University of São Paulo
"Luiz de Queiroz" College of Agriculture

Understanding hormonal and temporal factors associated with tomato (*Solanum lycopersicum* L. cv. Micro-Tom) acquisition of competence: key concepts for *in vitro* shoot regeneration

Marcela Morato Notini

Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

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Bachelor in Biological Sciences

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To my dear grandma Vilma (*in memoriam*), who supported my scientific gift since the early experiments in her backyard

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CONTENTS

RESUMO

Compreensão dos fatores hormonais e temporais associados à aquisição de competência em tomateiro (*Solanum lycopersicum* L. cv. Micro-Tom): conceitos-chave para a regeneração *in vitro* de gemas caulinares

A regeneração de plantas através da organogênese *de novo* é uma fase crítica para a maioria dos procedimentos de micropropagação e transformação genética. Recentemente, progressos significativos tem sido alcançados no entendimento dos mecanismos fundamentais à organogênese *de novo* de tomateiro (*Solanum lycopersicum*). Entretanto, fatores hormonais e moleculares envolvidos na aquisição de competência para formação de gemas caulinares na espécie, etapa essencial ao processo de regeneração, permancece desconhecido. O fracasso em adquirir competência pode ser associado a amplamente descrita incapacidade de tomateiro em regenerar brotos caulinares a partir de raízes. No primeiro capítulo, realizou-se uma caracterização temporal e hormonal das fases de aquisição de competência e indução de gemas caulinares usando a cultivar modelo Micro-Tom. A eficiência de regeneração foi melhorada através de pré-incubação em meio indutor de raízes (RIM) durante os dois primeiros dias de cultivo, período correspondente à fase de aquisição de competência em explantes cotiledonares. Diferentemente, a pré-incubação em outro meio rico em auxina, o meio indutor de calo (CIM), sob mesmo intervalo, aboliu completamente a regeneração. A pré-incubação de dois dias em RIM induziu uma intensa e extensa resposta a auxina endógena no explante, o que provavelmente aumentou a competência das células a induzir brotos caulinares em resposta a citocinina presente no meio indutor de gemas caulinares (SIM). A aplicação desse conhecimento na melhoria do procedimento de transformação genética via Agrobacteria levou a um eficiente, simples, barato e genótipo-independente protocolo. No segundo capítulo, nós desenvolvemos um método inédito de regeneração de tomateiro via explante radicular. A formação de brotos caulinares foi obtida por ajuste do pré-tratamento em CIM ao período de aquisição de competência, correspondente a quatro dias de cultivo em explantes radiculares. O número e qualidade dos brotos também foram elevados pela otimização do explante, composição do meio de cultivo, e condições de cultivo. Somando-se os dois capítulos, o conhecimento obtido a cerca da competência organogênica resultou em novos sistemas de regeneração e transformação genética, ferramentas importantes para processos biotecnológicos e estudos funcionais de genes específicos em tomateiro.

Palavras-chave: *Solanum lycopersicum*; Regeneração i*n vitro*; Auxina; Competência organogênica

ABSTRACT

Understanding hormonal and temporal factors associated with tomato (*Solanum lycopersicum* L. cv. Micro-Tom) acquisition of competence: key concepts for *in vitro* shoot regeneration

Plant regeneration through *de novo* organogenesis is a critical step in most of the plant micropropagation and genetic transformation procedures. In the last years, significant progress has been made in the understanding of the mechanisms underlying *de novo* organogenesis in the worldwide crop tomato (*Solanum lycopersicum*). However, the hormonal and molecular factors involving the acquisition of competence for tomato shoot formation, an essential step for the regeneration process, are still not known. The failure in acquire competence can be the reason for the widely described absence of shoot regeneration from tomato root explants. In the first chapter, we conducted a temporal and hormonal characterization of the tomato acquisition of competence and the shoot induction phases using the model system cv. Micro-Tom. Regeneration was improved by pre-incubation on root-inducing medium (RIM) during the early two days in culture, a period corresponding to acquisition of competence step in cotyledon explants. Conversely, the pre-incubation on another auxin-rich condition, the callus-inducing medium (CIM), under the same period, abolished the regeneration achievement. The 2d RIM pre-treatment induced an extensive and intense endogenous auxin response in the explant, probably improving the cells competence to produce shoots under further cytokinin induction on shoot-inducing medium (SIM). This knowledge was applied to improve the Agrobacterium-mediated tomato genetic transformation procedure, leading to an efficient, simple, inexpensive and genotype-independent protocol. In the second chapter, we developed an unprecedented method for tomato shoot regeneration from root explants. The shoot organogenesis was obtained by adjusting the CIM pre-treatment to the acquisition of competence period, corresponding to the initial four days in culture for root explants. The number and quality of shoots formed were also augmented by the optimization of explants properties, medium components, and culture conditions. Taken the two chapters together, the knowledge obtained about organogenic competence advanced and created new regeneration and genetic transformation systems, which are very useful tools for biotechnology and functional studies of specific genes in tomato.

Keywords: *Solanum lycopersicum*; I*n vitro* regeneration; Auxin; Organogenic competence

1. THE ACQUISITION OF COMPETENCE FOR SHOOT INDUCTION IN TOMATO (*Solanum lycopersicum* CV. MICRO-TOM) IS DRIVEN BY A TEMPORALLY PRECISE ENDOGENOUS AUXIN ACCUMULATION, WHICH CAN BE MANIPULATED TO IMPROVE GENETIC TRANSFORMATION SYSTEM

Abstract

Tomato (*Solanum lycopersicum* L.) is a worldwide crop with extensive economic importance. The miniature cv. Micro-Tom is a suitable model plant that shows an in vitro organogenic pathway different from Arabidopsis. Tomato "acquisition of competence" and "shoot induction" steps were temporally defined through progressive media transferences from the root-inducing medium (RIM) to the shoot-inducing medium (SIM) and from SIM to the basal medium (BM), respectively. Although the exogenous cytokinin present in SIM is sufficient for tomato shoot induction from cotyledon explants, the regeneration efficiency can be improved by RIM, but not by callus-inducing medium (CIM), pretreatments during the two days corresponding to the acquisition of competence phase. We demonstrated that the spatiotemporal expression of cytokinin response promoter (*pARR5::GUS*) during the acquisition of competence for tomato shoot organogenesis was similar among explant incubated on auxin- (RIM) or cytokinin-rich (SIM) medium. On the other hand, RIM pre-incubation induced an intense and extended expression of auxin response promoter (*pDR5::GUS*) in proximal and distal cut ends tissues of the explant. Conversely, cytokinin pre-incubation led to less intense *pDR5::GUS* staining, which was restricted to the proximal end. Taken together, the results indicate that RIM pre-incubation in tomato has similar properties of CIM pre-incubation in Arabidopsis, although bypassing the negative effect of callus formation on the further shoot induction in tomato. Hence, the RIM pre-incubation represents a fine-tuned auxin-rich medium that induces an extensive and intense auxin response, improving the explant competence to produce shoots under further cytokinin induction. This knowledge allowed the development of an efficient, simple, inexpensive and genotype-independent genetic transformation system for tomato.

Keywords: *Solanum lycopersicum*; Shoot regeneration; Organogenic competence; Auxin; Genetic transformation

1.1. INTRODUCTION

Plants can not use locomotion to escape or avoid herbivory or other environmental changes that cause tissue damage and affect their survival. In response to this challenge, plant cells have developed a remarkable ability to regenerate tissues and organs and even to form a complete body from a single somatic cell, a term coined as totipotency (Haberlandt, 1902). To perform this task, the plant regeneration system follows three alternative pathways: tissue repair, somatic embryogenesis and *de novo* organogenesis (Ikeuchi et al., 2016). Plant regeneration through *de novo* organogenesis is a critical step in virtually all plant transformation and micropropagation procedures (Perianez-Rodrigues et al., 2014; Motte et al., 2014). The organogenesis process is highly variable between species and requires optimization of multiple

factors that influence the regeneration capacity, such as the type of explant and hormonal balance (Motte at al., 2014). Since Skoog and Miller (1957) elucidated the influence of plant hormones and their interactions in adventitious organ formation, *in vitro* formation of organs is now a process which has been widely exploited in several plant species (Smith, 2013; Vasil and Thorpe, 2013). However, a series of previous events takes place on the explant before the visible organ formation (Christianson and Warnick, 1985; Che et al., 2007).

Adventitious organogenesis can be divided into three main phases: acquisition of competence, organ induction, and morphological differentiation. At the first phase, cells, which can be already responsive to hormonal stimuli, acquire the capacity to assume different developmental fates. In sequence, during the induction phase, competent cells are induced to form shoots or roots, according to the hormonal composition of the medium, which culminates in cells fated or determined for specific organ formation. After this step, the process becomes hormone-independent, and the cells can be called determined or committed to a developmental fate. Finally, cells undergo morphological differentiation resulting in visible organs, characterizing the differentiation phase (Christianson and Warnick, 1985; Lombardi-Crestana et al., 2012). Similar stages of *de novo* organogenesis have been described in different species, including *Nicotiana tabacum* (Attfied and Evans, 1991) and *Arabidopsis thaliana* (Valvekens et al., 1988; Zhao et al. 2002). In the last years, significant progress has been made in the understanding of the developmental and molecular mechanisms underlying *in vitro* organogenesis (Che et al., 2007; Sugimoto et al., 2010; Liu et a., 2014; Kareem et al., 2015; Iwase et al., 2017). Although, these advances were almost completely based on the model plant Arabidopsis (Motte et al., 2014; Ikeuchi et al., 2016).

The traditional design for *in vitro* organogenesis described in Arabidopsis involves two-step procedures: explants are incubated on auxin-rich callus-inducing medium (CIM) and subsequently transferred to a cytokinin-rich shoot-inducing medium (SIM; Valvekens et al., 1988). The CIM-induced callus is originated from pericycle cells in response to local auxin maxima and resembles a lateral root meristem (Atta et al., 2009; Sugimoto et al., 2010). Explants cultured on CIM upregulates the expression of *PLETHORA3* (*PLT3*), *PLT5* and *PLT7*, which subsequently promotes the acquisition of pluripotency through the induction of competence-related genes *PLT1*, *PLT2*, *CUPSHAPED COTYLEDON1* (*CUC1*) and *CUC2* (Gordon et al., 2007; Motte et al., 2011; Kareem et al., 2015).

Upon transfer to SIM, the assignment of organ identity to the pluripotent cell mass and the development of the organ are controlled by cytokinin (CK) (Che et al., 2002). SIM incubation promotes the uptake and transport of CK and the induction of CK signaling pathways, which

will ultimately result in the expression of genes involved in shoot meristem establishment (Che et al., 2002; Gordon et al., 2007). High CK response domains induce the shoot meristem regulator *WUSCHEL* (*WUS*) and the transcription factors *ENHANCER OF SHOOT REGENERATION1/DORNRÖSCHEN* (*ESR1/DRN*) and *ESR2/DRN-LIKE* (*DRNL*), stimulating the shoot regeneration (Kirch et al., 2003; Chatfield et al., 2013). *CUC2* expression becomes spatially confined to low CK response domains in which *PIN-FORMED1* (*PIN1*) and *SHOOT MERISTEMLESS* (*STM*) are upregulated and cells continue to proliferate to form dome-like structures called promeristems (Che et al., 2006; Gordon et al., 2007; Motte et al., 2011).

The *in vitro* organogenesis of important crops, such as tomato, pepper, potato, does not strictly follow the arabidopsis model describe above, thus hampering the development of a predictable and usual regeneration protocol that can be applied to all species (Duclercq et al., 2011). Therefore, the use of alternative model plants is necessary to generate a comprehensive understanding of organogenesis in complement to the two-step model described in arabidopsis. A species that perfectly meets these requirements is the tomato.

Tomato (*Solanum lycopersicum* L.) is a worldwide culture with extensive economic importance that has been attracted scientists attention due to its attributes of a suitable model plant: diploid species with a compact sequenced genome (950 Mb; http://solgenomics.net/), rich germplasm collections (http://tgrc.ucdavis.edu/), high self-fertility and possibility to regenerate whole plants from different explants (The Tomato Genome Consortium, 2012).The tomato has been considered an alternative model to A. thaliana due to its diverse developmental traits not found in arabidopsis, such as the photoperiod-independent flowering, formation of fleshy climacteric fruits, compound leaves and glandular trichomes (Frary et al., 2000; Lifschitz et al., 2006; Krieger et al., 2010; Bergau et al., 2015; Xu et al., 2015; Tal et al., 2017). In addition to those advantages, tomato has a miniature cultivar Micro-Tom (MT) widely used (Martí et al., 2006; Aoki et a., 2010; Carvalho et al., 2011; Garcia et a., 2016) which presents a small size and short life cycle (Scott and Harbaugh; 1989), making it an excellent handling genetic model system comparable to Arabidopsis.

Differently to Arabidopsis, the cultivated tomato has not the ability to regenerate shoots from root explants (Koornneef et al., 1993; Peres et al., 2001; Lima et al., 2004) and its organogenesis is negatively correlated to callus induction (Peres et al., 2001; Lombardi-Crestana et al., 2012). Hence, MT shoot organogenesis is normally achieved directly through SIM incubation (Lima et al., 2004; Pinto et al., 2017). Even though, the regeneration efficiency is improved by a pre-incubation onto an auxin-rich root-inducing medium (RIM) during the two-

days acquisition of competence phase (Pino et al., 2010; Pinto et al., 2017). Although wealthy information regarding tomato organogenesis is available, the molecular mechanisms underlying tomato organogenesis are poorly understood. Arikita et al. (2013) identified six loci, named as *RG3C*, *RG6A*, *RG7H*, *RG8F*, *RG9DE* and *RG10F*, whose alleles from the wild relative S. pennellii confer enhanced *in vitro* shoot and/or root regeneration. Additionally, Pinto et al. (2017) created Near-Isogenic Lines (NILs) harboring the S. pennellii alleles correspondent to the loci *RG3C*, *RG7H* and *RG8F* into the MT background and performed their physiological and genetic evaluation. They found that the NILs MT-Rg3C and MT-Rg8F acquired competence 24h before MT. This result is consistent with the early hypothesis that the genes *RG3C* and *RG8F* controls the phase of acquisition of competence (Arikita et al., 2013). Hence, since the S. pennellii alleles enhance both shoot and root regeneration, their action should be positioned before the phase of induction, which is specific for shoots or roots. Additionally, two putative genes involved in the control of organogenic competence in tomato, *PROCERA* (*PRO*) (Bassel et al., 2008) and *REGENERATION1* (*RG1*) (Koornneef et al., 1993), affect both root and shoot organogenesis (Lombardi-Crestana et al., 2012). Conversely, the NIL MT-Rg7H was considered to affect shoot induction specifically, which is in agreement with the enhanced expression of the shoot-related genes *WUS* and *STM* in this line (Pinto et al., 2017).

Manipulation of the plant genome by introducing foreign genes has become an indispensable tool in plant biology and biotechnology. The *Agrobacterium tumefasciens*-mediated plant transformation is the most extensively used method (van Larebeke et al., 1975; Gelvin, 2003; Pitzschke and Hirt, 2010). However, a challenge factor that limits the use of transgenesis and genome editing is the recalcitrance to tissue culture and transformation (Shrawat and Lörz, 2006; Hiei et al., 2014). Often, cells that are readily transformed cannot be regenerated, and vice versa. Despite all explants have the capacity to undergo shoot or root organogenesis, there are some genetic or developmental factors that prevent the explant from acquiring the competence necessary to respond to hormonal inductive signals (Christianson and Warnick, 1985, 1988; Gilissen et al., 1996; Auer et al., 1999) or to assume a new developmental fate (Lombardi-Crestana et al., 2012). The elucidation of the acquisition of competence mechanisms and the transposition of its blockade may allow a successful transformation system with competent explants able to give rise of organs (Cary et al., 2002). Since the first report of tomato transformation using Agrobacterium (McCormick et al., 1986), numerous tomato genotypes, bacterial strains and methods have been explored to improve transformation efficiency (Fillatti et al., 1987; Park et al., 2003; Sun et al., 2006; Gupta and Van Eck, 2016), although the direct manipulation of the phase of acquisition of competence were not addressed in such efforts.

Here, we characterize the phase of acquisition of competence for organogenesis in tomato using the model system, cv. MT. Through media transference experiments it was determined that the explants became committed to the shoot formation fate during the initial 6-7 days of culture. Within this period, the first two days were the time necessary to acquire organogenic competence. A short pre-incubation on auxin-containing RIM during the acquisition of competence phase, and subsequent incubation on SIM with a high CK concentration, significantly improved the shoot organogenesis. Based on auxin promoter *DR5::GUS* expression, it was proposed that RIM incubation promotes more competent cells by inducing auxin maxima along the proximal and distal explant cut ends. We also associated the knowledge about organogenic competence to the establishment of an efficient, simple, inexpensive and genotype-independent genetic transformation system useful for tomato biotechnology and functional studies of specific genes.

1.2. MATERIALS AND METHODS

1.2.1 Plant Material

Three genotypes, *Solanum lycopersicum* cv. Micro-Tom (MT) and the transgenic lines on MT background *pARR5::GUS* (D'Agostino et al., 2000) and *pDR5::GUS* (Ulmasov et al., 1997) were used for the regeneration and histochemical experiments. MT-*ARR5::GUS* is a transgenic plant produced from the construct donated by Dr. Joseph Kieber from University of North Carolina at Chapel Hill. MT seeds carrying the *pDR5::GUS* gene were kindly provided by Dr. José Luiz Garcia-Martinez from Universidad Politécnica de Valencia. Additionally, the tomato cultivars MT and M82 were used for transformation assays.

1.2.2. *In vitro* cultivation and explant preparation

Seeds of different tomato genotypes were surface-sterilized by shaking in 20 mL 30% (v/v) commercial bleach (2.7% sodium hypochlorite) added by two drops of commercial detergent for 15 min, followed by three rinses with sterile water. The seeds were germinated in glass flasks with 30 mL of germination medium (GM; Tab. 1) and incubated at 25 ± 1 °C in the dark for four days. After this period, cultures were transferred and maintained for additional four days at 25 ± 1 °C under long-day conditions (16 h light/ 8 h dark), 45 μmol photons m^{-2} s^{-1} PAR irradiance.

1.2.3. Determination of the time necessary for acquisition of competence and induction events during shoot regeneration

To determine when cells become competent and induced for shoot organogenesis, explant transfer experiments (Christianson and Warnick, 1983) were conducted. Shoot induction was settled by explants transference from shoot-inducing medium (SIM) to a basal medium without hormone supplementation (BM; Tab. 1) after different days of incubation (1-10d) on SIM (Fig. 2). Control explants were incubated on SIM for 21 days. Two distinct SIM composition were evaluated (SIMI and SIMII; Tab. 1). After a total of 21 days of culture, the percentage of explants showing shoot primordia was measured. Shoot induction was defined as the minimum period on SIM incubation necessary to induce shoots before transfer to non-inductive BM.

