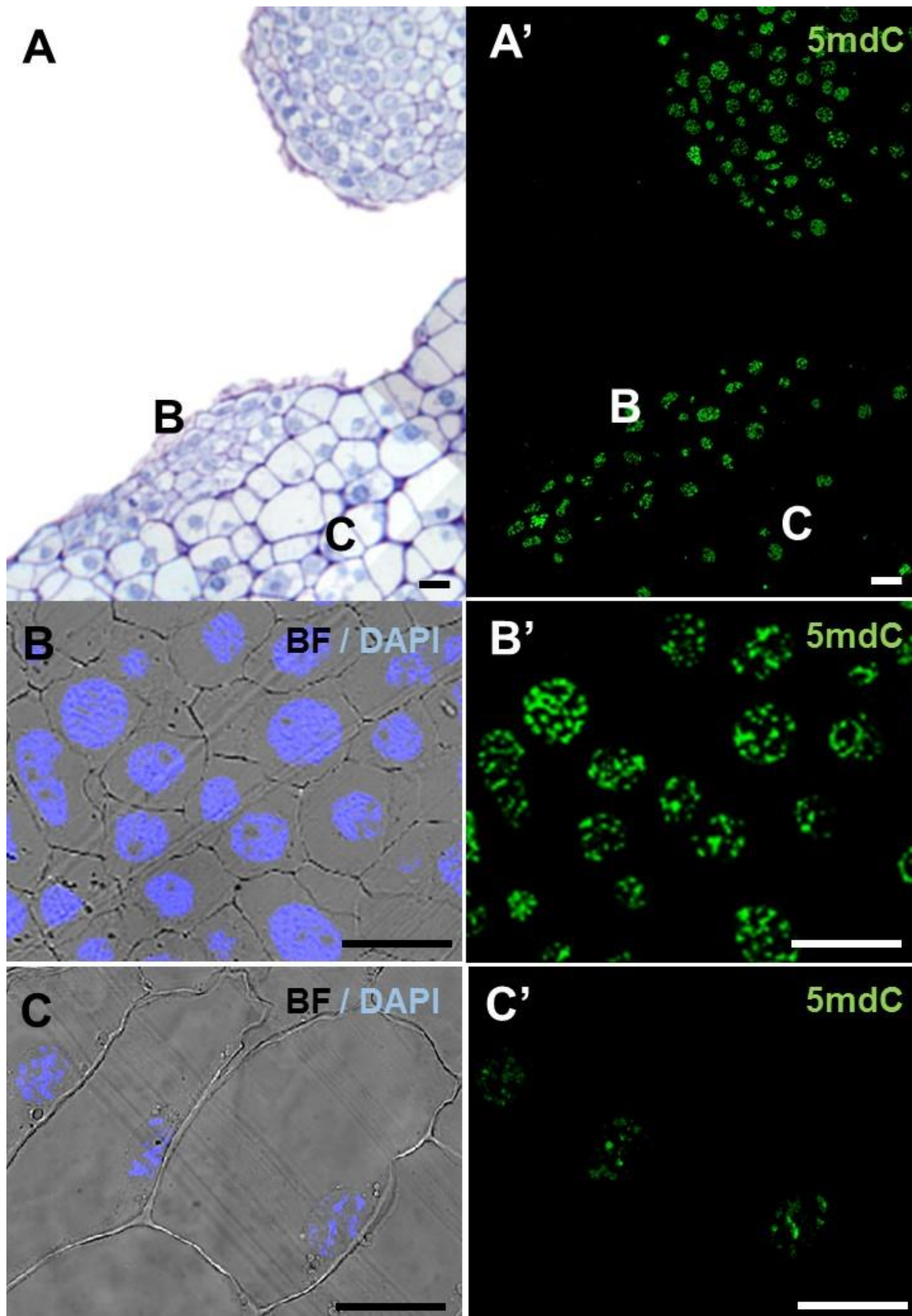


Figure 25 – Developing embryo stage. Focus on somatic embryo's distinct cell types, all pictures are from a three-week maturated embryo of YL2016a2 line. A – Toluidine blue staining; B, C – bright field / DAPI; A', B', C' – 5mdC immunofluorescence signal. Bars: 20 µm.