Table 1. Composition of culture media used in *in vitro* culture and regeneration experiments of *Solanum lycopersicum* cv. Micro-Tom. MS: Murashige and Skoog, 1962; B5: Gamborg et al., 1968; BAP: 6-benzylaminopurine; NAA: 1-naphthaleneacetic acid; 2,4-D:2,4-dichlorophenoxyacetic acid; GM: germination medium; BM: basal medium; SIM: shoot-inducing medium; RIM: root-inducing medium; CIM: callus-inducing medium

Item	GM	BM	SIMI	SIMII	RIM	CIMI	CIMII
MS salts	0.5X	1X	1X	1X	1X	1X	-
B5 salts	-	-	-	-	-	-	1X
B5 vitamins	0.5X	1X	1X	1X	1X	1X	1X
Sucrose (g L^{-1})	15	30	30	30	30	30	-
Glucose (g L^{-1})	-	-	-	-	-	-	20
Trans-zeatin (μM)	-	-	-	5	-	-	-
BAP (μM)	-	-	5	-	-	0.5	-
NAA (μM)	-	-	-	-	0.4	-	-
2,4-D (μM)	-	-	-	-	-	1	9.1
Kinetin (μM)	-	-	-	-	-	-	0.2
Agar (g L^{-1})	7	-	-	-	-	7	7
Phytagel (g L^{-1})	-	2.3	2.3	2.3	2.3	-	-

The acquisition of competence for shoot organogenesis was determined by explants transferences from a non-shoot-inducing medium to SIM, varying the number of days that the explants remained in each medium for up to 8 days (Fig. 1 and 2). After that, all explants were transferred to BM for up to 21 days of cultivation, when the percentage of explants with shoot primordia or roots was recorded. Distinct media were tested as the non-shoot-inducing medium:

root-inducing medium (RIM) and two callus-inducing media (CIMI and CIMII). Media compositions are described in table 1. Acquisition of competence was defined as the time at which CIM or RIM can substitute SIM without decreasing shoot formation efficiency or leading to root formation (recorded after 21 days of incubation).

1.2.4. Regeneration assays

MT cotyledon explants were placed with the abaxial side down onto four different treatments: SIMI and SIMII, with and without two days RIM pre-incubation. Fifteen explants were cultured per Petri dish (90 × 15 mm), with six plates per treatment (n=6). Plates were individually sealed with PVC film. After 21 days of culture, the percentage of explants with shoot primordia was recorded.

A

	Days of incubation											
SIM	0	1	2	3	4	5	6	7	8	9	10	21
BM	21	20	19	18	17	16	15	14	13	12	11	0
total	21											

B

	Days of incubation								
CIM / RIM	0	1	2	3	4	5	6	7	8
SIM	8	7	6	5	4	3	2	1	0
BM	13								
total	21								

Figure 1. Schematic design for the treatments used to determine the time necessary for shoot induction (A) and for acquisition of competence (B) in *Solanum lycopersicum* cv. Micro-Tom. Twelve treatments, each containing 6 Petri dish with 15 explants (n=6), were used in (A) and nine treatments, each containing 6 Petri dish with 15 explants (n=6), were used in (B). BM: basal medium; SIM: shoot-inducing medium; RIM: root-inducing medium; CIM: callus-inducing medium.

1.2.5. Histochemical assays

Spatiotemporal expression of auxin (DR5) and cytokinin (ARR5) responsive promoters were examined through GUS reporter signaling along the shoot organogenesis phases. *pARR5::GUS* and *pDR5::GUS* signals were evaluated in explants cultured on SIMI, SIMII and RIM at different incubation periods (6, 12, 18, 24, 30, 36, 42, 48 h) during the early 48 hours, which corresponds to the acquisition of competence phase (Fig.3). Additionally, GUS expression was verified at different incubation periods (1, 2, 3, 6, 9, 12, 15, 18, 21 d) in explants cultured on SIMI, with and without 2 d of RIM pre-incubation. For all time point analysis, 10 explants per genotype were collected at each six hours intervals and then transferred to staining buffer (80mM sodium phosphate buffer, pH 7.0; 0.4 mM potassium ferrocyanide; 8 mM EDTA; 0.05% Triton X-100; 0.8 mg/mL 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Gluc); 20% methanol) and incubated at 37°C (Jefferson et al., 1987). The stained tissues were incubated in 70% alcohol for 12 h, and then in the chloral hydrate solution (200 g chloral hydrate; 20 g glycerol; 50 ml H2O; Tsuge et al., 1996) at 65°C for approximately 12 h, until tissues became transparent. The explants were examined with a Leica S8AP0 stereomicroscope.

1.2.6. Bacterial strain and binary vector

The binary vector pK7WG2 (Life Technologies, USA; Karimi et al., 2002) was introduced into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The pK7WG2 contains an inducible marker gene *ccdB* (*killer* (*KIL*) gene) and *neomycin phosphotransferase II* (*nptII*) as a selective marker gene, both genes under the control of the *cauliflower mosaic virus* (*CaMV35S*) promoter.

Agrobacterium was cultured in 3 mL of liquid LB medium supplemented with 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin and incubated at 28ºC for 24 h at 120 rpm. From this culture, 500 µL were taken and added to 50 mL of fresh LB medium with the appropriate antibiotics, as above. The culture was incubated overnight at same conditions and centrifuged at 2000 g for 15 min, at room temperature. The pellet was re-suspended in liquid BM (Tab. 1) to an OD600 of 0.25-0.3 and added by 100 µM acetosyringone (AS) before inoculation.

1.2.7. Tomato transformation

MT cotyledon explants were placed with the abaxial side down onto a co-cultivation (CC) medium composed of MS salts, B5 vitamins, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 100 µM AS, supplemented with different hormone treatments (Table 2). Two drops of Agrobacterium suspension in liquid MS were applied per explants using a micropipette. After 10 min, the excess of bacterial suspension was removed with a sterile pipette and the explants were blotted dry on sterile filter paper. Plates were kept for two days at 25ºC in dark for co-cultivation. Explants were transferred to the shoot-induction (SI) selective medium containing MS salts, B5 vitamins, 30 g L^{-1} sucrose, 2.3 g L^{-1} phytagel, 100 mg L^{-1} kanamycin, 300 mg L^{-1} timentin, supplemented by different hormone treatments (Table 2). For each transformation experiment, 20 Petri dishes (90 × 15 mm) with 20 explants per plate were inoculated (n=20), corresponding to 400 explants per treatment. The percentage of explants with shoot primordia and the number of shoots produced per transformation experiment were recorded 21 days after transformation.

After 19 days from inoculation on selective SIM, shoot primordia (1-2 cm) started to be excised from explants and transferred to flasks containing 30 mL of BM (Tab. 1) supplemented with 100 mg L^{-1} kanamycin and 300 mg L^{-1} timentin. After three weeks, the number of well-developed shoots showing usual morphology, denominated as true shoots, was evaluated for each transformation experiment.

Table 2. Hormone supplementation at co-cultivation (CC) and shoot induction (SI) media used in *Solanum lycopersicum* cv. Micro-Tom transformation experiments. CC: co-cultivation phase medium; SI: shoot induction phase medium; BAP: 6-benzylaminopurine; NAA: 1-naphthaleneacetic acid.

	Transformation experiment (TE)							
	TE1		TE2		TE3		TE4	
	CC	SI	CC	SI	CC	SI	CC	SI
BAP (µM)	5	5	-	5	-	-	-	-
Trans-zeatin (µM)	-	-	-	-	5	5	-	5
NAA (µM)	-	-	0.4	-	-	-	0.4	-

Putative transgenic true shoots were confirmed by PCR (see item 2.8). The transformation efficiency was calculated by dividing the total number of transgenic rooted shoots by the number of explants inoculated and then multiplied by 100. Multiple shoots generated from a single callus were treated as a single transformation event. The confirmed transgenic true shoots

were transferred to a fresh medium, as described above. After three weeks, the developmental pattern of the plants was analyzed. Along with this step of analyses, all transgenic plants with usual development already presented roots. They were transferred to the greenhouse for acclimatization in 150 ml pots with a 1:1 mixture of commercial substrate Basaplant® (Base Agro, Artur Nogueira, SP, Brazil) and expanded vermiculite, supplemented with 1 g NPK 10:10:10 L^{-1} substrate and 4 g L^{-1} dolomite limestone (MgCO3+CaCO3). Acclimatized plants were allowed to self-pollinate, producing fruits. The plant and fruit morphologies and the seed formation were observed.

The tomato cultivar M82 was transformed using the TE2 combination (Tab. 2) of medium for CC and SI phases using the same procedures described above.

1.2.8. PCR analysis

To identify transformants, genomic DNA was extracted from leaves of putative transgenic events and controls (non-transformed) according to Fulton et al. (1995). The presence of nptII marker gene was confirmed using the gene-specific primers forward 5'-GAACAAGATGGATTGCACGC-3' and reverse 5'-GAAGAACTCGTCAAGAAGGC-3'. The diagnostic amplicon size expected with these primers is approximately 780 bp. The PCR program started with a one-step cycle of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C; 30 s at 60°C; and 2 min at 72°C, and a 10 min final extension at 72°C. DNA was separated and visualized by electrophoresis through a 1% agarose, SYBR Green-stained gel.

1.2.9. Flow cytometry analysis

To estimate the DNA C values, 2 cm^2 of fresh leaves from MT wild-type or transgenic plants was macerated with the same mass of the internal reference standard *Pisum sativum* cv. Citrat (9.09pg; Doležel, 1997). The maceration was performed on a Petri dish containing 1 mL of cold LB01 buffer using a scalpel blade to release the nuclei into suspension (Doležel et al. 1989). The nuclei were stained by adding 25 μL of a 1 mg mL^{-1} solution of propidium iodide (PI, Sigma, USA). Additionally, 5 μL of RNase (100 μg mL^{-1}) was added to each sample. The analysis was performed using the FACSCantoIITM (Becton, Dickinson and Company, USA) flow cytometer, and histograms were obtained with Flowing Software 2.5.1 software. The DNA 2C values of each sample were calculated by the relative fluorescence intensity of the sample and the internal reference standard (*P. sativum* 9.09 pg). A statistical evaluation was performed using the software

WinMDI 2.8. Three samples from each plant were analyzed, and the variation in DNA content between the plants was tested by an analysis of variance (ANOVA).

1.2.10. Statistical analysis

For statistical analysis to be performed on the percentage of explants showing shoot primordia, each Petri plate (with fifteen and twenty explants for regeneration and transformation experiments, respectively) was considered as a unit. All the data obtained were subjected to analysis of variance (ANOVA) and Tukey's post-hoc test using SISVAR (Ferreira, 2003). Only standard error was calculated for the percentage of explants responding.

1.3 RESULTS

1.3.1 Competence and induction for shoot organogenesis in Micro-Tom

Micro-Tom shoot regeneration from cotyledon explants usually follows 21 days of culture on medium supplemented by 5 μM 6-benzylaminopurine (BAP) or 5 μM trans-zeatin (tZ) denominated shoot-inducing medium I or II (SIMI or SIMII), respectively (Lima et al., 2004; Pino et al., 2010; Lombardi-Crestana et al., 2012). Experiments involving the transference of cotyledon explants from SIM to a medium without hormone supplementation (BM) at different days of culture were used here to establish the shoot induction phase (Fig. 1). Shoots were observed after a minimum of six and seven days of cultivation on SIMII and SIMI, respectively, followed by transference to BM in a total of 21 days of culture (Fig. 2). SIM incubations longer than 10 days in culture, followed by transference to BM, showed no significant difference in regeneration frequency (data not shown).

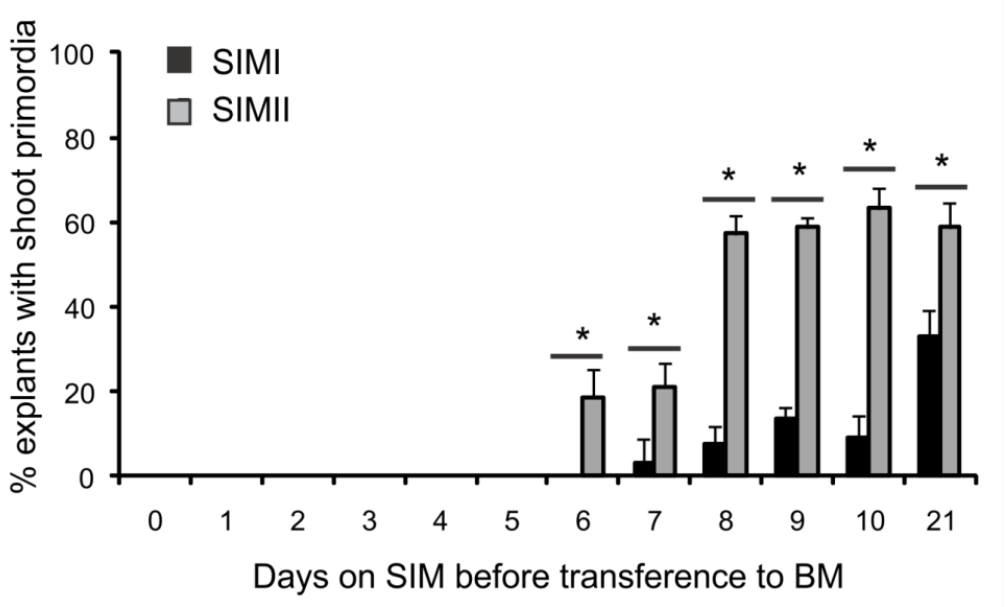


Figure 2. Media transfer experiment to determine the number of days to reach the induction for shoot organogenesis in cotyledon explants of Micro-Tom. Explants were cultured on SIMI or SIMII (0-10 days) before transfer to BM. Mean percentage of explants with shoot primordia was evaluated after 21 days in culture. 21 days of culture on SIM correspond to the positive control. Vertical bars indicate ± standard error of the mean (n = 6 Petri dishes with 15 explants each). Asterisks mean a statistical difference among the pair of means ($p \leq 0.05$; Tukey's test). SIM: shoot-inducing medium supplemented by 5μM 6-benzylaminopurine - BAP (SIMI) or 5μM trans-zeatin (SIMII); BM: basal medium.

SIMII induced a higher frequency of explants with shoot primordia compared to SIMI in all incubation periods resulting in shoots (Fig. 2). The percentage of explants showing shoot primordia reached control levels (21 days of culture on SIM) after 8 days of incubation on medium supplemented with tZ, but it remained lesser than the control when BAP was used. These results indicated that induction for shoot organogenesis takes place between days six and seven, depending on the CK added to SIM.

The acquisition of competence phase was defined through a new series of transfer experiments. Since the acquisition of competence precedes the shoot induction, cotyledon explants were pre-incubated on a non-shoot inducing medium for different intervals within 8 initial days (a period longer than that necessary for the explant to be induced) followed by transfer to BM (Fig.1). Two different callus-inducing medium compositions (CIMI and CIMII) and a root-inducing-medium (RIM) were applied as non-shoot inducing media. Explants cultured on CIMI for 0-8 days, moved to SIMI or SIMII (until to complete 8 days) and further transferred to BM, showed no shoot regeneration after 21 days of culture (Fig. 3). In such explants, only root induction was observed in 2-5 days pre-incubation on CIMI and a prominent callus formation at 6-7 days pre-incubation. CIMI composition was previously selected as the most efficient hormone combination to induce callus in MT cotyledon explants (data not published). Regardless

of the CK used on SIM, BAP (SIMI) or tZ (SIMII), shoots were formed only in explants without CIMI pre-incubation. A similar experiment was conducted using CIMII, a medium successfully applied in *Arabidopsis thaliana* tissue culture for shoot improvement (Sugimoto and Meyerowitz, 2013). However, no shoot formation was verified regardless the period of pre-incubation on CIMII (Fig. 4).

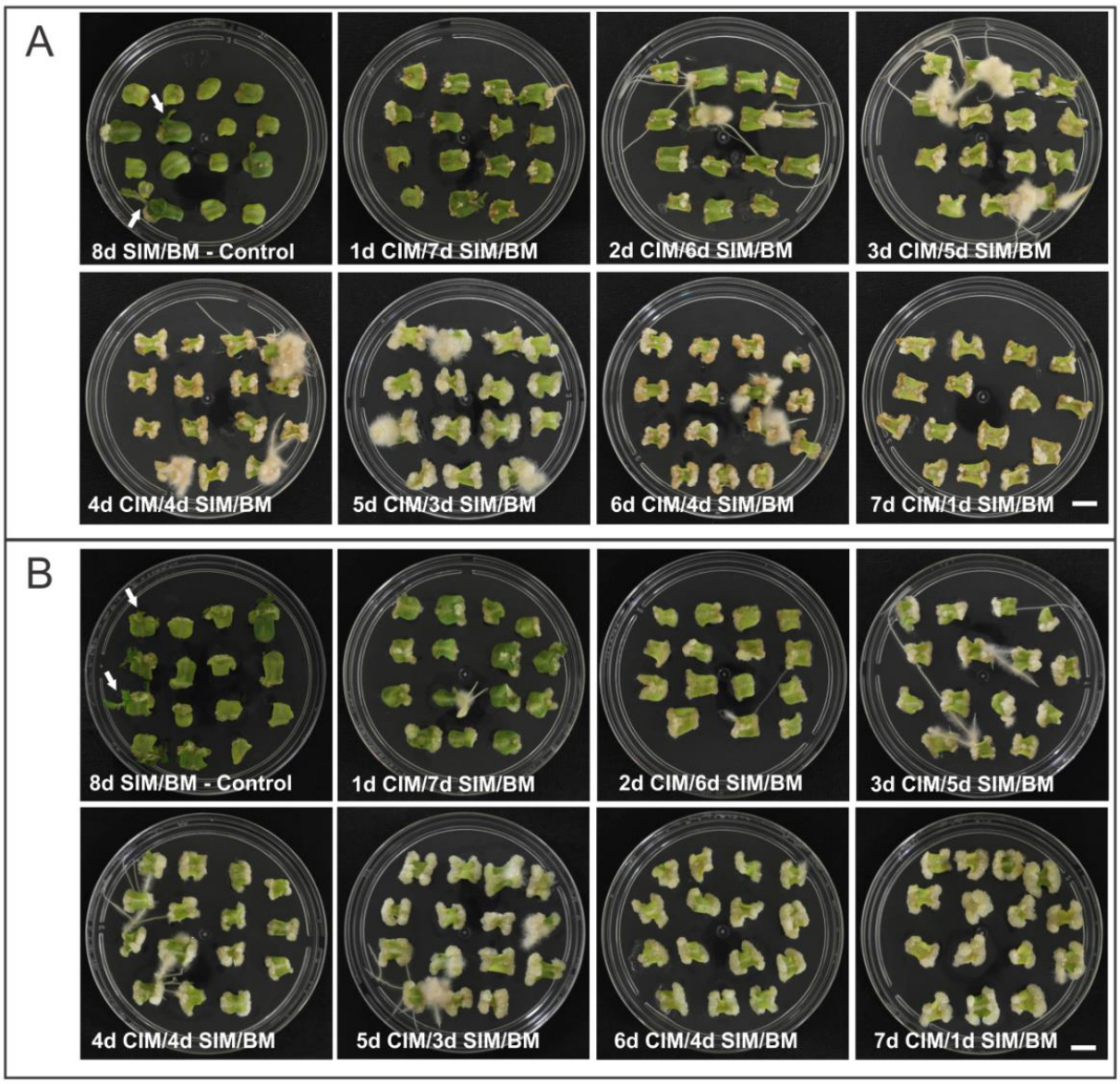


Figure 3. *In vitro* regeneration of cotyledon explants of Micro-Tom at 21 days in culture. Explants were submitted to pre-incubation on CIM I (0-7 days), follow by SIMI (A) or SIMII (B) until complete the shoot induction phase (8 days in culture). After this period, explants were incubated on BM. Shoot primordia are indicated by arrows. CIMI: callus-inducing medium I; SIM: shoot-inducing medium supplemented by 5 μM 6-benzylaminopurine - BAP (SIMI) or 5 μM trans-zeatin (SIMII); BM: basal medium. Bars: 1cm.

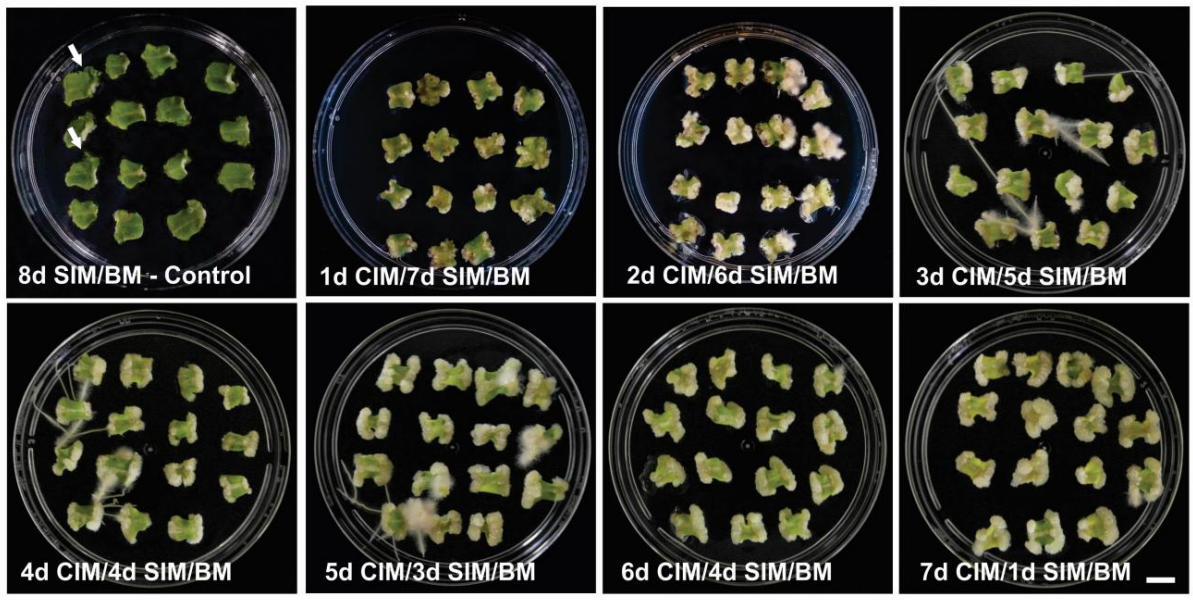


Figure 4. *In vitro* regeneration of cotyledon explants of Micro-Tom at 21 days in culture. Explants were submitted to pre-incubation on CIMII (0-7 days), follow by SIMI until complete the shoot induction phase (8 days in culture). After this period, explants were incubated on BM. Shoot primordia are indicated by arrows. CIMII: callus-inducing medium II; SIMI: shoot-inducing medium I; BM: basal medium. Bars: 1cm.

Alternatively, the root-inducing medium (RIM) was used for pre-incubation (0-8 days) before 8 initial days on SIM, followed by BM until complete 21 days of culture (Fig. 5). The pre-incubation on RIM for two days before transfer to SIM (6 days) induced the higher percentage of explants showing shoot primordia. This pre-treatment on RIM before SIMI and SIMII increased the number of explants with shoot primordia five and two times, respectively, when compared to the control 8 days on SIM. Longer RIM pre-incubation resulted in root induction and fast reduction of shoot formation frequency, independently of the CK used at SIM. These data suggest that two days of incubation in a culture medium is the time required to reach competence for shoot organogenesis. In addition, the results suggest that NAA pre-treatment (present in RIM medium) improves the shoot regeneration efficiency when explants are further transferred to SIM.

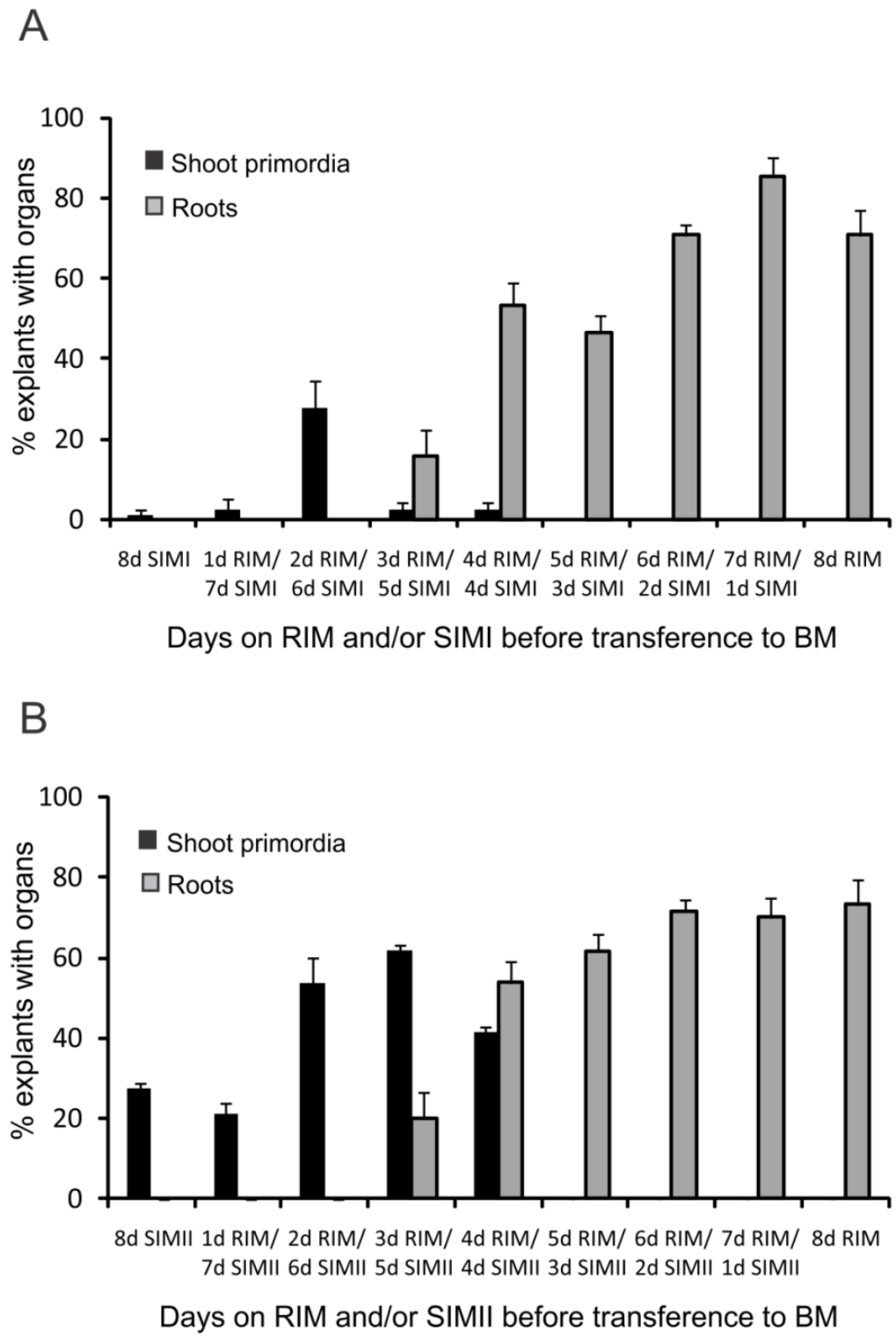


Figure 5. Media transfer experiment to determine the number of days to reach competence for shoot organogenesis in cotyledon explants of Micro-Tom. Explants were cultured on RIM (0-7 days), follow by SIMI (A) or SIMII (B) until complete the shoot induction phase (8 days in culture). After this period, explants were incubated on BM. Mean percentage of explants with shoot primordial and roots was evaluated after 21 days in culture. Vertical bars indicate ± standard error of the mean (n = 6 Petri dishes with 15 explants each). RIM: root-inducing medium; SIM: shoot-inducing medium.

1.3.2. NAA pre-treatment improves the shoot organogenesis in Micro-Tom cotyledon explants

To confirm the ability of NAA pre-treatment to improve the shoot formation, explants were cultured for 21 days on SIMI and SIMII, with and without two days of pre-incubation on RIM (0.4 μM NAA). An initial exposure on RIM improved the shoot organogenesis regardless of the SIM (I or II) used later (Fig. 6). However, a RIM pre-culture previously to SIMI increased the frequency of shoot formation in almost three-fold, which is superior to the 1.5-fold verified in explants transfer to SIMII. Furthermore, the percentage of explants with shoot primordia induced by the combination of two days on RIM followed by BAP-rich medium (SIMI) was statistically similar to that verified in explants incubated on a tZ-rich medium (SIMII), regardless of the pre-incubation on RIM.

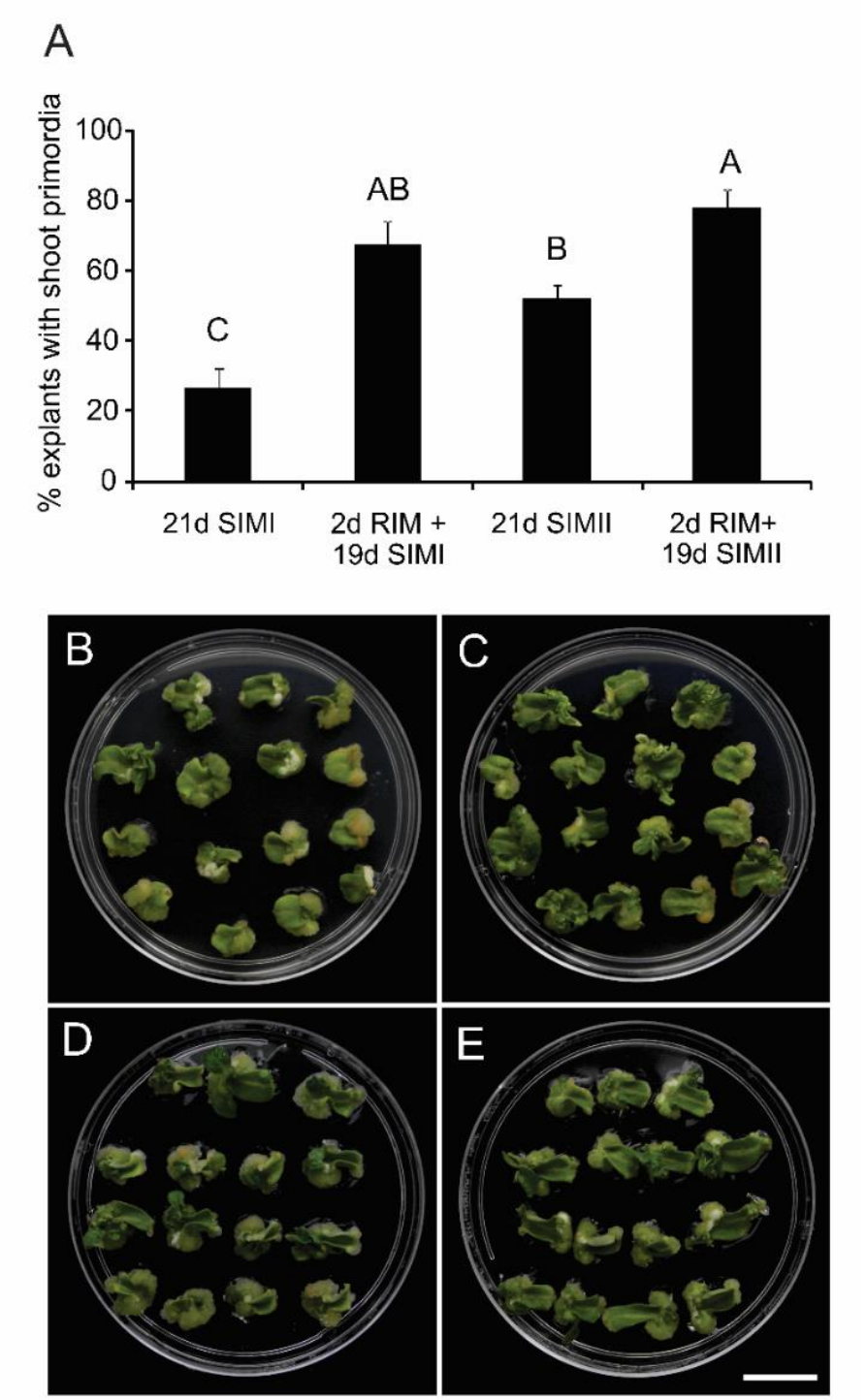


Figure 6. Shoot regeneration from cotyledon explants of Micro-Tom (MT).A: Shoot regeneration frequency (% explants with shoot primordia) of MT cotyledon explants cultured on SIMI and SIMII with or without two days of pre-incubation on RIM. Vertical bars indicate ± standard error of the mean (n = 6 Petri dishes with 15 explants each). Different letters indicate significant differences at $P \leq 0.05$ (Tukey's test). B-E: Morphology of explants cultured for 21 days on SIMI without (B) or with (C) two days of pre-incubation on RIM, and explants cultured on SIMII without (D) or with (E) two days of pre-incubation on RIM. SIM: shoot-inducing medium; RIM: root-inducing medium.

1.3.3. The pattern of *pDR5::GUS* and *pARR5::GUS* expression is dependent on media composition during the acquisition of competence

Auxin and CK response markers *pDR5::GUS* and *pARR5::GUS*, respectively, were analyzed in cotyledon explants during the initial two days of culture on NAA-, BAP- and tZ-rich medium (Fig. 7 and 8).

Explants cultured on RIM showed *pDR5::GUS* signal in both cut ends of the explant, proximal and distal, starting from the earlier six hours of exposition (Fig. 7A). At this time point, the auxin response was spread for all cut ends, with higher intensity near to the main vein region. After longer incubations, the expression of *pDR5::GUS* was stronger and not differentiated between central and peripheric tissues at excised ends. On the other hand, in explants cultured on CK-rich media SIMI and SIMII, the GUS staining was barely detectable at initial 18 hours of incubation. The reporter *DR5* expression was limited to the main vein region at the proximal end (bottom side) (Fig. 7B and C), following the expected accumulation of auxin due to its known basipetal transport at the vascular parenchyma (Petrasek and Friml, 2009). *pDR5::GUS* expression progressively increased at later time points and GUS signal was detectable in central and peripheral tissues in the proximal cut pole. At 48 hours of incubation, GUS staining was spread to the inner tissues of the explant, near to the proximal end and not visualized at distal ends. SIMII (Fig. 7C), supplemented by tZ, induced a more intense expression of the auxin response reporter compared to the media supplemented by BAP (SIMI; Fig. 7B), despite both media induce signal with temporal and local similarities.

pARR5::GUS expression (Fig. 8) indicates that CK response is more similar amongst the two early days of incubation on RIM and SIM than verified for auxin (Fig. 7). GUS staining evidenced the ARR5 promoter response restrict to the vascular tissues on the beginning of incubation (0-18h) on RIM (Fig. 8A) and SIMI (Fig. 8B), spreading throughout the whole explant at later time points. At 48 hours of incubation, the signal concentrated mainly in the excised ends, proximal and distal. Additionally, tZ induced higher expression of *pARR5:GUS* in parenchymal and vascular tissues starting from the first 6 hours of incubation on SIMII (Fig. 8C). However, the explants exhibited a similar response to CK on both media at the end of the two days of incubation on SIMI and SIMII (Fig. 8B-C).

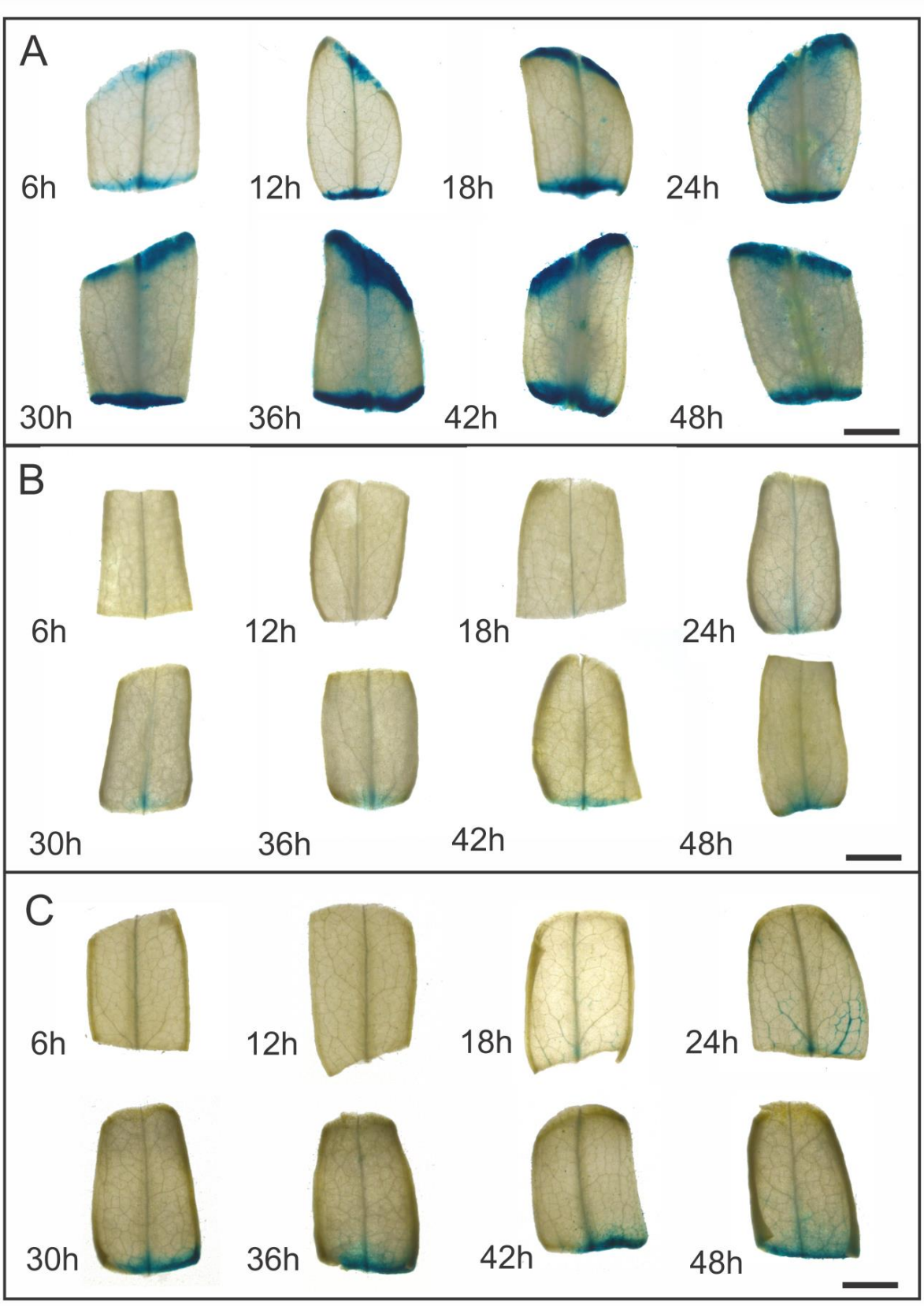


Figure 7. Expression of the synthetic auxin response marker pDR5::GUS during the early 48h of incubation, consisting of the acquisition of competence phase, on root-inducing medium (RIM; A), shoot-inducing medium I (SIMI; B) and shoot-inducing medium II (SIMII; C). Explants are positioned with distal end to the top and proximal end to the bottom. Bars= 5mm.