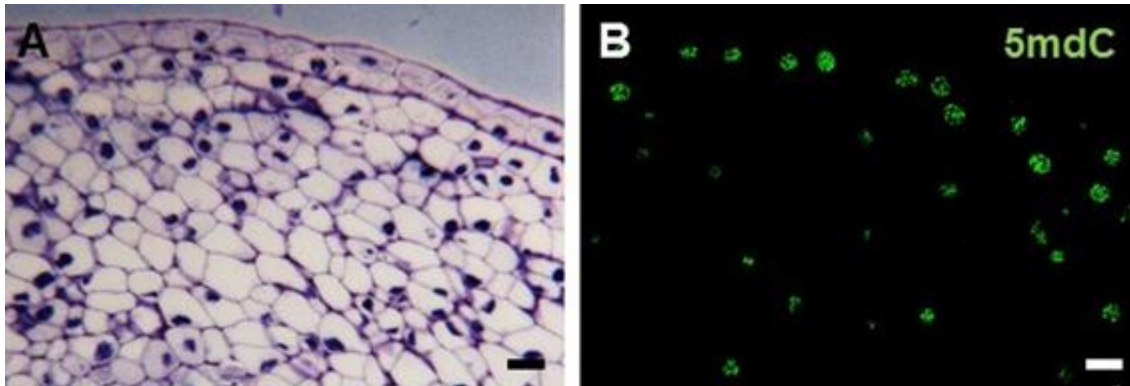
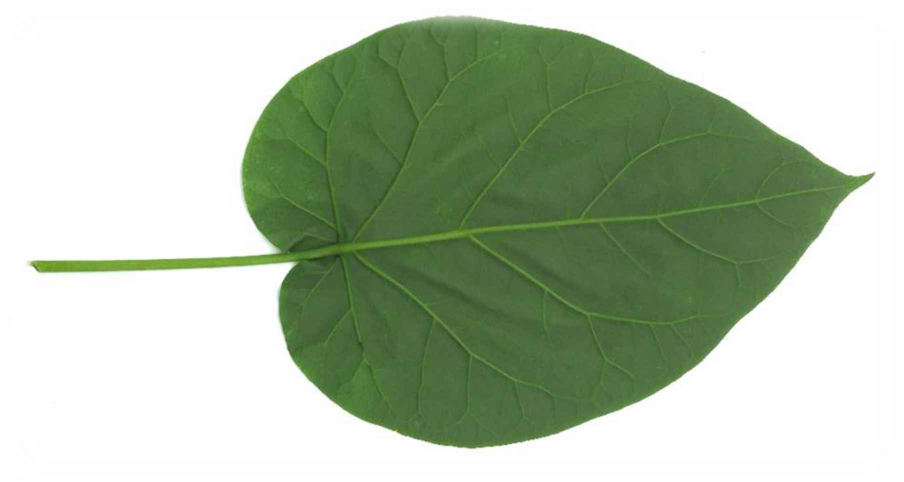


Figure 26 – Developing embryo stage, protoderm differentiation. Protoderm differentiation in a somatic embryo converted from YL2014 line. A – toluidine blue staining; B – 5mdC immunofluorescence signal. Bars: 20 µm.

At later stages of the maturation phase, young embryos separated from the embryogenic masses show very distinct and delimited zones constituted by cells with clear differences on both toluidine blue appearance and 5mdC signal intensity. There are zones with abundant dividing cells, possessing dense cytoplasm, large nuclei and typical mitotic figures (Fig. 24A, 24D and 25B), whereas other zones are constituted mainly by larger and highly vacuolated cells with small dark nuclei (Fig. 24B and 25C). The former are located in presumably meristematic regions, whilst the latter are probably part of the ground tissue.

These different cellular regions can be observed throughout during maturation stage, from masses with embryogenic initiation (Fig. 23) to embryos at various developmental stages (Fig. 24 and 25). 5mdC immunofluorescence assays showed higher signal intensity in the small nuclei of vacuolated cells (Fig. 23B', 24B and 25C') than in the interphasic nuclei of proliferating cells (Fig. 23C' and 24C), although in meristematic regions, mitotic chromosomes exhibited an intense fluorescence signal (Fig. 23c', 24A and 25B').

As embryo maturation progresses, cell differentiation can be observed, specifically at the embryo periphery a clear protoderm is formed as a linear array of isodiametric cells (Fig. 26). These differentiated epidermal cells showed an intense 5mdC immunofluorescence signal, higher than the rest of embryo cells (Fig. 23, 24B and 26).



Discussion

1. DIFFERENCES IN ACQUISITION OF EMBRYOGENIC COMPETENCE, PROLIFERATION RATES AND EMBRYO CONVERSION YELDS IN CULTURES OF DIFFERENT GENOTYPE AND AGE

1.1. Acquisition of embryogenic competence by somatic cells is affected by explant genotype

The yield of the SE process starts with the yield of the induction phase. In this work we observed that the genotype of the leaf explant had strong influence in the ability to form embryogenic cell masses, what had already been documented by Correia (2011).

For instance, leaf explants proceeding from TDJ3 seedlings had a significantly lower response to induction treatment (*i.e.* exposure to auxin medium and stresses) than the leaves from TV310 and TSM10 seedlings.

The three micropropagation lines were all established from *in vitro* germination of seeds, however these seeds had different origin and establishing time. For instance, TV310 was started in first place, TDJ was the second and TSM10 the last to be established.

The time a certain micropropagation line has spent being subcultured might influence the responsiveness of its explants to the SE induction treatment, as well as its origin, however, the results of the present work are insufficient to advance any conclusion on this aspect.

1.2. Embryo conversion competence strongly decays with long-term culture

A recurrent observation in previous works is that embryogenic tissues subcultured for prolonged time may lose the ability to develop into morphologically normal somatic embryos (Fellers et al. 1997; Salajova et al. 1999; von Arnold et al. 2002; Currais et al. 2013; Correia et al. 2009; 2011; Park et al. 2011), often due to somaclonal effects accumulating in the successive subcultures.