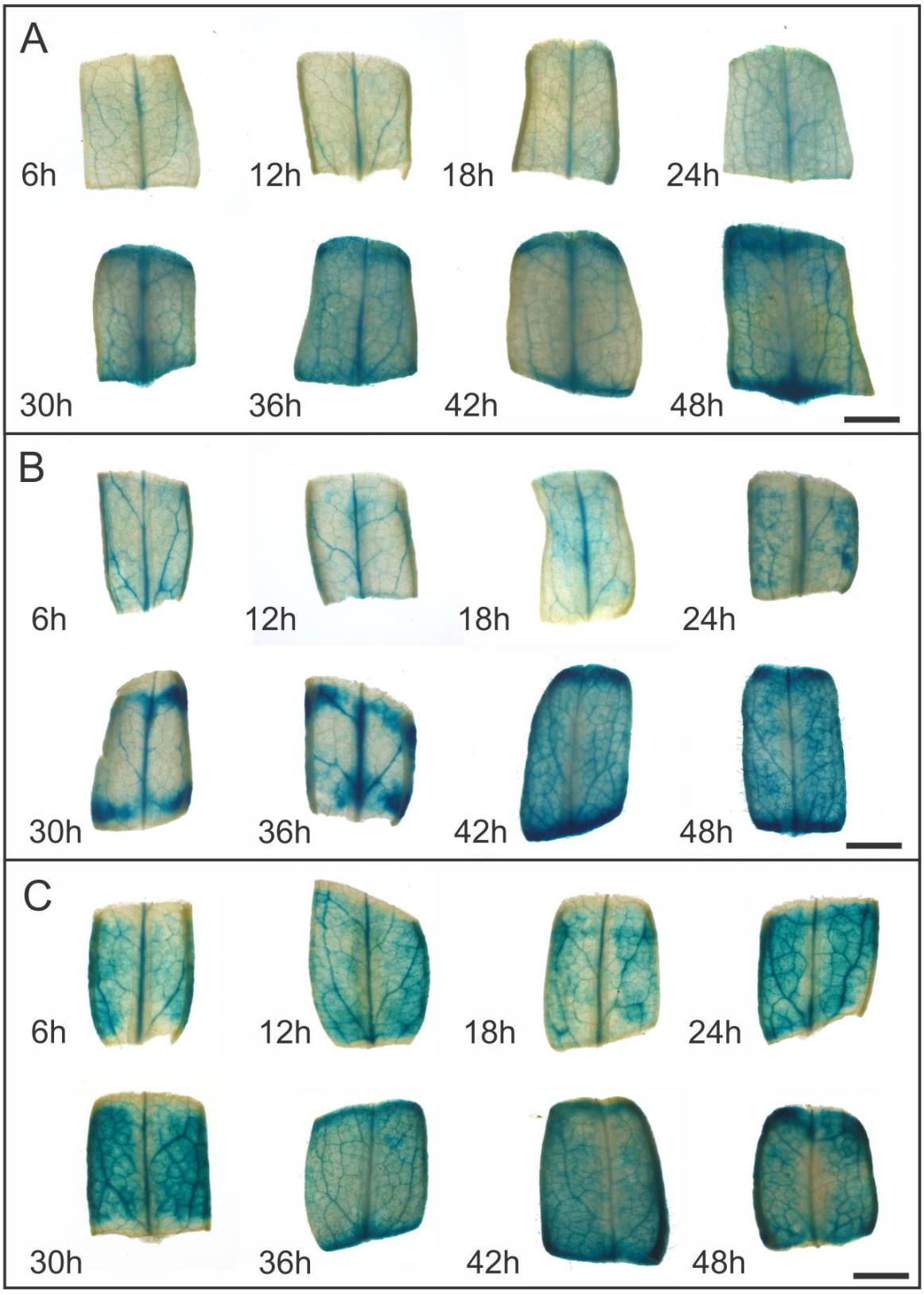


Figure 8. Expression of the cytokinin response marker pARR5::GUS during the early 48h of incubation, consisting of the acquisition of competence phase, on root-inducing medium (RIM; A), shoot-inducing medium I (SIMI; B) and shoot-inducing medium II (SIMII; C). Explants are positioned with distal end to the top and proximal end to the bottom. Bars= 5mm.

1.3.4. Auxin-rich pretreatment does not affect the pattern of *pDR5::GUS* and *pARR5::GUS* expression along shoot induction and differentiation

The expression of auxin and CK responsive promotors were also analyzed during the shoot induction and differentiation phases in explants cultured on SIMI, with or without RIM pretreatment (Fig. 9 and 10).

As previously demonstrated, MT-*DR5::GUS* explants cultured on RIM for the early two days showed extended and intense auxin response in proximal and distal ends, in contrast with the restricted and less intense signal verified in explants cultured on SIM during this period (Fig. 9). Explants were initially cultivated on SIM or RIM and after 48 hours of incubation, they were transferred to SIM. At this point, both treatments were maintained under the same medium composition conditions (SIMI). In general, from the 3rd to the 15th day of culture, auxin response started to increase in explants directly incubated on SIMI and to be attenuated in explants pre-incubated on RIM along the initial two days, reaching similar expression pattern at the 18th day of culture (Fig. 9). For explants cultured exclusively on SIMI (Fig. 9A), auxin signaling started on distal and proximal explant ends starting from the 3rd day of culture. GUS staining increased in intensity and area, following the callus growth until day 15. Conversely, pre-incubation on RIM before transference to SIMI (Fig. 9B) resulted in auxin signaling mitigation starting at day 3 (first 24 hours on SIMI) along all SIMI culture. From the 18th day of culture, for both cultivation conditions, *DR5::GUS* staining is barely detected in the prominent callus on the proximal explant end. At 21st day of culture, shoot buds emerged from callus and GUS signal was sorely expressed in the meristematic zone and the tip of leaf primordium (Fig. 9).

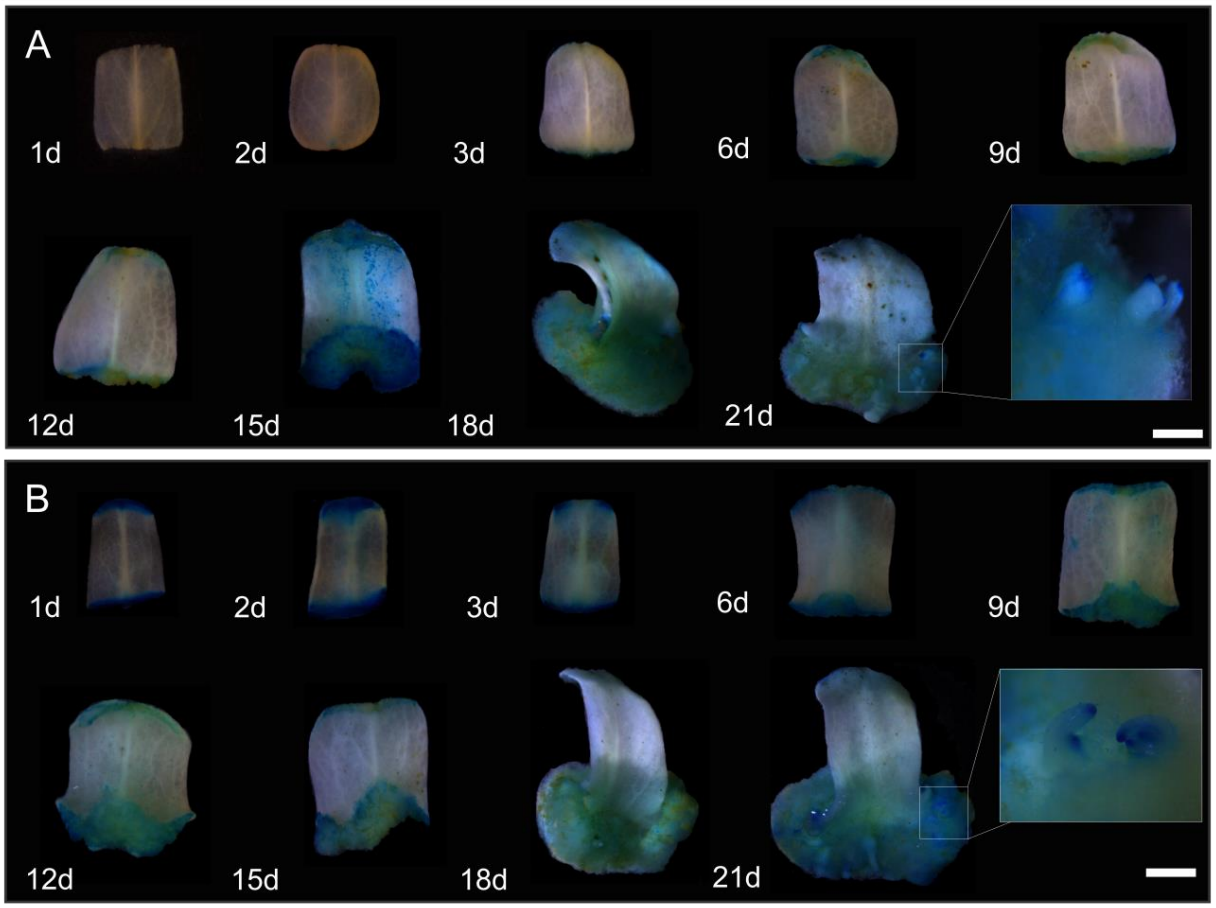


Figure 9. Expression of the synthetic auxin response marker pDR5::GUS during the shoot organogenesis process on shoot-inducing medium (SIM) without (A) or with (B) 2 days RIM pretreatment. Explants are positioned with distal end to the top and proximal end to the bottom. Bars= 1 cm.

Analysis of MT-*ARR5::GUS* signaling showed similar CK response in explants cultured on SIMI regardless of the medium composition applied during the first two days of culture (Fig. 10). As described above, CK response was expressed throughout the whole explant cultured on RIM and SIMI during the 48 hours corresponding to the acquisition of competence period, especially in vascular tissues and proximal and distal ends (Fig. 10). From the second day of culture onwards, in which all explants were incubated on SIMI, GUS signal became less intense in parenchymatic and vascular tissues of the explant middle section. At day 12, CK response was verified only in the explant cut ends, especially in the large callus tissue developed at the proximal end. At later incubations, *ARR5*::GUS signal maintained intense and confined to the callus, with CK response barely detected in primordia and more developed shoots (Fig. 10).

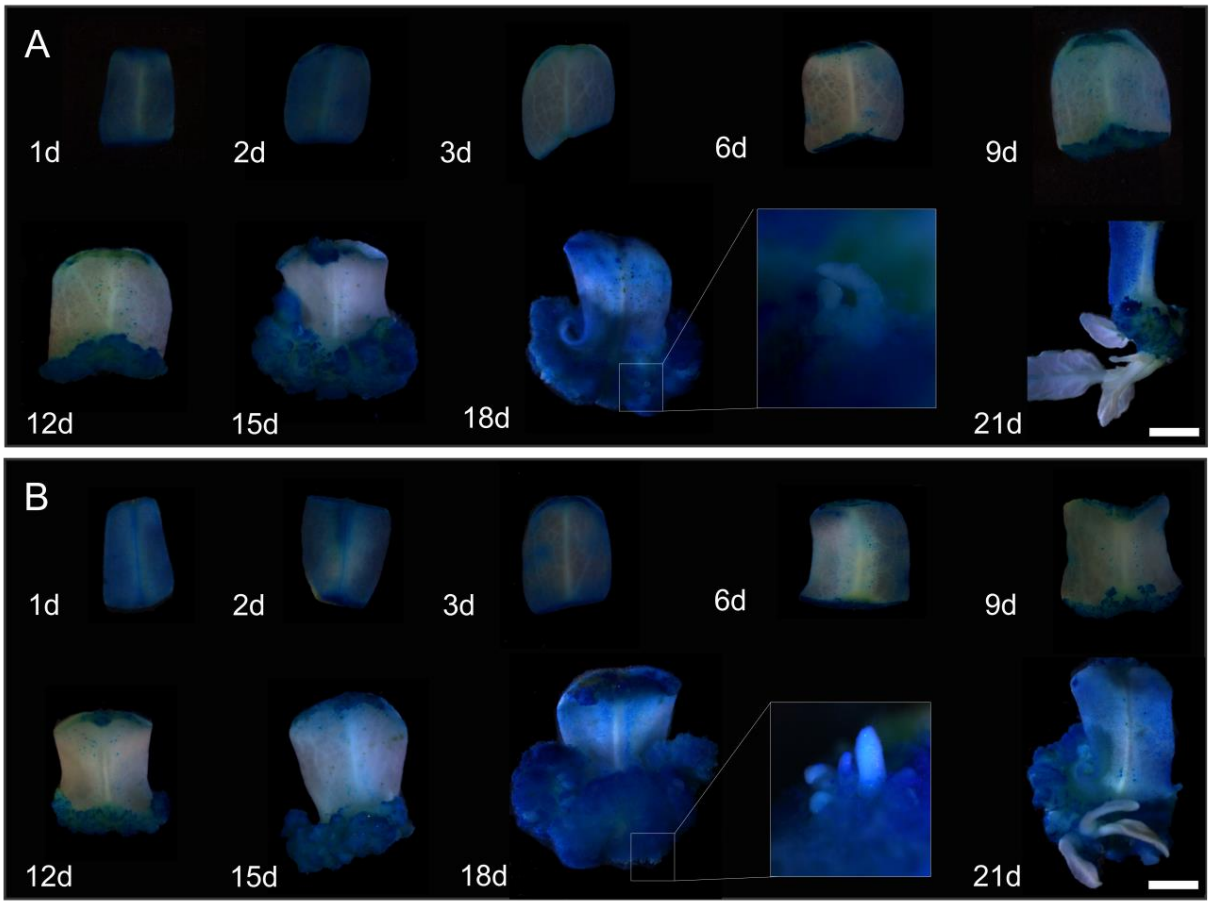
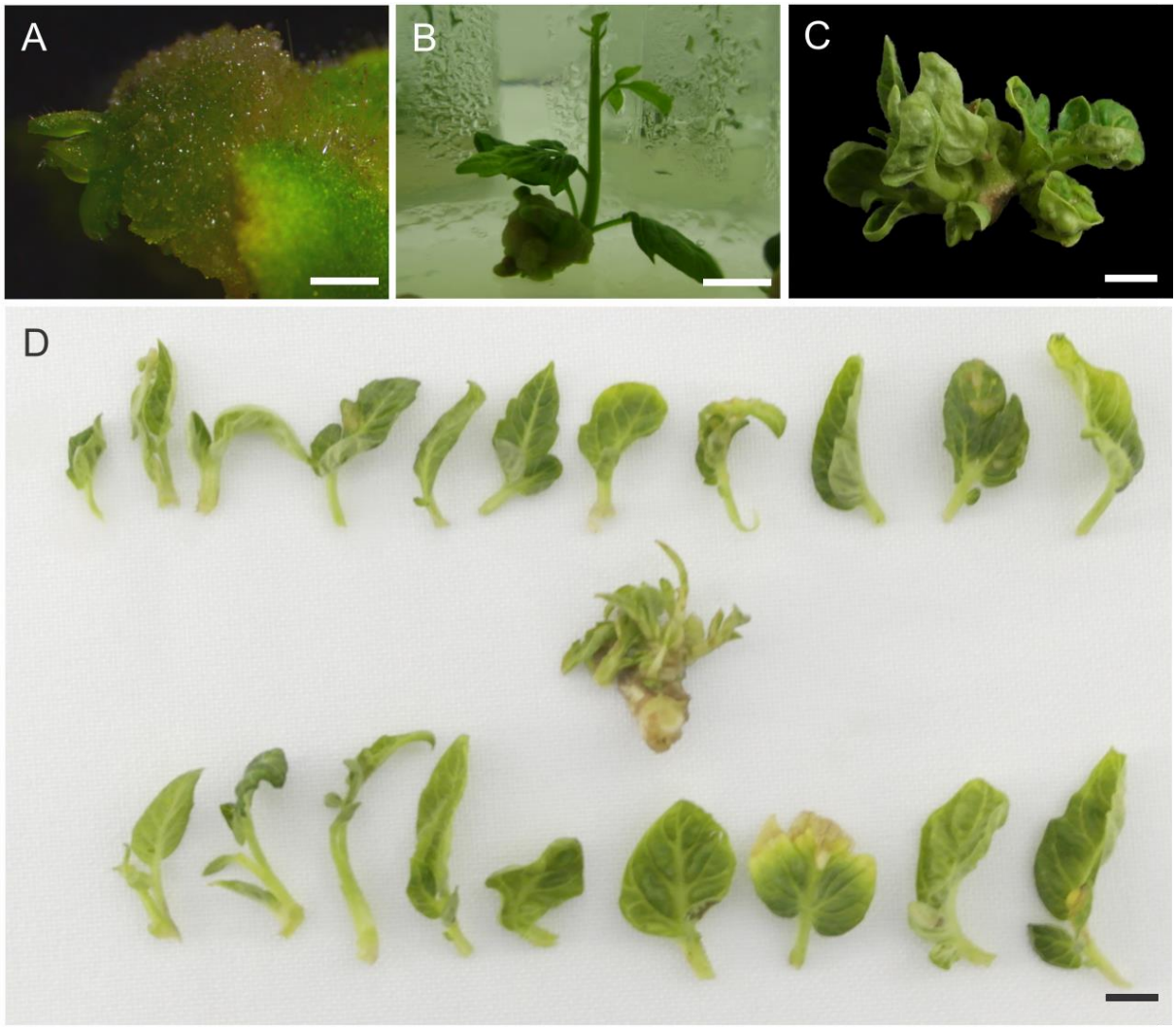


Figure 10. Expression of the cytokinin response marker *pARR5::GUS* during the shoot organogenesis process on shoot-inducing medium (SIM) without (A) or with (B) 2 days RIM pretreatment. Explants are positioned with distal end to the top and proximal end to the bottom. Bars= 1 cm.

1.3.5. Effect of NAA and different CKs in shoot regeneration during genetic transformation experiments

The possibility that an initial pulse of NAA, and its enhancement in shoot formation, could improve the efficiency of genetic transformation procedures was tested. Cotyledon explants infected with Agrobacterium tumefaciens strain LBA4404 harboring the binary vector pK7WG2 were cultured under different media combinations, named as "transformation experiment" (TE) 1 to 4 (Tab. 2). At 21 days after transformation, shoot primordia (Fig. S1A) and some abnormal structures, such as isolated leaf raised from the callus tissues, were obtained. Only the shoot primordia were taken into account in the analyses of the regeneration efficiency.



Supplementary Figure S1. Morphology of tomato Micro-Tom structures arising from transformation procedures. A: Shoot primordia obtained 21 days of culture after transformation B - D: Shoots derived from shoot primordia maintained at selective basic media for three weeks. Shoots with usual development (true shoots; B) and shoots with proliferative leaf formations without the development of a stem or main axis (C and D) were observed. D: All leaves lined at the upper and lower rows were originated from the central structure, derived from single shoot primordia. Bars= 5mm (A), 2cm (B) and 1cm (C and D).

Shoot regeneration medium supplemented with tZ induced a higher percentage of explants with shoot primordia, regardless the medium used during the co-cultivation phase, as verified in TE3 and TE4 (Tab. 2; Tab. 3). Additionally, an initial exposure to NAA during the co-cultivation applied in TE2 did not improve the regeneration frequency when compared to cotyledon explants cultivated on BAP-rich media during both phases of transformation (TE1; Tab. 3). The same response was verified comparing TE3 and TE4.

Table 3. Regeneration and transformation parameters obtained after *Solanum lycopersicum* cv. Micro-Tom genetic transformation through a different combination of media, denominated transformation experiment (TE) 1 to 4. Transformation efficiency was calculated by dividing the total number of genetic transformation events by the total number of inoculated explants (400 explants). Different letters indicate significant differences at $P \leq 0.05$ (Tukey's test).

Transformation experiment	% explants with shoot primordia	Nº of shoot primordia	Nº of true shoots	Nº of transgenic events	Transformation efficiency (%)
TE1 (BAP/BAP)	21.0 (±1.9) B	84	14	10	2.5
TE2 (NAA/BAP)	28.5 (±2.4) B	114	30	26	6.5
TE3 (tZ/tZ)	59.3 (±2.3) A	237	107	65	16.3
TE4 (NAA/tZ)	57.5 (±2.8) A	230	108	75	18.8

Shoot primordia were isolated from callus tissue and transferred to a selective basal medium (containing kanamycin) to develop and elongate for more three weeks. After this interval, the number of true shoots (shoots in late stage, well developed and showing usual morphology; Fig. S1B) was measured for each combination of media applied on the transformation procedure (Tab. 3). On this step of analyses, tZ maintained the superior capacity to induce regeneration, whereas explants co-cultivated on tZ-rich (TE3) or NAA-rich (TE4) medium, gave rise to 107 and 108 true shoots, respectively. Although BAP shows lower inductive potential, co-cultivation on NAA-rich medium (TE2) improved the number of these well-developed shoots in two-fold compared to those incubated on BAP-rich medium in both transformation phases (TE1).

The considerable decrease in the number of true shoots compared to the number of shoot primordia induced (Tab. 3) is closely related to the high proportion of shoot primordia that do not develop in a normal vegetative structure: a main vertical axis, basically constituted of leaves, internodes and a terminal meristem (Fig. S1B). Instead of this usual pattern, several primordia only develop a sequence of leaf series in a radial fashion, without a main vertical axis, which was unable to form roots and be acclimatized (Fig. S1C-D).

1.3.6. Transformation efficiency in different set of media

PCR analysis for the presence of the *neomycin phosphotransferase II* (*nptII*) selectable marker gene was made to confirm the transgenic true shoots (Fig. 11A). The number of confirmed

transgenic events (transgenic true shoots in late stage) and the transformation efficiency were shown in table 3. The transformation efficiency was substantially higher in cotyledons transformed using tZ to induce shoots (TE3 and TE4 methods) compared to those in which shoots were induced by BAP (TE1 and TE2; Tab. 3). However, the difference amongst the tZ and BAP potentials decreased when the co-cultivation step took place on NAA-rich medium.

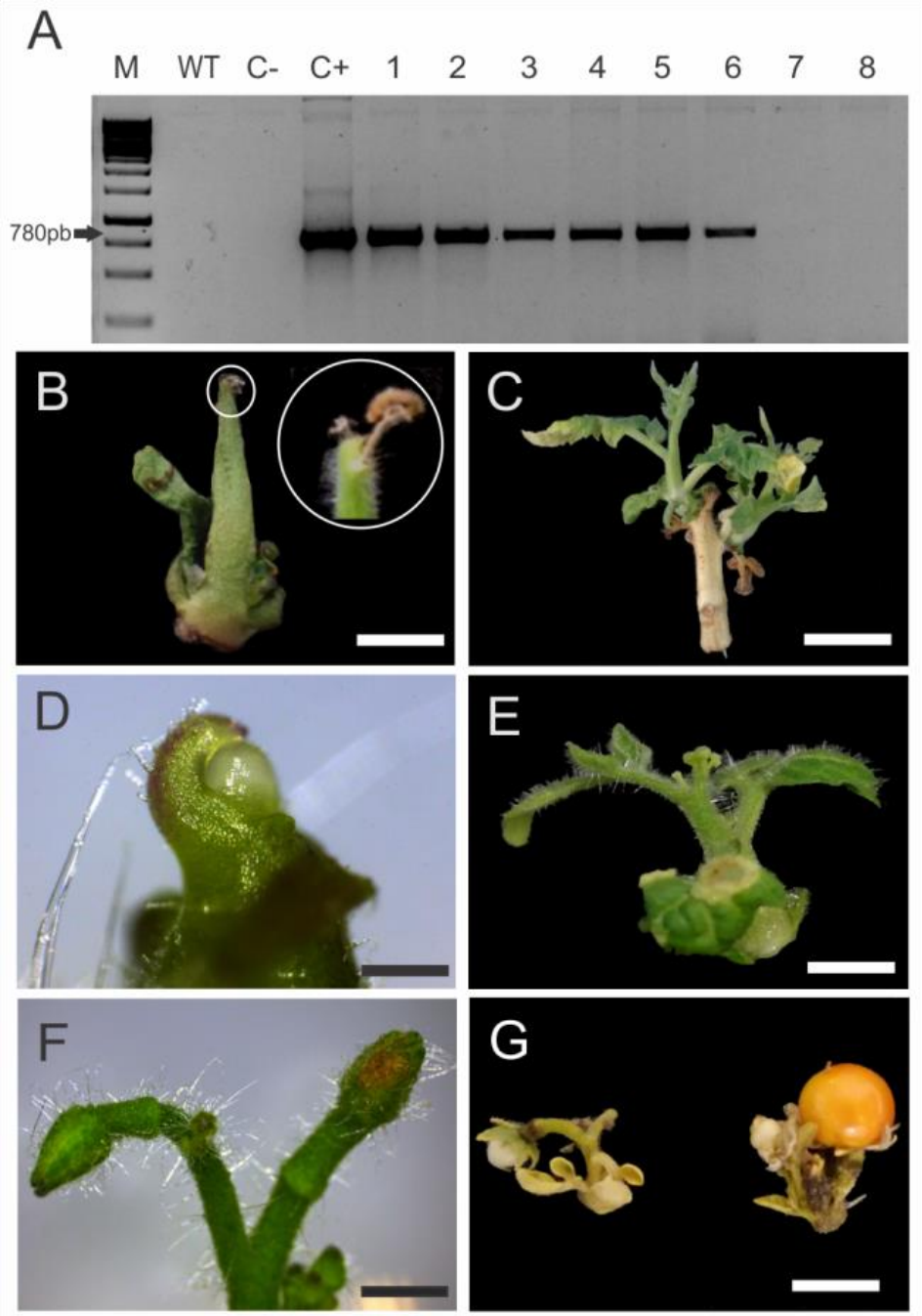


Figure 11. PCR detection of *Solanum lycopersicum* cv. Micro-Tom transgenic plants and their developmental characteristics. A: Representative electrophoresis analysis of PCR amplification of the *neomycin phosphotransferase II* (*nptII*) gene (780-bp fragment) in putative Micro-Tom transgenic plants. Lane M: molecular weight marker (1-kb DNA ladder). Lane WT: genomic DNA from an untransformed tomato plant. Lane C-: negative control (water was used as sample). Lane C+: positive control of plasmid DNA. Lanes 1 to 8: putative transgenic events kanamycin-resistant. B – C: Transgenic plants showing dead shoot apical meristem (SAM), without (B) and with (C) development of lateral buds. D: Detail of an apical meristem during the transition from vegetative to reproductive development. E: Transgenic plantlets with SAM determined into floral meristem. F: Detail of floral buds developed *in vitro* from plants which SAM determined into floral meristem. G: Transgenic plants showing fruits at different stages of development. Bars = 1 cm (B – C, E), 5 mm (D and F) and 2 cm (G).

1.3.7. Trans-zeatin induces an expressive premature differentiation of the shoot apical meristem

Transgenic shoots confirmed by PCR were placed onto a fresh selective basal media for more three weeks. After this period, the developmental pattern of these plants was analyzed. Transgenic plants originated from TE3 and TE4, in which the shoots were induced by tZ, showed a high proportion of plants with developmental disorders. Amongst them, a premature differentiation of the shoot apical meristem (SAM) leads to the death of the meristem (Fig. 11B-C) or the untimely determination in floral meristem (Fig. 11D-F). Some of these flowers developed in seedless fruits *in vitro* (Fig. 11G). At all, only 25 in a total of 65 transgenic plants (38%) obtained by TE3 method showed usual development. Similarly, considering the 75 transgenic events produced through TE4, only 29 developed in a usual structure (39%). On the other hand, none transgenic event originated from TE1 or TE2 exhibited dead SAM, although some of these vegetative meristems had differentiated into floral meristems. Consequently, the proportion of transgenic plants with usual development induced by BAP (TE1 and TE2) was not so reduced as verified in events derived from tZ shoot induction (TE3 and TE4).

All transgenic plants showing usual development were able to form roots along the incubation on BM and were acclimatized. After 45 days of culture in the greenhouse, events originated from a different combination of media (TE1-4) showed no clear difference related to morphology, flowering time and fruit development (Fig. S2A). All fruits collected exhibited seeds (Fig. S2B). Micro-Tom wild-type and transgenic plants showed similar vegetative and reproductive characteristics (Fig. S2A-B).

Supplementary Figure S2. Morphology of *Solanum lycopersicum* cv. Micro-Tom (MT) wild-type and transgenic plants (A) and fruits (B) 45 days after acclimatization. Transgenic events were generated by a different combination of medium, denominated transformation experiment (TE) 1 to 4. Left to right: Wild-type (MT); TE1; TE2; TE3; TE4. Bars= 2cm.

1.3.8. A combination of co-cultivation on NAA and shoot induction by BAP ensures the most cost-effective genetic transformation protocol

After elucidating the total number of high-quality transgenic events obtained, the transformation cost-effectiveness of different combination of media was verified. Considering the cost and concentration of hormones supplemented on the co-cultivation and shoot induction media used in each transformation experiment (Tab. S1), the hormone costs to realize a transformation and to produce a single transgenic event were measured for each method (Tab. 4).

Supplementary Table S1. Cost and concentration of hormones supplemented on 20 plates of co-cultivation and shoot induction media used in Micro-Tom transformation procedures. Pricing according to Sigma-Aldrich website (http://www.sigmaaldrich.com; March / 2017. USD: United States Dollar; tZ: Trans-zeatin; BAP: 6-benzylaminopurine; NAA: 1-naphthaleneacetic acid.

Hormone supplemented	Price per milligram (USD)	Amount used per liter	Price per transformation phase (0.6L; USD)
tZ (Sigma, Z0876)	4.32	5 μM (1.1 mg L^{-1})	2.85
BAP (Sigma, B3408)	0.03	5 μM (1.13 mg L^{-1})	0.02
NAA (Sigma, N0640)	0.001	0.4 μM (0.07 mg L^{-1})	0.000043

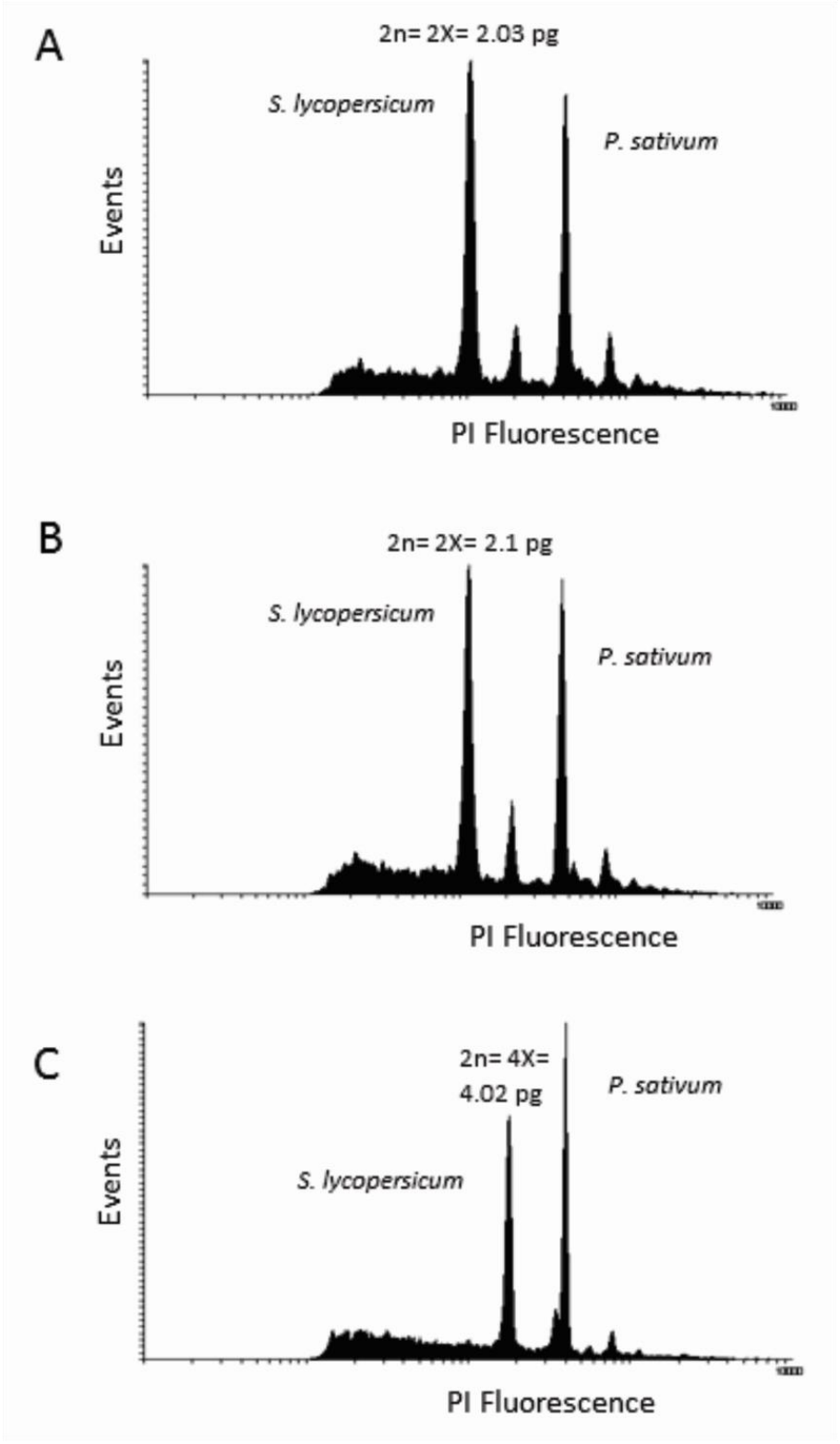
In general, the cost spent in hormones at a transformation experiment (20 Petri plates or 400 explants) was vastly inferior in protocols using BAP (TE1 and TE2), compared to those supplemented by tZ (TE3 and TE4), regardless of the medium used during the co-cultivation step (Tab. 4). Notwithstanding, TE3 and TE4 showed highest transformation efficiency (Tab. 3), the superiority of tZ in transgenic shoot recover seems to be insignificant on the cost-effectiveness of the transformation process. Amongst transformations applying CK-rich medium on the co-cultivation and shoot induction phases, hormone cost expenses to obtain a single transgenic plant by TE1 (using BAP supplementation) represented 4.3% of the value spent in the tZ based protocol, TE3 (Tab. 4). As verified in table 4, the supplementation of NAA on the co-cultivation media improved the cost-effectiveness of the transformation, regardless of the CK added on shoot induction medium. Under this condition, the hormone cost spent to generate a single high-quality transgenic event through TE2 (with BAP shoot induction) was one hundred-fold less than the value spent in procedures applying TE4, a tZ shoot induction method. Therefore, the hormonal cost and concentration, besides the number of high-quality events, were considered to indicate TE2, a combination of co-cultivation on NAA-rich medium and shoot induction on BAP-rich medium, as the most cost-effective genetic transformation protocol.

Table 4. Comparative analysis of hormonal cost-effectiveness of different transformation experiments. Hormone cost was calculated to perform 20 Petri plates of co-cultivation and shoot induction medium, and to generate a single transgenic event with usual development. The value between parentheses represents the number of transgenic events with usual development generated by each transformation experiment. USD: United States Dollar.

	Hormone cost (USD[a])	
Transformation experiment	Transformation (20 plates)	Transgenic event with usual development
TE1	0.04 (6)	0.01
TE2	0.02 (20)	0.001
TE3	5.70 (25)	0.23
TE4	2.85 (29)	0.10

1.3.9. The optimized transformation protocol does not induce polyploidy and is not cultivar-specific

Ploidy levels of transgenic plants with usual development generated by the most cost-effectiveness transformation protocol were determined using flow cytometry. Among 20 events analyzed, we found that a higher proportion of transgenic plants were diploids (75%) and the other 25% were identified as tetraploids (Fig. S3). We did not estimate the proportion of polysomatic cells in explant tissue (cotyledons) of parental lines used for transformation, although parental plants were diploids, as expected, based on analysis of leaf samples by flow cytometry.



Supplementary Figure S3. Representative flow cytometry histograms of *Solanum lycopersicum* cv. Micro-Tom wild-type and transgenic plants. A: WT diploid plant (2.03 pg); B: diploid transgenic plant (2.1 pg); C: tetraploid transgenic plant (4.02 pg). The number in parenthesis represents 2C values.

To determine the adaptability of the transformation protocol optimized for *Solanum lycopersicum* cv. MT across other genotypes of tomato, we applied the TE2 protocol to one of the most adopted tomato cultivar, the M82. Our study indicated a high percentage of M82 regenerating explants induced by TE2 transformation procedure compared to MT, generating a total of 201 shoot primordia (from 400 transformed explants). As verified in MT, only a low percentage of shoot primordia (15%) develop in true shoots. A PCR screening for the presence of nptII selectable marker gene confirmed 13 transgenic true shoots, resulting in a transformation efficiency of 3.3%. In a different fashion to MT, M82 transgenic events showed no vegetative meristem death or differentiation in floral meristem. Transgenic events were acclimatized, producing fruits with seeds and advancing generations (data not shown).

1.4. DISCUSSION

According to Christianson and Warnick (1983; 1985), the developmental process of de novo organogenesis is composed by the acquisition of competence, organ induction, and organ differentiation phases. In this study, we established the organogenesis phases for the tomato cultivar Micro-tom (MT). Along the organ induction, a specific pathway for organ development is triggered inside the explant resulting in explant determination (Christianson and Warnick, 1983; 1985). This phase has an experimentally recognizable endpoint: If the explant has passed through induction and reached determination, it will go into the differentiation phase and produce shoots even if it is moved from shoot-inducing medium (SIM) to a hormone-free basal medium (BM; Zhao et al., 2002). MT cotyledon explants need six and seven days of culture on SIMII and SIMI, respectively, to reach induction and determination (Fig. 2). A similar result has already been described for MT in which shoots developed on BM after five days of initial incubation on SIMII (Pinto et al., 2017). Explants originated from *Arabidopsis thaliana* cotyledon seems to require a longer SIM incubation to be induced and determined and the shoot induction phase corresponded to the early 12 days of culture (Zhao et al.; 2002).