In order to assess the extent in which this happens in the case of tamarillo *calli*, and following methodologies described in previous works (Correia, 2011; Currais et al., 2013), we tested embryogenic masses induced seven, three and less than one year ago for the quantity of somatic embryos that are converted upon auxin removal.

As expected, substantial differences among the various lines were observed: only recent embryogenic masses (*i.e.* with less than one year) produced normal embryos, capable of germinating into normal plants which complete development. Three-year

-old embryogenic mass only rarely produced embryos, most of which showed impaired development and incapacity to fully regenerate new plants. The oldest lines had completely lost their embryogenic ability. Moreover, these differences in conversion rates and embryo quality were evident by simple observation of maturating *calli* at naked eye: one could perceive, after only three weeks, that while embryogenic masses induced in 2016 (*i.e.* with less than 1 year spent in subculture) had several and evident whitish embryos arising (Fig. 5B), older embryogenic masses barely had visible embryo-like structures (Fig. 7).

1.3. Proliferation rates are higher in non-embryogenic lines and habituated long-term embryogenic lines

It was observed (no analytic data) that non-embryogenic *callus* present a typically fast-growing behavior, according to what had already been reported (Correia et al., 2012b).

Regarding embryogenic lines, proliferation rates were measured in lines with different culturing ages and explant origin. A general tendency of higher proliferation in older *calli* was observed. The most reliable comparison is made between YL 2009 and YL 2014 embryogenic lines: since they were induced from the exact same clonal tamarillo (TV310), and same type of explant (young excised leaves), with a difference of 5 years in subculture time, the 'explant type' and 'genotype' variables are eliminated. And indeed, the oldest line (YL 2009) proliferated almost twice as much than the more recent line (YL 2014). One can

therefore assume that aging of the embryogenic cultures is a contributing factor to the increase in proliferation rates.

When establishing comparisons among other embryogenic lines with distinct proliferation behavior, however, not only the subculturing age but also the influence of genotypes shall be taken into account. For instance, Canhoto et al. (2005) reported substantial differences among the proliferation rates of different *callus* lines and cultivars of tamarillo.

Distinct proliferation behavior of *calli* may also correlate with differences in their embryogenic competence and epigenetic state, among other factors decurrently of culturing conditions (see Neelakandan & Wang 2012; Us-Camas et al. 2014).

Correia (2011) described non-embryogenic *calli* of tamarillo as generally fast-growing, in opposition to embryogenic lines, which were slow-growing. Facing this, and confronting with the previously discussed results, it can be summarized that proliferation rates are inversely correlated with embryogenic capacity.

2. DNA METHYLATION LEVELS IN EMBRYOGENIC AND NON-EMBRYOGENIC CULTURES: DIFFERENCES WITHIN PAIRS AND OVER CULTURE TIME

We determined the global DNA methylation levels of both embryogenic and non-embryogenic lines of tamarillo *calli*, induced from young leaves or from zygotic embryos and subcultured for different periods. Within a pair (lines originated in the same year), differences in global DNA methylation level were only statistically significant in the 7-year-old zygotic embryo-induced line, in which embryogenic masses were more methylated than the non-embryogenic *callus*, and in the leaf-induced line with less than 1-year-old, whose non-embryogenic *callus* was more methylated than the embryogenic mass.

That indicates that, as culture time goes by, the within-pair relation concerning DNA methylation levels tends to reverse, especially due to the accumulation of DNA methylation in the embryogenic cells.

Regarding the fact that non-embryogenic *calli* did not suffer a proportional accumulation of cytosine methylation (and therefore the initial within-pair relation of embryogenic *vs* non-embryogenic methylation levels was not maintained), we can hypothesize that DNA methylation in non-embryogenic cells is at an intermediate level that allows maintenance of regular proliferation rates and constant cellular features.

2.1. In recently induced lines, embryogenic masses have lower levels of methylated cytosines and H3K9 than non-embryogenic *calli*

In the global DNA methylation assay performed, the non-embryogenic *callus* with less than 1-year-old in proliferation had higher levels of cytosine methylation than the embryogenic mass, which in turn, as already addressed, presented good embryo conversion rates. That may indicate that, in recently induced *calli* in proliferation, the epigenetic state of the DNA molecule in the cells with embryogenic competence is typically associated to a more readable and accessible structure than in the cells without embryogenic competence.

To confirm that, we performed a H3K9 methylation quantification assay on the most recently induced embryogenic/non-embryogenic pair. H3K9 methylation is a repressive epigenetic mark which generally occurs in association with DNA methylation (Rosa and Shaw, 2013; Munshi et al., 2015). For example, H3K9me2 has been showed to participate in the change of developmental program of the microspore towards embryogenesis in *Brassica napus*, accompanying changes in DNA methylation (Rodríguez-Sanz et al., 2014b).

The results obtained supported our hypothesis, showing a relation that fully agrees with the DNA methylation assay, *i.e.* the embryogenic mass was significantly less methylated than the non-embryogenic *callus*.

In non-embryogenic lines from both gimno and angiosperms, high methylation levels are consistently reported in the literature (De-la-Peña et al., 2015), whilst embryogenic lines show low 5mdC signal (Fraga et al., 2002). Global DNA methylation on embryogenic *callus* of Siberian gingseng (induced from leaves with 2,4-D) was significantly lower than in the non-embryogenic *callus* from the

same induction even (Chakrabarty et al., 2003). In *Pinus nigra* cell cultures, low methylation is associated with embryogenic capacity (Noceda et al., 2009).