Competence is achieved when a tissue is able to move into the phase of induction and, in response to the media, become able for a specific developmental pathway such as de novo shoot formation (Christianson and Warnick, 1985; Lombardi-Crestana et al., 2012). The time at which an auxin-rich callus-inducing medium (CIM) or root-inducing medium (RIM) can still substitute SIM without decreasing the final shoot formation (and without inducing root formation) marks the period of acquisition of competence. In Arabidopsis, the acquisition of competence occurs during the early two days of incubation, regardless the explant been

originated from cotyledons or roots tissues (Zhao et al., 2002; Che et al. 2007; Motte et al., 2011). In this model plant, it is generally assumed that the pre-incubation on auxin-rich CIM improves the acquisition of competence to generate shoots in SIM (Valvekens et al., 1988). However, CIM incubation, and the callus formation, is negatively related to shoot organogenesis in cultivated tomato explants (Peres et al. 2001; Lima et al. 2009; Lombardi-Crestana et al., 2012). Conceivably, CIM pre-incubation to establish the MT acquisition of competence failed to form shoots in all treatments tested (Fig. 3; Fig. 4). Using RIM pre-incubations, it was determined that MT explants acquire competence at two days of incubation (Fig. 5), a period similar to the 2-3 days recently described for near isogenic lines harboring high regenerating alleles introgressed into this cultivar (Pinto et al., 2017). Although Arabidopsis and tomato belongs to two distinct clades (Rosids and Asterids, respectively), it is remarkable that these two species presented similar periods necessary for acquisition of organogenic competence.

Along the organogenesis assays, we observed that trans-zeatin (tZ) is more effective in shoot induction than 6-benzylaminopurine (BAP; Fig. 2; Fig. 5). This is in agreement with previous reports that zeatin (or its derivatives) is more effective than BAP to induce MT organogenesis protocols (Park et al., 2003; Pino et al., 2010; Cruz-Mendívil et al., 2011). For other tomato cultivars, zeatin-supplemented media also prone to give the higher number of shoots primordia and shoots per explant (Ichimura and Oda, 1995; Gubis et al., 2004). This superiority of tZ can be ascribed to its natural occurrence, which is the most active kind of CK in all plant species (Gajdošová et al., 2011). Hence, although the synthetic BAP is more stable than tZ (Kieber and Schaller, 2014), the CK receptor Arabidopsis Histidine Kinase3 (AHK3) was shown to have the highest affinity for tZ and the lowest affinity for BAP (Lomin et al., 2012). The other sensors AHK2 and AHK4 also demonstrated highest affinities for tZ followed by BAP and cis-zeatin (Stolz et al., 2011).

We verified that an initial pulse of auxin and subsequent transference to SIM resulted in higher regeneration frequency for tomato (Fig. 5; Fig. 6). This is similar to the successful Arabidopsis two-step organogenesis method (Valvekens et al.; 1988), which is based on auxin-rich CIM pre-incubation. However, since excess callus proliferation is negatively correlated with shoot regeneration in tomato (Peres et al., 2001; Lombardi-Crestana et al., 2012; Fig 3; Fig. 4), a pre-incubation on NAA-rich RIM, instead of CIM, was used. Despite the fact that auxin supplementation has been long used in tomato regeneration media (Kut and Evans, 1982), the restrict use of auxin to the acquisition of competence phase as a shoot induction strategy was only described by Pino et al. (2010). This approach can substitute callus-mediated shoot regeneration that is difficult to achieve in some plant species and negatively correlated to

organogenesis in others. Recently, an efficient protocol for direct conversion of lateral root primordia into shoots based on pre-incubation on media containing NAA was published in Arabidopsis (Kareem et al., 2016). This suggests that the strategy of using NAA pre-incubation in a defined window of acquisition of competence can be extended to other species for successful shoot induction.

DR5::GUS is a synthetic promoter linked to a reporter gene that was designed to be expressed in response to auxin (Ulmasov et al. 1997). By using this reporter as an auxin status indicator (Benková et al., 2003), we compared the auxin distribution in explants cultured on SIM and RIM during the two days corresponding to acquisition of competence phase. We demonstrated that auxin response started strictly in vascular tissues after 18 h of incubation on SIM, regardless of the CK used. At two days of incubation, competent explants cultured on BAP- or tZ-rich medium showed auxin accumulation on vascular and parenchymatic tissues exclusively on the proximal cut end, but tZ was more efficient to promote an intense and widely distributed auxin response compared to BAP (Fig. 7). The auxin accumulation on proximal side of the explant can be at least in part explained by the basal cell localization of the main auxin efflux carrier PIN1 (Gälweiler et al., 1998) that allows an intercellular auxin flow from the cotyledon tip (distal region) to the proximal region (Michniewicz et al., 2007). On the other hand, NAA-rich media cultivation induced auxin response in all tissues of both poles of explant, proximal and distal, starting in the early hours of culture (Fig. 7). Besides this NAA-induced additional auxin accumulating region on the explant, the intensity and amplitude of these auxin maximum were superior to those induced by CKs. Under auxin-rich RIM incubation, the DR5 expression in the explant might be explained not only by endogenous auxin accumulation related to PIN polarity but also to the NAA influx. According to Delbarre et al. (1996), the NAA is very efficient to generate multiple auxin maxima throughout the root explants in Arabidopsis, since it can enter the cells independent of the presence of AUX/LAX auxin influx carriers.

The differential auxin distribution in the explant described above is a relevant data since Arabidopsis studies suggested that founder cell specification and the acquisition of organogenesis competence are mediated by auxin maxima (Atta et al., 2009; Sugimoto et al., 2010) and the concomitant local activation of auxin responses in specific pericycle cells (Che et al., 2007; Dubrovsky et al., 2008). On the other hand, the role of CK is supposed to be more relevant for the assignment of organ identity, after competence takes place (Che et al., 2002). This may explain the absence of variation in CK response in explants incubated on different media composition along the acquisition of competence (Fig. 8). Taking these into account, we suggest that 2d NAA pretreatment is likely to promote a wide region of competent cells in both cut ends

of the explants. These cells, which are able to further respond to CK stimuli for shoot formation, would improve the regeneration efficiency. This hypothesis on the positive effect of NAA improving specifically the phase of acquisition of competence is supported by the fact that NAA pretreatment did not lead to significant changes in auxin or cytokinin response along the shoot induction and differentiation phases of organogenesis (Fig. 9; Fig. 10). The pattern of *DR5::GUS* expression under SIMI and SIMII incubations can also explain the superiority of tZ in tomato shoot induction, compared to BAP, since it produces a more intense auxin response, and probably more competent cells, during the acquisition of competence phase.

Since McCormick et al. (1986), several Agrobacterium-mediated transformation protocols have been developed for tomato (Van Eck et al., 2006; Sharma et al., 2009; Pino et al., 2010). Based on the superiority of zeatin and its derivatives for inducing tomato shoots (Fig.4; Gubis et al., 2004; Pino et al., 2010), most of the transformation protocols suggested the application of this CK to recover transgenic shoots (Fillatti et al., 1987; Park et al., 2003; Van Eck et al., 2006; Cruz-Mendívil et al., 2011). Indeed, MT transformation applying tZ supplementation in SIM induced higher regeneration efficiency compared to BAP supplementation (Tab. 3). It was also demonstrated that supplementation of BAP and NAA concomitantly (Park et al. 2003) or NAA separately during the 2d co-cultivation (Pino et al., 2010) improve transformation efficiency. This data suggests that co-cultivation medium composition also affects transformation success and that the hormone supplementation has different influences during co-cultivation and shoot induction phases of genetic transformation.

Here, a comprehensive analysis of genetically transformed explants regeneration and subsequent growth of transgenic plants was performed. Different from most tomato transformation and regeneration reports, we evaluated not only the shoot emergency (regeneration frequency) but also the development of shoot primordia into adult reproductive plants (Tab 3). In our preliminary analysis, the NAA-rich incubation during the two days of co-cultivation phase, instead of a CK-rich medium, did not improve the percentage of explants recovering shoot primordia after transformation, regardless of the CK used in the shoot induction phase (Tab. 3). However, co-cultivation on NAA generates a considerable superior number of transgenic events when compared to the use of BAP or tZ without NAA pre-incubation (Tab. 3).

Morphological aberrations, such as isolated leaf, stem deformation, and regenerants without buds, are common on *in vitro* organogenesis (Pratta et al., 1997; Gubis et al., 2004; Steinitz et al., 2006), as also reported in this work (Fig. S1D-E). Shoot-like regenerants with no stem were unable to recovery growth and to complete the life cycle. Consequently, we

demonstrated an intense decrease on the number of well-developed shoots in comparison to the number of shoot primordia recovered for all transformation experiments tested (Tab. 3). Considering the number of true shoots recorded, the NAA capacity to induce superior parameters among transformation applying BAP-shoot induction remained. This data suggests an advantageous use of NAA during the co-cultivation aiming transgenic shoot formation.

A literature search shows that transformation efficiencies depend on numerous parameters, such as the plant genotype and the Agrobacterium strain (Park et al., 2003; Chaundhry et al., 2007; Chetty et al. 2013). For MT protocols, the transformation efficiency ranges from 19 to 56% (Park et al., 2003; Sun et al., 2006; Qiu et al., 2007; Pino et al., 2010; Cruz-Mendívil et al., 2011; Chetty et al., 2013). Despite EHA105 has been efficiently used in numerous protocols (Qiu et al., 2007; Pino et al., 2010; Cruz-Mendívil et al., 2011), previous MT cotyledon transformations using this super-virulent strain led to an uncontrolled bacterial growth during the co-cultivation phase, followed by oxidation and death of explants (data not shown). Moreover, an experimental procedure generating moderate transformation efficiencies allows more visible distinction between the treatments to be compared. Based on these statements, the less-virulent Agrobacterium strain LBA4404 (Ellul et al., 2003) was selected as the more indicated bacterial strain for this study. Besides the bacterial strain, the lower transformation efficiencies obtained here can be also associated with the absence of any development or hormonal related gene or promoter on the vector used on transformation, differently from that performed by Pino et al. (2010).

Despite transformations based on tZ shoot induction promoted a higher number of transgenic shoots, compared to that procedures based on BAP (Tab. 3), it was demonstrated that tZ was associated to the disruption on apical meristem development, leading to the meristem death or precocious conversion into reproductive meristem (Fig. 11B-G). Unexpected changes in meristem development were also verified in transformations based on BAP shoot induction; however, this disruption frequency was reduced. Since CK activates cell-cycle genes and interact with genetic regulators of stem cell number within the shoot apical meristem (SAM), CKs play an important role in SAM formation and maintenance, acting as a positive regulator of cell proliferation (Rupp et al., 1999; Yanai et al., 2005; Gordon et al., 2009). CKs have also been reported in flowering modulation and timing for different species (Bernier et al., 1993; He and Loh, 2002; D'Aloia et al., 2011; Bartrina et al., 2017; Wen et al., 2017). Thus, the higher frequency of early flowering and disruption on stem cells maintenance carrying on meristematic death induced by tZ might be related to the superior perception of this natural CK, in comparison to

BAP (Lomin et al., 2012; Stolz et al., 2011), by the CK signal transduction pathway present in the meristematic cells.

In most of the cases, plants showing meristematic death or differentiation do not develop their axillary buds to recover the vegetative growth and present high mortality frequency (data not shown). Thus, the comparison between transformation efficiencies should be done through evaluation of the number of transgenic presenting *in vitro* usual development and maintenance of vegetative meristem. This profile of transgenic plants is totally able to produce a vigorous plant, be acclimatized and advance generations. According to this approach, transformation procedure based in co-cultivation on NAA-rich medium and BAP shoot induction generates a similar number of transgenic events with usual development compared to transformations based on tZ, regardless of the medium used for co-cultivation (Tab. 4).

Although high transformation efficiencies are relevant to minimize the effort required to produce sufficient numbers of independent transgenic plants, there are many other desirable features of transformation systems. Simplicity and low cost are desirable to allow maximum access to the technology. The protocol suggested here comprehend few and simple steps and requiring fewer media changes. In special, this process is uncoupled of feeder layer, explant pre-culture previous to bacterial inoculation, and auxin supplementation for rooting is not necessary. Besides these advantages, the replacement of the highly expensive tZ by the low-cost combination of NAA and BAP provided the achievement of a similar number of transgenic lines under a reduced budget.

Changes in ploidy level have been described as a negative consequence of transformation and regeneration experiments in tomato (Van Roekel et al. 1993; Joubes and Chevalier 2000; Ellul et al. 2003). Polyploidization induces several disadvantages, such as disrupting effects of nuclear and cell enlargement and the epigenetic instability that results in transgressive gene regulation (Comai, 2005). Analyses of ploidy variation of tomato transgenic plants showed proportion of nuclei corresponding to 2C level ranging from 20 to 72% (Ellul et al., 2003) and from 64 to 79% (Sigareva et al., 2004). Taking this into account, the high proportion of diploid transgenic plants obtained through the most cost-effectiveness protocol suggested here (75%) is a valuable advantage to be considered on the transformation protocol choice.

Despite one key remaining challenge for most crop transformation systems be the genotype dependence (Harwood, 2012), we demonstrated that the cost-effective protocol suggested here is not cultivar specific. Although we had obtained M82 transgenic events, the transformation efficiency achieved was inferior to that observed recently by Gupta and Van Eck

(2016) for the same cultivar. Many factors such as superior bacterial concentration (OD) and tZ concentration in selective regeneration medium can be related to this difference. Apart of the aforementioned, our experiments were able to demonstrate the suitability of our methods for others tomato cultivars, taking in consideration the necessity of small modification as the OD value and CK concentration.

1.5. CONCLUSION

In this work, we evaluated the time taken for tomato shoot induction and acquisition of competence by quantifying organogenesis after transferring explants, respectively, from the shoot-inducing medium (SIM) to the basal medium (BM) and from root-inducing medium (RIM) to the SIM. Additionally, regeneration was improved by incubation of explants onto RIM for the early 48 h, corresponding to the acquisition of competence period, before cytokinin treatment. Our results indicate that RIM pre-incubation in tomato has similar properties of callus-inducing medium (CIM) pre-incubation in Arabidopsis, although bypassing the negative effect of callus formation on the further shoot induction in tomato. We also demonstrated that the fine-tuned auxin-rich medium induces an extensive and intense auxin response, improving the explant competence to produce shoots under posterior cytokinin induction. The coincidence between the minimum incubation period required for gene transfer from Agrobacterium to plant cells (Ziemienowicz, 2013) and for the acquisition of organogenic competence of cells in the explant, both corresponding to the early two days, facilitated the evaluation of NAA pretreatment in transformation parameters. Such analysis allowed the development of a simple, rapid and inexpensive tomato transformation method, a relevant ambition shared by basic science researchers and large-scale biotech crop industry, based on co-cultivation on NAA-rich medium. Tomato regeneration and genetic transformation steps are summarized in Figure 12.

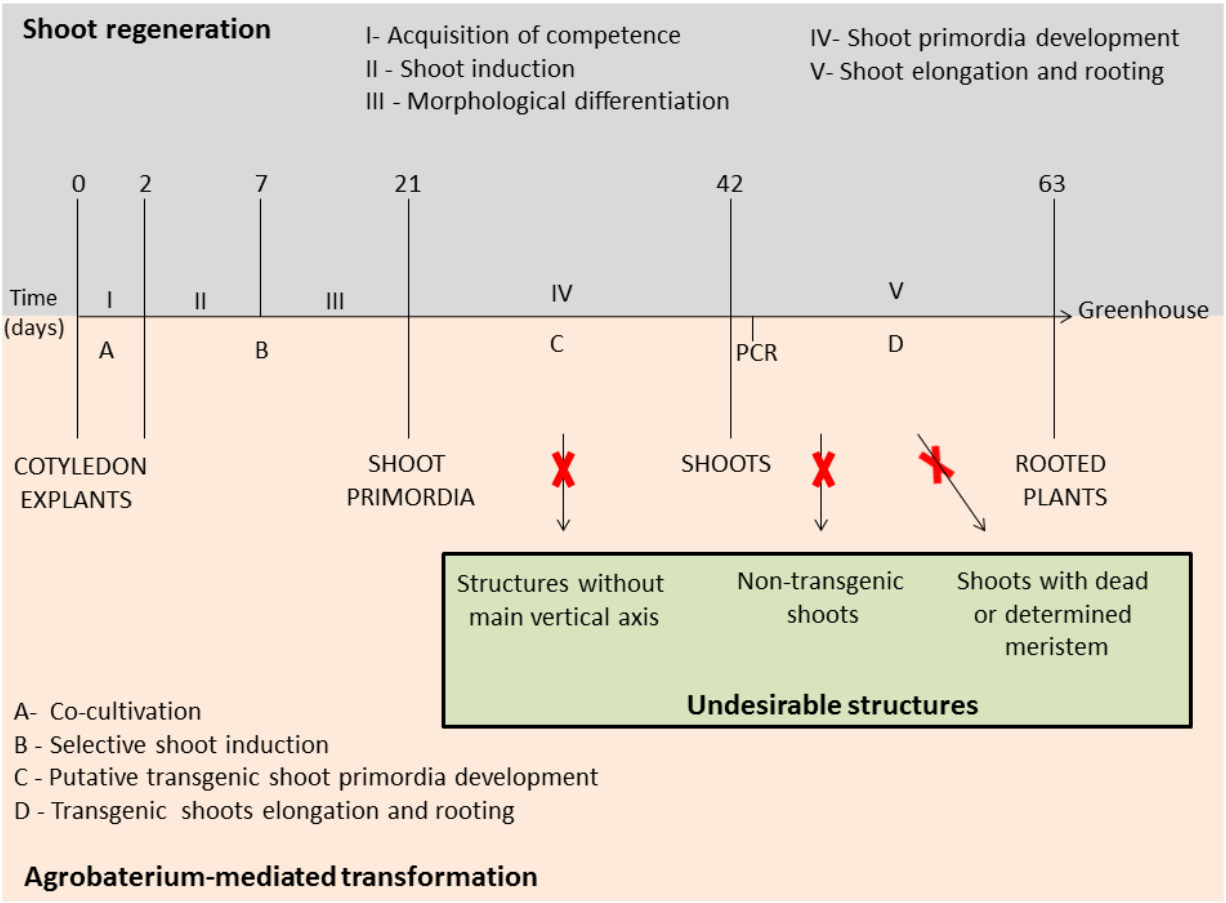


Figure 12. Schematic representation of the optimized shoot organogenesis and Agrobacterium - mediated transformation methodology for *Solanum lycopersicum* cv. Micro-Tom.

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2. THE RECALCITRANCE OF TOMATO (*Solanum lycopersicum* L.) FOR SHOOT REGENERATION FROM ROOT EXPLANTS CAN BE BROKEN BY TEMPORALLY PRECISE PRE-INCUBATION ON CALLUS-INDUCING MEDIUM: EVIDENCES FOR THE AUXIN CONTROL OF ORGANOGENIC COMPETENCE

Abstract

Tomato (*Solanum lycopersicum* L.) is a genetic model system important for both basic and applied research. However, genetic manipulation of cultivated tomato still have some limitations, such as its recalcitrance to regenerate shoots from root explants, a rich source of meristematic tissues. The goal of this study was to investigate this recalcitrance and the way to break it. Root tip explants of the cultivar Micro-Tom (MT) were pre-cultured on callus-inducing medium (CIM) for different intervals (0, 2, 4, 7, 10 and 60 days) before transferring to shoot-inducing medium (SIM). Distinct media compositions were also investigated. Several factors, such as root explant age and region isolated, gas exchange conditions, temperature, and type of cytokinin present on SIM, affected the regeneration frequency and the quality of shoots recovered. However, we demonstrated that the main limiting factor related to shoot regeneration success was a temporally precise pre-incubation on auxin-rich CIM, regardless of the type of cytokinin (BAP or zeatin) supplementation in SIM. Although two days pre-incubation on CIM, previously to SIM, were sufficient to recover shoots from root explants, four days incubation on CIM induced higher percentages of regenerating shoots and number of shoots per explants. Longer incubation periods, which increased callus proliferation, drastically decreased regeneration and pre-incubation on CIM for more than 10 days completed blocked shoot formation when explants are transferred to SIM. Taken together, the results suggest that the 2-4 days pre-incubation on CIM, which spans the period of acquisition of competence for shoot organogenesis in tomato, defines a fine tuned 2,4-D exposition necessary for cell fate reprograming, without inducing detrimental callus proliferation. This knowledge will provide for both the better understanding of the organogenic competence process and for the development of new protocols for tomato biotechnology.

Keywords: *Solanum lycopersicum*; Root explant; Shoot regeneration; Callus induction; Organogenic competence; Auxin

2.1. INTRODUCTION

De novo organogenesis is the process in which adventitious organs regenerate from detached or wounded plant tissues (Duclercq et al., 2011). An extensive investigation concerning this process has been developed over the past decades since the early fundamental discoveries made by Skoog and Miller (1957). The organogenesis can be divided into three main phases: acquisition of competence, organ induction, and morphological differentiation (Christianson and Warnick, 1983). During acquisition of competence, cells develop the ability to assume different developmental fates and to respond to organ induction. Along organ induction, an auxin-to-cytokinin balance favoring cytokinin in the shoot-inducing medium (SIM) directs the cell fate to

shoot formation; while an auxin-to-cytokinin balance favoring auxin induces roots in the auxin-rich root inducing medium (RIM) (Skoog and Miller, 1957). In the third phase, determined cells differentiate and develop visible organs (Christianson and Warnick, 1985; Lombardi-Crestana et al., 2012). Numerous factors including the explant characteristics, culture media composition, and culture conditions vastly affect organ development (Bathia et al, 2004; Sugimoto and Meyerowitz, 2013). Regardless the efforts to standardizing the above-mentioned factors, the unequal response to tissue culture conditions between different species and even among genotypes of a particular species represents one of the main challenges in plant regeneration (Motte et al., 2014) and its application in plant biotechnology.

Most of the knowledge about the molecular control of *in vitro* regeneration was obtained in the genetic model plant *Arabidopsis thaliana* (Motte et al., 2014). In Arabidopsis, the traditional design for *in vitro* organogenesis is based on indirect organogenesis from root explants, which involves a previous treatment on callus-inducing medium (CIM) to form a regenerative mass of cells (callus) (Feldmann & Marks, 1986; Valvekens et al., 1988). In the early CIM incubation, auxin activates four *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*) transcription factors that act downstream to *AUXIN RESPONSE FACTOR 7* (*ARF7*) and *ARF19* to initiate callus formation (Fan et al., 2012). Further, the pluripotent state of callus is established by the root stem cell maintenance regulators *PLETHORA 1* (*PLT1*) and *PLT2* (Kareem et al., 2015). Both *AP2/ERF* transcription factors are regulated by the redundantly acting of *PLT3*, *PLT5*, and *PLT7* (Prasad et al., 2011), establishing pluripotency in the callus derived from periclycle and procambium adult stem cells (Atta et al., 2009; Sugimoto et al., 2010; Duclercq et al., 2011). *PLT3*, *PLT5*, and *PLT7* are rapidly induced upon external auxin application and their expression also induces the NAC family transcription factors *CUP-SHAPED COTYLEDON 1* (*CUC1*) and *CUC2* which have been strongly associated with cellular pluripotency (Cary et al., 2002; Gordon et al., 2007; Kareem et al., 2015). In the end of the CIM incubation, the pluripotent callus has the ability to respond to cytokinin media supplementation that induces the shoot formation (Cary et al., 2002; Che et al., 2007).

Tomato roots were the first organ successfully cultured *in vitro* when White (1934) reported the potentially unlimited growth of excised root tips in renewed fresh media. The stability of these organs *in vitro* coincides with their inability to form shoots, even when transferred to SIM (Peres et al., 2001). Hence, despite the facility for tomato *in vitro* regeneration of shoots from cotyledons, hypocotyls and leaves (Schutze and Wieczorrek, 1987; Duzyaman et al., 1994), its roots, including those from the miniature cultivar Micro-Tom, seem to lack the competence to regenerate shoots (Garcia-Reina and Luque, 1988; Koornneef et al., 1993; Peres et

al., 2001; Lima et al., 2004; Zhang et al., 2012). Conversely, root segments were successfully used as explants for shoot regeneration in tomato wild related species, such as *S. peruvianum*, *S. chilense*, *S. habrochaites*, *S. arcanum*, *S. corneliomulleri*, and *S. huaylasense* (Koornneef et al., 1993; Peres et al., 2001; Trujillo-Moya et al., 2014).

The *S. peruvianum* ability to regenerate shoots from root explants was attributed to two loci, *Rg-1* and *Rg-2* (Koornneef et al., 1987). The *S. peruvianum´*s allele for *Rg-1* was introgressed into a tomato cultivar resulting in the MsK genotype, which is able to regenerate shoots in cultured roots (Koornneef et al., 1993). In a comparative analysis, root explants from the parental *S. peruvianum* and the F1 hybrids between *S. lycopersicum* and *S. peruvianum* showed higher regeneration capacity compared to the cv. MsK (Peres et al., 2001). This data suggest that, in addition to *Rg-1*, other genes may also regulate the shoot regeneration from root explants (Peres et al., 2001).

Moreover, different patterns of shoot morphogenesis from root explants were verified comparing the mentioned genotypes (Peres et al., 2001). *S. peruvianum* and *S. chilense* regenerated shoots without previous callus formation, in a direct process. Conversely, *S. habrochaites*, the progeny between *S. lycopersicum* and *S. peruvianum* and the genotype MsK had a previous callus proliferation, with the size of the callus formed inversed proportional to the capacity to form shoots. All tomato cultivars tested, including MT, only formed non-regenerating large calli, which later became brown (Peres et al., 2001). Histological analysis conducted in MsK roots showed that the initial cell divisions for shoot formation occured in the pericycle of the basal part of the explant after about four days in culture (Koornneef et al., 1993).

In agreement to the described for primary roots, regeneration of transgenic plants from hairy roots induced by *Agrobacterium rhizogenes* has been obtained in *S. peruvianum*, S. *habrochaites* and *S. chilense* (Morgan et al., 1987; Smith et al., 1996; Peres et al., 2001). The data suggest that both primary and hairy roots from same genotypes have similar organogenic capacity. The recovering of transgenic plants from hairy roots was verified in some tomato cultivars (Shahin et al., 1986; Moghaieb et al., 2004). The hairy root syndrome is a plant disease caused by *A. rhizogenes* Conn (Chiton et al., 1982) which have been used in several practical applications, including functional analysis of genes, expression of foreign proteins, and production of secondary metabolites (Hu and Du, 2006). The fast growth associated with the genetic and biosynthetic stabilities during further subculturing and plant regeneration make the hairy root an advantageous system for transgenic plants production (Giri et al., 2000). Tomato transformation has been obtained through the use of cotyledons and other shoot explants and disarmed *A. tumefaciens* harboring binary vectors since Fillati et al. (1987). However, the efficiency of such

procedures is generally low (Chapter 1, Frary and Earle, 1996; Park et al., 2003; Sun et al., 2006). In contrast, inoculation of tomato hypocotyls with *A. rhizogenes* harboring the native pRi plasmid plus binary vectors produce large numbers of transformed hairy root clones (Shahin et al., 1986; Hashimoto et al., 1999; Peres et al., 2001). Thus, the readily recovering of plants from hairy roots makes this system an important advance in transgenic tomato production.

In this report, we investigated the causes of the recalcitrance of cultivate tomato to form shoot from root explants. We used the tomato miniature cultivar Micro-Tom (MT), which has been considered an alternative model plant to study plant development, due to its small size, rapid life cycle, sequenced genome (The Tomato Genome Consortium, 2012) and relatively efficient transformation protocols (Sun et al., 2006; Pino et al., 2010). This cultivar also has a large collection of mutants affecting plant development, including mutation in hormone metabolism and signaling (Carvalho et al., 2011), besides to contain near isogenic lines (NILs) harboring natural allelic variation affecting *in vitro* regeneration capacity (Lombardi-Crestana et al., 2012; Pinto et al., 2017). In such studies, the time for acquisition of competence for shoot regeneration was defined for MT (Cap 1, Pinto et al., 2017) and it was also established that auxin-induced callus proliferation is detrimental for further shoot formation in tomato (Lima et al., 2009). Here, we provide evidences that a fine-tuned auxin application restricted to the period of acquisition of competence allows shoot regeneration from MT root explants. This also paves the way for further applications in tomato genetic engineering.

2.2. MATERIALS AND METHODS

2.2.1 Plant material and culture conditions

Seeds of *Solanum lycopersicum* cv. Micro-Tom (MT) were surface-sterilized by shaking in 20 mL 30% (v/v) commercial bleach (2.7% sodium hypochlorite) added by two drops of commercial detergent for 15 min, followed by three rinses with sterile water. The seeds were germinated in glass flasks with 30 mL of medium containing half strength MS salts (Murashige and Skoog, 1962), half strength B5 vitamins (Gamborg et al., 1968), 15 g L-1 sucrose and 7 g L-1 agar. The media pH was adjusted to 5.8 before autoclaving. Cultures were sealed with polyvinyl chloride plastic (PVC) and incubated at 25 ± 1°C in the dark for four days, followed by transference to long-day conditions (16 h light/ 8 h dark), 45 μmol photons m−2 s−1 PAR irradiance.

2.2.2. Effect of explant type, callus induction and media composition on indirect shoot regeneration

Root tissues originated from different root portions and ages were used as explants. Segments of 1 cm were taken from both the root tip and the root median portion (central region between root tip and hypocotyl) of *in vitro* seedlings eight days after sowing under sterile conditions. Root tip segments of 21 days after sowing *in vitro* seedlings were also tested. Explants were immediately placed on the medium making sure that the entire explant contacts with the surface. To study the effect of callus induction on shoot morphogenesis from root explants, explants were cultured on callus-inducing media (CIM) during different periods of incubation (0, 2, 4, 7, 10 or 60 days), followed by transference to shoot-inducing media (SIM). Combinations of distinct CIM and SIM compositions (culture medium (CM) 1 to 3; Table 1) were also evaluated on indirect shoot induction. The media pH was adjusted to 5.8 before autoclaving. Five replicates of 10 explants per Petri dish (90 × 15 mm) were cultured for each treatment (n=5). Cultured root explants were incubated for 60 days under 16 h photoperiod and 45 μmol m^{-2} s^{-1} irradiance. One subculture was performed at day 30^{th}. Petri dishes were sealed with two layers of Micropore gas-permeable filter tape (3M). After 60 d of culture, the percentage of explants with shoots and the number of shoots per explant were recorded for each treatment.

2.2.3. Temperature and gas exchange conditions

During the two-step shoot organogenesis experiments described above, two temperatures of incubation 21 ± 1 and 25 ± 1 ºC were used separately. Additionally, distinct gas exchange conditions were evaluated during the culture. Petri dishes were sealed with two layers of 3M tape or Parafilm.

Table 1. Composition of culture medium used in in vitro shoot regeneration experiments of *Solanum lycopersicum* cv. Micro-Tom root explants. MS: Murashige and Skoog, 1962; B5: Gamborg et al. 1968; MES: 2-(N -Morpholino) ethanesulfonic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; IBA: Indole-3-butyric acid; BAP: 6-benzylaminopurine; SIM: shoot-inducing medium; CIM: callus-inducing medium.

	Culture medium 1		Culture medium 2		Culture medium 3	
	CIM	SIM	CIM	SIM	CIM	SIM
MS salts	-	1X	1X	1X	1X	1X
B5 salts	1X	-	-	-	-	-
B5 vitamins	1X	1X	1X	1X	1X	1X
Biotin (mg L^{-1})	-	1	-	-	-	-
Sucrose (g L^{-1})	-	10	30	30	30	30
Glucose (g L^{-1})	20	-	-	-	-	-
MES (g L^{-1})	0.5	0.5	-	-	-	-
2,4-D (μM)	9.1	-	1	-	1	-
IBA (μM)	-	2	-	-	-	-
BAP (μM)	-	-	0.5	-	0.5	5
Trans-zeatin (μM)	-	9.1	-	5	-	-
Kinetin (μM)	0.2	-	-	-	-	-
Agar (g L^{-1})	7	-	7	-	7	-
Phytagel (g L^{-1})	-	2.3	-	2.3	-	2.3

2.2.4. Shoot elongation, rooting and acclimatization

After 60 days of inoculation, regenerated shoots of about 1-3 cm were excised from calli clumps and transferred to glass flasks with basal medium (BM) containing MS salts, B5 vitamins, 30 g L^{-1} sucrose and 7 g L^{-1} agar for three weeks. Elongated shoots were induced to form roots on glass flasks containing root inducing medium, composed of BM supplemented with 0.4 μM 1-naphthaleneacetic acid (NAA). After three weeks, plantlets with well-developed roots were transferred to the greenhouse for acclimatization in 150 ml pots with a 1:1 mixture of commercial substrate Basaplant® (Base Agro, Artur Nogueira, SP, Brazil) and expanded vermiculite, supplemented with 1 g NPK 10:10:10 L^{-1} substrate and 4 g L^{-1} dolomite limestone ($MgCO_3$+$CaCO_3$). Acclimatized plants were allowed to self-pollinate, producing fruits. The plant and fruit morphologies and the seed formation were observed.

2.2.5. Statistical analyses

For statistical analysis, each Petri plate (with ten explants each) was considered as a unit. All the data obtained were subjected to analysis of variance (ANOVA) and Tukey's post-hoc test using SISVAR (Ferreira, 2003).

2.3 RESULTS

2.3.1. Tomato root explants are able to regenerate shoots through two-step organogenesis protocol

The current literature shows that cultivated tomato (*Solanum lycopersicum*) has not the ability to regenerate shoots from root explants (Garcia-Reina and Luque, 1988; Koornneef et al., 1993), including the tomato model system Micro-Tom (MT) (Peres et al., 2001; Lima et al., 2004). This organogenic capacity was described to be restricted to wild related species, such as *Solanum peruvianum*, *S. chilense* and *S. hirsutum* (Koornneef et al., 1993; Peres et al., 2001; Trujillo-Moya et al., 2014). Interestingly, none of the reports that failed in regenerate shoots from tomato root tissues attempted to use the efficient shoot regeneration method described for *Arabidopsis thaliana* roots (Valvekens et al., 1988). Thus, MT root explants were submitted to the shoot regeneration protocol described for Arabidopsis root explants.

In the most widely used *A. thaliana* two-step regeneration procedure (Feldmann and Marks, 1986; Valvekens et al., 1988), root tip explants are first incubated on an auxin-rich callus-inducing medium (CIM) and subsequently transferred to a cytokinin-rich shoot-inducing medium (SIM). As a first attempt, named culture medium 1 (CM 1), MT root tip explants were pre-incubated on CIM for different intervals and then transferred to SIM, applying the culture media composition described for Arabidopsis (Table 1; Sugimoto and Meyerowitz, 2013). Evaluation of regeneration parameters after 60 days in culture showed that explants direct cultivated on SIM, without pre-culture on CIM (0 days), induced shoots only in 8% of explants (Fig. 1A). The figure 1B illustrated that explants incubated directly on SIM developed a low-pronounced callus along the original root tissue, varying the color from yellow/green to brown (oxidized). In this treatment presenting a low percentage of regeneration, the shoots formed strictly at the meristematic end of the explant (Fig. 1B-E). Four days CIM pre-treatment, previously to SIM incubation, highly improved shoot regeneration efficiency, showing almost 60% of explants regenerating (Fig. 1A). Tip roots pre-incubated on CIM during the early four and seven days exhibited well-developed compact green calli and shoot emergency was observed along all

explant area (Fig. 1F-M). Longer CIM exposition critically reduced the explant organogenic capacity and total loss of morphogenetic potential was verified after 10 days of CIM incubation (Figure 1A, N-Q).