2.2. Culture time modifies DNA methylation state of originally embryogenic cells, what may account for loss of the embryogenic competence

The artificial environment can significantly account to phenomena such as somaclonal variation, which is a manifestation of the epigenetic instability which tends to increase with culture time (reviewed by Neelakandan & Wang 2012). The tissue culture conditions (Phillips et al., 1994), the chemicals, some PGR's like 2,4-D, GA_3 and 6-BAP (Valledor et al., 2007), the osmotic stress (Kovarìk et al., 1997; Guangyuan et al., 2007), the wounding (Kaeppler and Phillips, 1993) and even some organic molecules released by plant cell cultures to the culture medium, such as phenolic compounds, alkaloids, organosulfur compounds and terpenoids (De-la-Peña et al., 2015; Nic-Can et al., 2015), often result in changes in DNA methylation, thus leading to break down of normal controls and produce genome instability effects (Jaligot et al., 2000; Kaeppler et al., 2000).

An evidence of such instability is the increase in transposon activity: normally transposons are methylated *i.e.* stabilized and unexpressed, but culture conditions may cause this regions to demethylate (Kaeppler et al., 2000; Liu et al., 2004). Some studies, though, point out that the frequency of this DNA methylation variations might decline in a more advanced time of the culture regenerates, possibly associated to the loss of totipotency and regeneration potential of extremely mutated cells (Neelakandan and Wang, 2012).

All of these factors may influence the global DNA methylation level of cultured cells is opposite ways (for example, phenolic compounds seem to have a demethylating effect (De-la-Peña et al., 2015), while 2,4-D is associated with an hypermethylating effect (Us-Camas et al., 2014)), and the totality of interactions and variables (such as species, genotype and tissue-specific effects) are far from being fully understood. Nevertheless, some reports have described a tendency of global increase in DNA methylation levels along with culture time, not

necessarily associated with visible morphologic changes (LoSchiavo et al., 1989; Valledor et al., 2007). That agrees with the results of the present work.

The loss of differentiation/regeneration capacity over time in culture has been justified with the accumulation of DNA methylation and other epigenetic changes (Russo et al., 1996; Valledor et al., 2007). Somaclonal variation in cultures of *Torenia* sp. is affected by PGR's, antibiotics and number of subcultured generations, probably via epigenetic modifications (Sun et al., 2013).

3. EMBRYOGENIC *VS* NON-EMBRYOGENIC *CALLI* AT A GLANCE: A CYTOLOGICAL AND EPIGENETIC PERSPECTIVE

The general macroscopic appearance of embryogenic and non-embryogenic *calli* obtained in this work match with previous descriptions published by our laboratory in University of Coimbra (Canhoto et al., 2005; Correia et al., 2009, 2011; Guimarães et al., 1988; Lopes et al., 2000). Aiming to better characterize these *calli*, their structural and cytological *nuances* were analyzed in this work by simple cytochemical and bright field microscopy techniques.

Regarding the epigenetic characterization of this lines, the methylation quantification discussed so far is insufficient to fully address the issue. Indeed, by global DNA methylation quantification it is not possible to clarify the epigenetic state of individual cells, which can be very different from cell-to-cell in *callus* (Krizova et al., 2009), nor to find out the 5mdC distribution and differences among specific cell types within a sample, for whose purpose bioimaging techniques are more adequate. The 5mdC immunofluorescence technique, for instance, is a useful approach that have been providing new insights into DNA methylation dynamics (Testillano et al., 2013).

Taken altogether, these data help us clarify the cytological activity and epigenetic state of distinct cell groups within embryogenic and non-embryogenic lines, as it will be discussed below.

3.1. Embryogenic *calli* show a very heterogeneous cellular structure and differential 5-methyl-cytidine distribution

Bright-field microscopy analysis of embryogenic masses revealed very similar structures to the ones described by previous works (Guimarães et al., 1988; Pasternak et al., 2002; Correia, 2011; Fraga et al., 2012), particularly the occurrence of protrusion cell arrangements had already been described (Steinmacher et al., 2011; Corredoira et al., 2017). The small, isodiametric cohesive cells with prominent nuclei, numerous starch grains and phenolic compounds form a structure with typical proliferative appearance (Solís et al., 2016). Reis and co-workers (2008) suggested that these phenolic-rich cells might form a barrier between the future somatic embryos and the mother tissue, blocking symplastic transport that could lead to inadequate development of the embryo independently from the subjacent mass (Pfluger and Zambryski, 2001; Fraga et al., 2012).

As already stated, these embryogenic masses had great microscopic heterogeneity, not only from a histologic point of view, but also regarding immunocytochemical data (even though macroscopically they do not appear to have such heterogeneity): both intensity and patterning of the 5mdC in the nuclei was uneven within the same mass. Generally speaking, one can speculate that the differential pattern of methylated cytosines would be on the basis of differential gene expression and, consequently, of different morphologic aspect and proliferation rates of the cells (Santos and Fevereiro, 2002).

When protrusion zones are observed, for example, they often reveal a less intense 5mdC signal, in comparison with zones more internal to the mass.

Previous works using the equivalent bioimaging techniques for immunolocalization of 5mdC (Bárány et al. 2005; Seguí-Simarro et al. 2011; Testillano et al. 2005; 2013) associated low 5mdC signal and thin reticulate, decondensed chromatin (DAPI) with proliferating cells from root meristems and developing microspores, whilst quiescent and anther's *tapetum* cells tipically presented high 5mdC signal and thick reticulate chromatin with condensation spots (DAPI).

Many genes known to be involved in meristem initiation and organ formation are controlled by DNA methylation (Valledor et al., 2007). Meristems in resting organs that defer growth seem to be persistently methylated (Pérez et al., 2015).

Therefore, we may suppose that these protrusions are often the embryogenic mass's most proliferative zone, and probably the one more prone to initiate somatic embryos (Steinmacher et al., 2011).

It was observed, although it is difficult to obtain and present numerical data to support such observation, that the most recently-induced embryogenic lines exhibit more of these protrusion zones than the older *calli.* That could correlate with the bigger tendency to form somatic embryos.

As culture time increases, embryogenic structures might progressively lose the original compact appearance, as more cells accumulate methylation and increase chromatin condensation and DNA methylation level, as it is evidenced by 5mdC signal higher intensity in zones more internal to the mass, composed generally by larger cells with larger vacuoli than in the protrusional or peripheral zones of the mass.

3.2. Non-embryogenic *calli* are constituted by sparse cells with large vacuoli and condensed chromatin

Non-embryogenic cells, when observed by microscopy, are usually large, irregularly shaped and sparsely distributed, not appearing to form any type of organized and compact tissue as the embryogenic cells do. Non-embryogenic cells seem less metabolically active than embryogenic ones, since the former show barely any cytoplasmic activity.

One of the observations that most contrasts with embryogenic cells' general appearance is that in the small nuclei of these non-embryogenic, isolated cells, DAPI and 5mdC patterns indicate a more condensed state of the chromatin.

Such information agrees with the global DNA methylation quantification data, which has showed that non-embryogenic lines generally have and maintain (through successive subcultures) high levels of DNA methylation. For instance,

DNA methylation strongly correlates with the heterochromatization process (Valledor et al., 2007; De-la-Peña et al., 2015).

It is a well-established concept that, the more condensed is the chromatin, the more difficult it is for the genome to be read (Miguel and Marum, 2011; Testillano et al., 2013) and, naturally, in the context of SE, that is equivalent to saying that the cell has less plasticity and is less prone to changing its developmental program.

Among young leaves' derived lines, the 5mdC signal intensity of non-embryogenic cells looks similar to signals of most of the cells of embryogenic lines. The ones which are indeed may exist some variability (*i.e.* less 5mdC signal) are often located on the periphery of embryogenic masses.

These data supports the fact that epigenetic modifications play a central role in the 'management' of gene expression throughout developmental pathways (Miguel and Marum, 2011). Some genes have already been identified as key markers of the embryogenic process (reviewed in Mahdavi-Darvari et al., 2015; Zeng et al., 2007), but a definite epigenetic 'signature' that inequivocally indicates the embryogenic and organogenic potential of a cell or group of cells is still lacking (Mahdavi-Darvari et al., 2015).

4. FROM PLANT EXPLANTS TO SOMATIC EMBRYOS: UNDERSTANDING DNA METHYLATION DYNAMICS THROUGHOUT INDIRECT SOMATIC EMBRYOGENESIS OF TAMARILLO

4.1. Induction treatment and dedifferentiation process

4.1.1. Global DNA methylation level decreases during dedifferentiation

In tamarillo SE system, picloram is included in the induction medium where explants are left to dedifferentiate and form embryogenic clumps. Mechanical stress (wounding) of the leaf explants is also used as an additional induction factor.

Results showed a considerable decrease in DNA methylation levels when comparing leaf explants before and during the dedifferentiation process.

As already mentioned in the introduction of this work, plant's response to stress agents is notable at an epigenetic level. Therefore, stresses are often used as inducing factors of SE *in vitro* (Karami and Saidi, 2010; Fehér, 2015).

In addition, global levels of DNA and histone methylation are expected to respond intensely to the presence of auxin in the medium where SE is induced *in vitro*, thus accounting for the developmental program switch (LoSchiavo et al., 1989; Leljak-Levanić et al., 2004; Yamamoto et al., 2005; Us-Camas et al., 2014). Through DNA methylation, exposure to auxin results in stimulation of cell division and dedifferentiation (von Aderkas and Bonga, 2000; Fehér et al., 2003; Karami and Saidi, 2010).

This positive correlation between DNA methylation and dedifferentiation (from the auxin's point of view), at the same time that stress triggers demethylation and, through it, SE induction and genetic reprogramming (Fehér, 2015) might seem, in a first moment, contradictory. An experiment by Santos & Fevereiro (2002) with embryogenic and non-embryogenic lines of *Mendicago trunculata* may help with some insights to clear out the 'methylation vs demethylation' duality during SE induction: testing the hypothesis that certain DNA methylation levels or patterns were necessary for acquisition of embryogenic competence, they included AzaC (demethylating drug) in the induction medium and discovered, in one hand, that embryogenic *calli* would lose their embryogenic capacity and, in other hand, that non-embryogenic *calli* did not progress proliferation, turned brown and died (Santos and Fevereiro, 2002).

What has been proposed by LoSchiavo and co-workers (1989) is that each and every tissue has its own DNA methylation level and pattern, required for the maintenance of a basal differentiative state of the embryogenic *callus*, which might be equivalent to the methylation level on the absence of auxin.

Facing this, what we propose is, although in a first instance of the induction phase, significant demethylation occurs (strongly triggered as a stress-response mechanism) and accounts for a general tissue dedifferentiation, the continued maintenance in the auxin medium will stimulate the acquirement of specific DNA

methylation patterns that differentiate non-embryogenic from embryogenic *calli.* Therefore, a basal level of DNA methylation is necessary for maintaining cell division and embryogenic competence. Either very low (in the first weeks of the induction treatment) or too high (in long-term cultures) cytosine methylation levels are associated with absence of embryogenic competence.

During acquisition of cell totipotency and embryo differentiation, large-scale chromatin reorganization occurs (Tessadori et al., 2007; Miguel and Marum, 2011). Therefore, it is expectable that not only DNA methylation global levels but also its patterns change throughout the process (El-Tantawy et al., 2014; Corredoira et al., 2017).

4.1.2. Cytochemical analysis show heterogeneity in leaf cell response to the induction treatment; 5-methyl-cytidine immunofluorescence is globally less intense in the dedifferentiating *callus*

Concerning the bioimaging aspects, the heterogeneity observed in the dedifferentiating material showed that the cells in the explant do not respond equally to the induction treatment. The majority of cells presented small nuclei and large vacuoles, presumably evolving into non-embryogenic *calli*. However, some regions constituted by cells with dense cytoplasms and larger nuclei were also found. We suppose these regions are more prone to form embryogenic clumps at the end of the induction process.

We have also observed that in general, both types of cells in the dedifferentiating tissue have a lower 5mdC signal intensity than the ones constituting the precursor fresh leaf.
This observation is in agreement with the results of the global DNA methylation assay.

Another observation is, while at the beginning of the culture almost all leaf cells have numerous starch granules, the presence of this polysaccharide within the cells decreases during the *callus* formation. Such aspect had been reported in previous works of SE induction in tamarillo (Lopes et al., 2000).

According to Correia and Canhoto (2012) and Lopes and co-workers (2000), in zygotic embryos put onto induction medium, the formation of the slow growing *callus* starts with divisions of the cells next to the vascular bundles. The sub-epidermal and epidermal cells acquire meristematic characteristics, forming a continuous peripheral layer of densely cytoplasmatic cells. Some of these cells organize into PEM that keep on proliferating and in some areas of the *callus,* by 8th-10th week, whitish clusters of embryogenic cells form (Correia and Canhoto, 2012).

In young leaves, at its turn, it's in the one-cell thick palisade layer that the first cell divisions occur, then spreading throughout the mesophyll cells with the consequent formation of *callus* tissue. Later on, within this *callus*, some densely cytoplasmatic cells originate PEM that keep on proliferating (Correia and Canhoto, 2012).

4.1.3. Further approaches may give more insights about the general demethylation tendency during dedifferentiation

Taken together, data from both DNA methylation global quantification and 5mdC immumofluorescence assay lead us to the supposition that it would be possible to improve efficiency of SE induction phase by using a demethylation drug, as it could favour the deactivation of the gene expression program of differentiated cells and acquisition of totipotency.

A preliminary assay to test this hypothesis was carried out during this work (data not shown), in which some tamarillo leaf explants were treated with a 6-day pulse of 5-azacytidine (AzaC) 10μM previous to the induction phase with auxin. AzaC is a nucleoside analog which can substitute 5-cytosine in DNA strand during replication and inhibit methyltransferase activity on those places (Jones and Taylor, 1980; Friedman, 1981). It has been widely used as a demethylation agent, producing varied effects on plant development context, depending on dose, time, species, genotype and process (LoSchiavo et al., 1989; Li et al., 2001; Santos and Fevereiro, 2002; Yamamoto et al., 2005; Fraga et al., 2012; Teyssier et al., 2014; Solís et al., 2015).

This might be an interesting approach to further investigation. Dose and time-dependent effects might be obtained and induction rates improved in the tamarillo's SE induction system in the future.

4.2. Embryo conversion and differentiation

4.2.1. An hypomethylation moment precedes embryo initiation

Global DNA methylation assays performed during the early stages of embryo conversion phase, after removal of the auxin from the medium, revealed that at least in the YL2014 embryogenic line, very early embryos collected and separated from the embryogenic mass had lower global DNA methylation levels than the surrounding mass. However, this difference was not statistically significant. More studies need to be conducted to reinforce the data, and embryos in an even younger stage should be sampled and assayed.

Nevertheless, this apparent tendency to a hypomethylation moment on the onset of embryo conversion is in agreement with the literature: in plants, as in animals, there is an important tendency to a hypomethylation moment before somatic cells embark on specific differentiating programs, such as before zygotic embryogenesis, organogenesis, flowering and rooting (von Aderkas and Bonga, 2000; Valledor et al., 2007).

Triggering a pronounced hypomethylation moment by applying a cold-shock treatment, for instance, will cause embryogenic cultures or seeds of *Quercus suber* to start germinating embryos (Pérez et al., 2015). The global demethylation which occurs at seed germination reflects the transition of a quiescent to an active-growing and developmental state (Zluvova et al., 2001). This has been reported to occur in other species, for example *Brassica napus* (Guangyuan et al., 2006) and *Triticum aestivum* (Meng et al., 2012).

Also, previous works in microspore embryogenesis induction in *Brassica napus* (Solís et al., 2012; Testillano et al., 2013), *Hordeum vulgare* (El-Tantawy et al., 2014) and *Quercus suber* (Rodríguez-Sanz et al., 2014a) consistently showed that the activation of cell proliferation and switching of developmental program

towards embryogenesis is accompanied by a global decrease in DNA methylation.

The necessity of a specifically hypomethylated state for triggering SE was reviewed by Tchorbadjieva & Pantchev (2004).

Furthermore, and since exogenous addition of 2,4-D is positively correlated to DNA methylation in many SE systems (LoSchiavo et al., 1989; Santos and Fevereiro, 2002; Leljak-Levanić et al., 2004; Fraga et al., 2012), it is intuitive to think that DNA methylation maintains a basal level during the proliferation phase of *in vitro* SE, falling abruptly when auxin is removed (Fraga et al., 2012; Mahdavi-Darvari et al., 2015).

4.2.2. DNA methylation levels gradually increase with embryo differentiation

The results obtained on global DNA methylation levels during the maturation phase progression on YL2016 embryogenic line, seem to reveal a gradual increase of total DNA methylation throughout embryos' differentiation process.

Many previous works recurrently state that embryo's development seem to come along with progressive increase in cytosine methylation (LoSchiavo et al. 1989; Munksgaard et al. 1995; Leljak-Levanić et al. 2004; Fraga et al. 2012; Solís et al. 2012; 2015; El-Tantawy et al. 2014; Pérez et al. 2015). During SE, DNA methylation levels are determinant factors not only to morphogenesis but also for cell proliferation (Bernacchia et al., 1998; Chakrabarty et al., 2003).

In fact, regulation by DNA and histone methylation is generally concurrent with the differentiation phenomena in many developmental processes in plants (Costa and Shaw, 2007; Jarillo et al., 2009; Malik et al., 2012; Solís et al., 2015) such as zygotic embryo development (von Aderkas and Bonga, 2000; Gehring and Henikoff, 2007; Solís et al., 2012), gametogenesis (Saze et al., 2003), organogenesis, vernalization and flower bud formation (Finnegan et al., 2000; Zluvova et al., 2001; Sung et al., 2006; Valledor et al., 2007; Meijón et al., 2009). The importance of DNA methylation throughout differentiation processes is easy to address, once that this mechanism is one of the most important in regulation

of chromatin accessibility and gene readability (Li et al., 2002; Valledor et al., 2007; Meijón et al., 2009), and also ensures silencing of transposons and repetitive elements, assuring stability (Tariq and Paszkowski, 2004; Köhler et al., 2012).

The crucial importance of DNA methylation in plant embryogenesis is reinforced by the observation that *Arabidopsis* mutant embryos with non-functional MET1 and CMT3 DNA methyltransferases show impaired development (Xiao et al., 2006).

A lot of studies have been conducted, in various DSE and ISE systems, in which AzaC, applied to the culture medium at different time points and concentrations, was employed as strategy to help clarify the oscillations in global DNA methylation levels along embryo maturation process. Since all systems are different, it is sometimes difficult to correlate and assemble data from different experiments. Nevertheless, some consistency has been found regarding the fact that the addition of AzaC to the culture medium during maturation of the embryos prevents globular embryo development to further stages. This has been observed in DSE of *Daucus carota* L. (LoSchiavo et al., 1989; Yamamoto et al., 2005), *Acca sellowiana* (Fraga et al., 2012) and other species (Santos and Fevereiro, 2002; Teyssier et al., 2014). The effect is dose-related and may be counterbalanced by presence of auxin (LoSchiavo et al., 1989; Fraga et al., 2012).

In microspore (direct) embryogenesis of *Brassica napus* and *Hordeum vulgare*, although short AzaC treatments increased proembryo formation (concomitantly with the DNA demethylation moment), when AzaC application was conducted for longer period, then embryo production diminished (Solís et al., 2015). This indicates that embryo differentiation requires *de novo* DNA methylation and is prevented by AzaC.

Importantly however, provoking hypermethylation (by applying auxins for example) at this embryo-development stage is equally deterrent to successful finishing of the process (LoSchiavo et al., 1989).

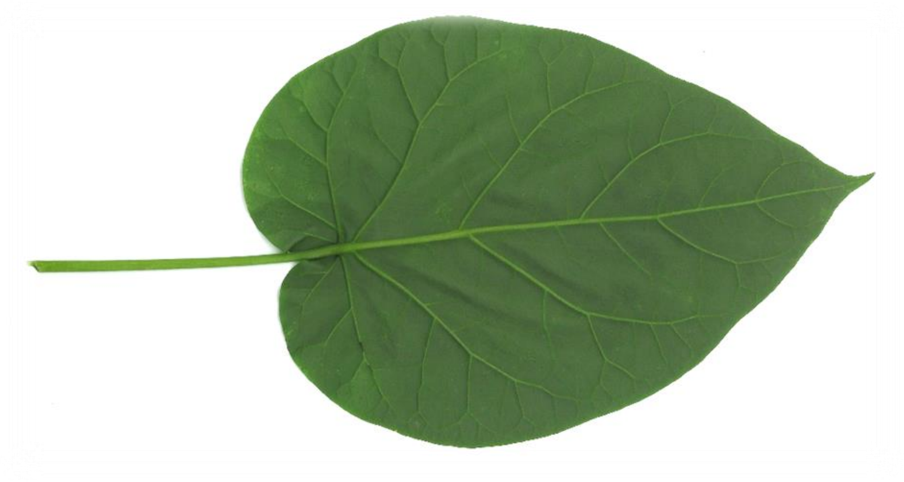
4.2.3. 5-methyl-cytidine immunofluorescence analysis provides insights on the importance of DNA methylation throughout embryo differentiation

Though global DNA methylation might reach a stable level during embryo development, it is important to stress that localization of 5mdC signal may change a lot (Pérez et al., 2015).

In this work, the 5mdC and chromatin patterns observed in embryos at different developmental stages and their surrounding embryogenic masses showed, once more, a notable heterogeneity. We have observed zones with many actively dividing cells, in which an intense DAPI and toluidine staining allowed the identification of mitotic figures. Previous studies in different systems had indeed correlated high proliferation activity of cells with low levels of DNA methylation, along with a higher transcriptional activity (Testillano et al., 2013). Large and high vacuolated cells with small dark nuclei were found in the parenchymal region, showing a generally more intense 5mdC signal than more peripheral cell layers, probably retaining some plasticity.

Works comparing chromatin patterns and *in situ* localization of 5mdC in the gametophytic *vs* embryogenic pathway of microspores in pepper, tobacco, rapeseed and barley consistently revealed some defined nuclear changes which occur throughout the process of microspore embryogenesis, such as the fact that proembryos have a generally decondensed chromatin pattern, characteristic of proliferating cells of several plant species (Testillano et al. 2000; 2002; 2005; Bárány et al. 2005; Seguí-Simarro et al. 2011). Further on the multicellular embryo development, big round nucleus with great heterogeneity in 5mdC signal has been observed (Testillano et al., 2013), attributed to the different phases of the cell cycle with different chromatin condensation state. Nucleolus always appear dark, with no 5mdC signal (Testillano et al., 2013).

The somatic embryos originated by ISE might have either a unicellular and/or multicellular origin (Fernandez et al., 1999; Queiroz-Figueroa et al., 2006). In previous studies on tamarillo, it is the unicellular origin which has been referred as the most likely hypothesis (Lopes et al., 2000; Correia and Canhoto, 2012). However, the present work does not provide enough evidence, based on microphotographs, which may lead to that conclusion.



Conclusions

Comparisons among embryogenic and non-embryogenic *calli* of tamarillo maintained in culture for different amounts of time led to the conclusion that proliferation rates are higher in non-embryogenic lines and habituated long-term embryogenic lines, and embryo conversion yield strongly decays with long-term culture, similarly to what had already been described in other systems.

Epigenetic variation, and particularly DNA methylation accumulation on the subcultured embryogenic *calli*, may significantly account for the loss of embryogenic competence.

Recently induced embryogenic masses showed both lower DNA methylation and lower H3K9 methylation levels, relative to non-embryogenic *calli*.

Heterogeneity in the distribution of methylated cytosines and chromatin patterns of cell masses may explain physiologic and morphologic differences between embryogenic and non-embryogenic lines, and may provide some insights concerning the most likely cellular origin of the future somatic embryos: the cells with decondensed chromatin, low 5mdC immunofluorescence signal and an active proliferation state.

Global DNA methylation level decreases at some moment during the dedifferentiation of leaves towards *calli* formation. Stresses and auxins present in the medium are important inducing factors in the tamarillo system; their manipulation triggers cell dedifferentiation through an epigenetic-related response.

A hypomethylation moment precedes embryo initiation, although the subsequent differentiation and development of the somatic embryos is accompanied by a gradual increase in DNA methylation levels.

The issues addressed in this work could be further explored and complemented with more assays and sampling moments, namely more time points along leaf dedifferentiation phase and formation of the first embryogenic clumps, as well as at earlier stages of embryo differentiation. Also, experiments employing methylation inhibitors (e.g. AzaC) at specific time points and concentrations might result in interesting and enlightening new data.

The acknowledgement of the changes in level and pattern of DNA methylation throughout the SE of tamarillo will allow, in the future, not only the development of strategies to improve the yields of the process, but also the progress towards a more complete and useful model of embryogenesis in woody plant species.

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