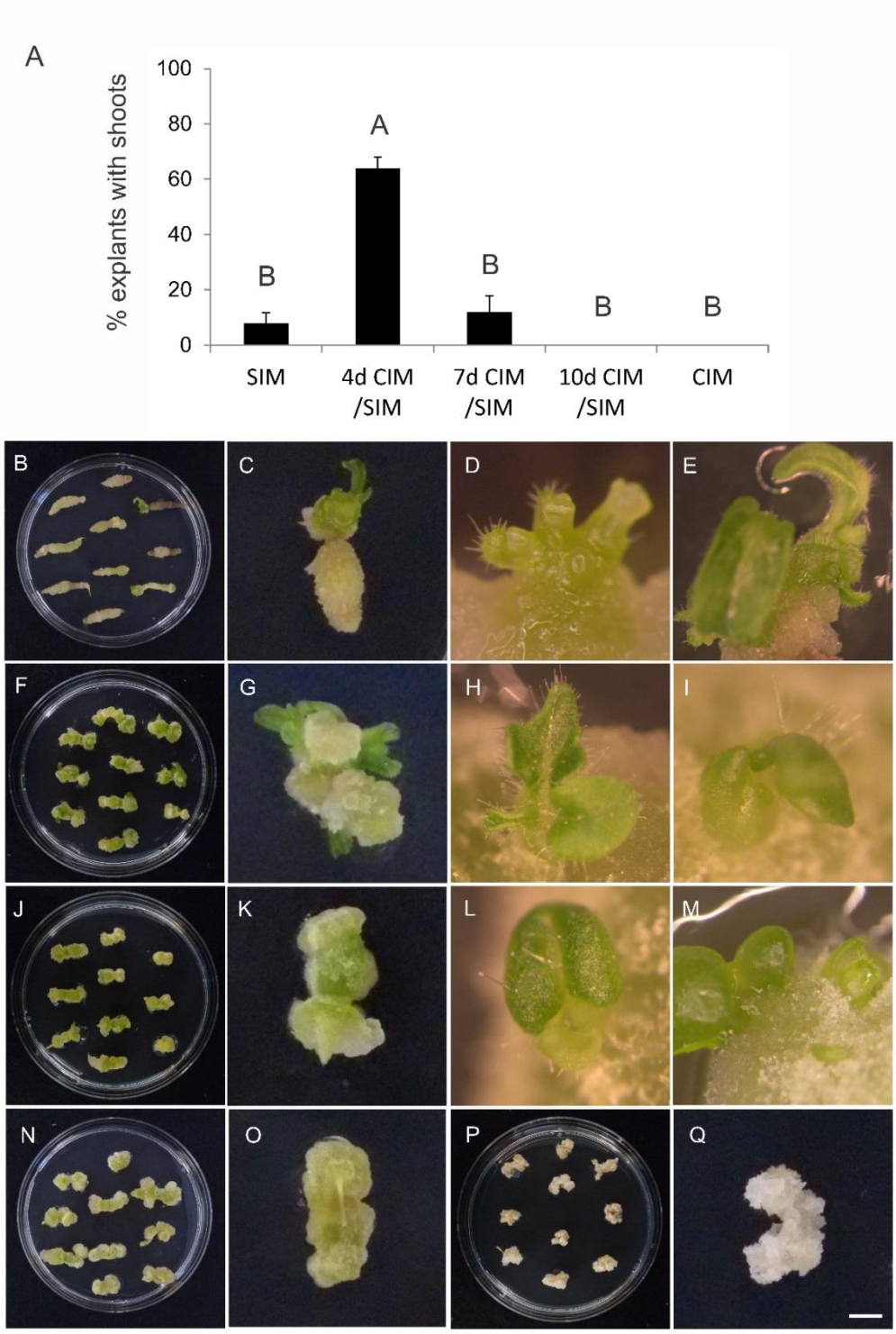

Figure 1. *In vitro* shoot regeneration from tomato (cv. Micro-Tom) root tip explants incubated on the culture medium 1 (see Table 1). Explants were isolated from 8-days old (after sowing) seedlings and cultured for 0, 4, 7, 10 and 60 days on CIM, follow by SIM. (A) Percentage of explants producing shoots at 60 days after explant inoculation is shown. Vertical bars indicate ± standard error of the mean (n = 5 Petri dishes with 10 explants each). Different letters indicate significant differences at $P \leq 0.05$ (Tukey's test). (B-Q) Morphology of explants pre-incubated on CIM for 0 (B-E), 4 (F-I), 7 (J-M), 10 (N, O) and 60 (P, Q) days, followed by SIM, at 60 days after explant inoculation. Bar= 1 cm (B, F, J, N, P), 5 mm (C-E, G-I, K-M, O, Q).

MT shoot regeneration from root explants was achieved using Arabidopsis medium (CM 1). Thus, we decided to test the regeneration capacity using CIM and SIMII described for MT regeneration from cotyledon explants assays (Pino et al., 2010; Lombardi-Crestana et al., 2012), which we named as culture medium 2 (CM 2; Tab. 1). Shoot regeneration was observed in explants cultivated on CIM for four, seven and 10 days before transference to SIMII (Fig. 2). Under this method, root tip explants developed in green and yellow organogenic calli formed along the explant (Fig. 2B-M). Well-developed shoots, with usual meristem and stem formation, arose from these calli (Fig. 3B-M). Higher regeneration capacity was obtained in explants cultivated for four days on CIM, decreasing in longer pre-incubation periods (Fig. 2A). The four days on CIM followed by SIMII induced shoot recovering in 67% of explants, a regeneration frequency superior to the 60% verified for the traditional tomato regeneration system based on cotyledon explants (Chapter 1). Explants directly cultured on SIMII showed reduced callogenesis, presenting posterior tissue oxidation (noticed by brown color) and absence of shoot recover (Fig. 2A; Fig. 2N-O), as previously described (Koornneef et al., 1993; Lima et al., 2004). Explants maintained exclusively on CIM developed into a white and friable mass of non-organogenic calli, regardless of the culture medium used (Fig. 1P-Q; Fig. 2P-Q).

Despite four days incubation on CIM induced similar organogenesis efficiencies under the CM 1 and 2 (Fig. 1A; 2A), shoots produced through the system based on Arabidopsis medium (CM 1; Fig. 1) presented distinct morphological characteristics from those induced by MT medium (CM 2; Fig. 2). Most of shoots originated from CM 1 had thicker and less developed leaves, abnormal shoot apical meristem and rarely developed stems (Fig. 1). Conversely, shoots induced by CM 2 presented usual morphological phenotype (Fig. 2) similar to the structures originated from cotyledon explants (Chapter 1; Lombardi-Crestana et al., 2012).

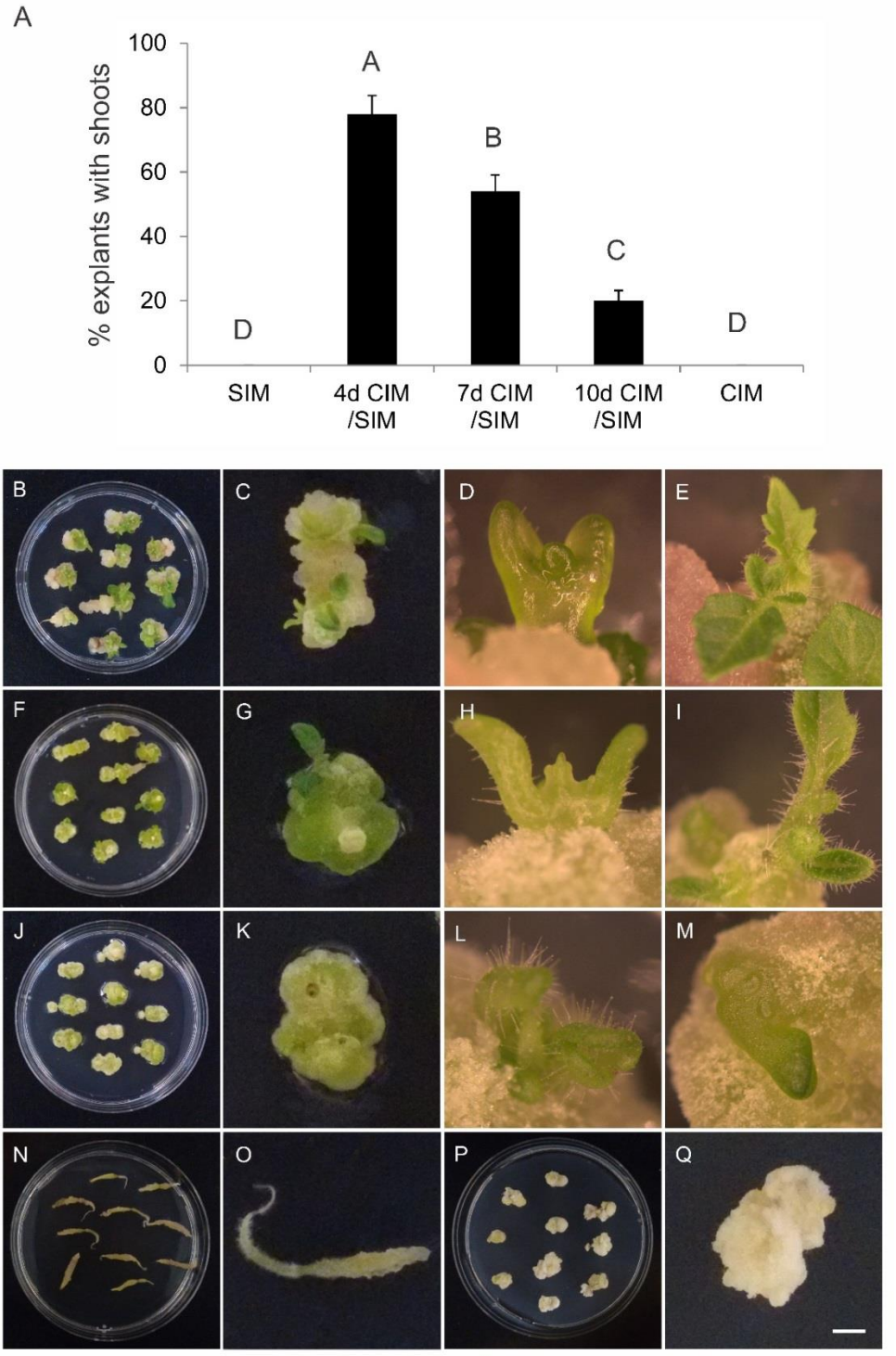

Figure 2. *In vitro* regeneration of tomato (cv. Micro-Tom) root tip explants incubated on the culture medium 2. Explants were isolated from 8-days old (after sowing) seedlings and cultured for 0, 4, 7, 10 and 60 days on CIM, follow by SIM. (A) Percentage of explants producing shoots at 60 days after explant inoculation is shown. Vertical bars indicate ± standard error of the mean (n = 5 Petri dishes with 10 explants each). Different letters indicate significant differences at $P \leq 0.05$ (Tukey's test). (B-M) Morphology of explants pre-incubated on CIM for 4 (B-E), 7 (F-I) and 10 days (J-M) followed by SIM, at 60 days after explant inoculation. Explants cultured exclusively on SIM (N, O) and CIM (P, Q) were also showed. Bar= 1 cm (B, F, J, N,P), 5 mm (C-E, G-I, K-M, O, Q).

2.3.2. Shoot regeneration from tomato root explants is not zeatin-dependent

For both CM tested (1 and 2) that successfully induced shoot organogenesis from tomato root explants, the cytokinin supplemented in SIM were trans-zeatin (tZ). Additionally, it was determined that the acquisition of competence for shoot organogenesis in cotyledon explants correspond to the first two days in culture (Chapter 1; Pinto et al., 2017) and that the regeneration efficiency is improved by RIM pre-incubation during this period and by posterior use of BAP in SIM (Chapter 1; Pino et al., 2010). Then, an investigation of shoot induction efficiency by using distinct cytokinin supplementation on SIM (tZ in CM 2 and BAP in CM 3, Tab. 1), was conducted.

Table 2 showed that CM 3, applying CIM pre-treatment and BAP supplementation in SIM, was effective in shoot induction from root explants. Although BAP was less efficient in the induction of shoot regeneration compared to tZ (CM 2), pre-incubation on CIM for four days followed by SIM presented higher percentage of explants with shoots and number of shoots per explant for both CM (Tab. 2). Under the CM 2, CIM pre-incubation for two and four days induced similar regeneration parameters. However, applying BAP shoot induction (CM 3), the organogenic capacity of explants cultured for four days on CIM was 60% superior to the shorter incubation (two days). Longer CIM pre-treatments decreased shoot organogenesis significantly, showing similar regeneration parameters regardless of the cytokinin used in SIM (CM 1 and 2). These results suggest that acquisition of competence for shoot organogenesis in root explants comprehend the initial four days of culture, a longer period than verified to cotyledon explants (Chapter 1; Pinto et al., 2017).

Root tip explants submitted to CM 3 under different CIM pre-incubation (two, four, seven and 10 days), followed by SIM until complete 60 days in culture, developed in a green and yellow prominent callus (Fig. 3A-E), similar to those described for CM 2 (Fig. 2). Several shoots were formed on short and compact callus (Fig 3B-C). Conversely, explants showing elongated shapes did not regenerate shoots or developed only a single shoot (Fig 3D-E). For both explant phenotypes, well-formed shoots with a regular main axis structure raised from the calli (Fig. 3F-G).

Table 2. Effect of different cytokinins on shoot organogenesis from root tip explants. Explants were isolated from 8-days old (after sowing) seedlings and cultured on CIM (0, 2, 4, 7, 10 and 60 d) before transferred to SIM supplemented by 5 μM trans-zeatin (Culture medium 2) or 5 μM BAP (Culture medium 3). At 60th day in culture, the percentage of explants producing shoots and the mean number of shoots per explant (± SE) were recorded (n = 5 Petri dishes with 10 explants each). Different letters indicate significant differences at $P \leq 0.05$ (Tukey's test).

	Culture medium 2		Culture medium 3	
Days on CIM	% explants with shoots	Mean nº of shoots per explant	% explants with shoots	Mean nº of shoots per explant
0	0 C	0.0 ± 0.0 B	0 C	0.0 ± 0.0 C
2	63 A	1.2 ± 0.2 A	30 AB	1.1 ± 0.5 AB
4	68 A	1.2 ± 0.3 A	50 A	1.6 ± 0.2 A
7	30 AB	0.7 ± 0.2 AB	33 AB	0.5 ± 0.1 BC
10	5 BC	0.1 ± 0.1 B	8 BC	0.2 ± 0.1 BC
60	0 C	0.0 ± 0.0 B	0 C	0.0 ± 0.0 C

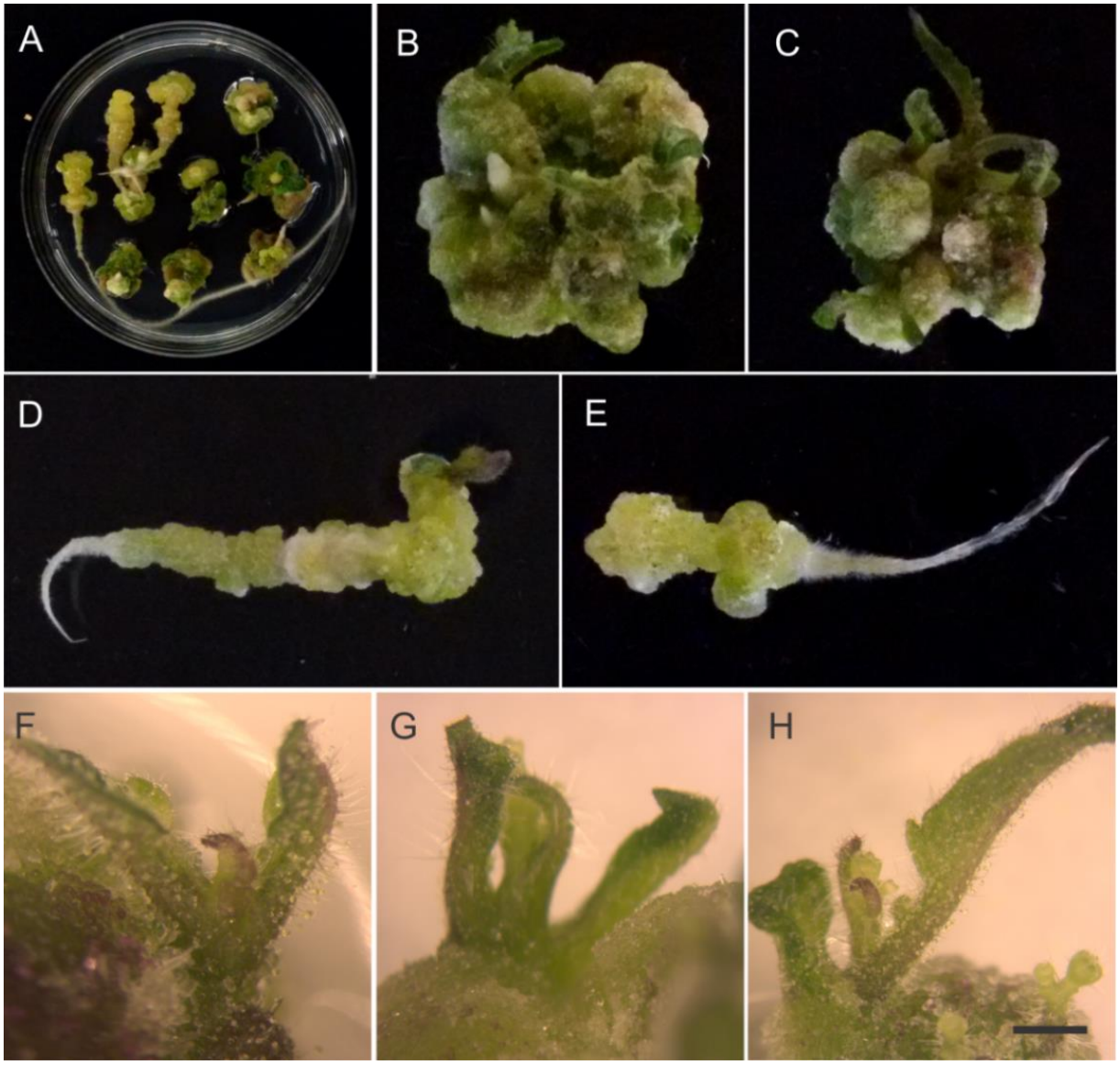

Figure 3. *In vitro* regeneration of root tip explants incubated on culture medium 3. Explants were isolated from 8-days old (after sowing) seedlings and pre-incubated on CIM for 4 days, followed by SIM. Petri dishes (A), root explants individualized (B - E) and details of shoots regenerated (F - H) at 60 days after explant inoculation are shown. Bar= 2 cm (A), 5mm (B - E), 2mm (F - H).

2.3.3. Root explant sections and age affect shoot organogenesis

The influence of explant on the growth and development of shoots depends on several factors, including the tissue isolated, the age and the size of the explant (Bathia et al., 2004). In Arabidopsis, explants isolated from apical side of the roots (the tip part) have superior capacity to regenerate shoots than those derived from basal side of the root (proximal to the hypocotyl) (Sugimoto and Meyerowitz, 2013). These authors also described that root explants 3–10 days after germination are in the optimal stage to make explants for efficiently yielding shoots.

However, no systematic evaluation of the effect of explant characteristics in the regeneration capacity was conducted in previous publications involving *Solanum* root explants (Koornneef et al., 1993; Peres et al., 2001; Lima et al., 2004; Trujillo-Moya et al., 2014). Explants originated from different ages (8- and 21- days after sowing) and root segments (tips, medium sections or a mix of different sections) were employed on these works. Based on this, we investigated the effect of root explant variations in tomato organogenic capacity induced by the most effective shoot regeneration system, the CM 2 applying four days CIM pre-incubation.

The effect of different root sections (tip including meristematic zone, and median region between tip and hypocotyl) from 8-days old (after sowing) seedlings were compared using the two-step tissue culture method described above. The regeneration parameters, including the percentage of explants developing shoots and the number of shoots formed per explant, were similar in explants originated from root tip and median section of the root (Tab. 3). Although the similar organogenic capacity, explant originated from different root tissues developed in distinct morphologies at 60 days in culture (Fig. 4A-H). Explants derived from root tips (Fig. 4A-D) were shorter and showed more pronounced callogenesis in comparison to those originated from median portions (Fig. 4E-H). Shoots developed from both types of explants had usual morphology and development (Fig. 4C-D; G-H). The organogenesis process was completely abolished in explants from 21-days old seedlings (Tab. 3). Under this condition, explants only differentiated into friable white calli and no shoot regenerated was observed (Fig. 4I-J).

Table 3. Effect of explant tissue source and seedling age on shoot organogenesis of root explants incubated on culture medium 2. Root tip and median section were isolated from 8- and 21-days (after sowing) seedlings. Explants were cultured on CIM for four days, followed by SIM. After 60 days in culture, the percentage of explants producing shoots and mean number of shoots per explant (± SE) are shown (n = 5 Petri dishes with 10 explants each). Different letters indicate significant differences at $P \leq 0.05$ (Tukey's test).

Explant characteristics		Regeneration parameters	
Explant type	Age (days)	% explants with shoots	Mean nº of shoots per explant
Root tip	8	35 A	0.8 ± 0.2 A
Median section	8	33 A	0.6 ± 0.1 A
Root tip	21	0 B	0.0 ± 0.0 B

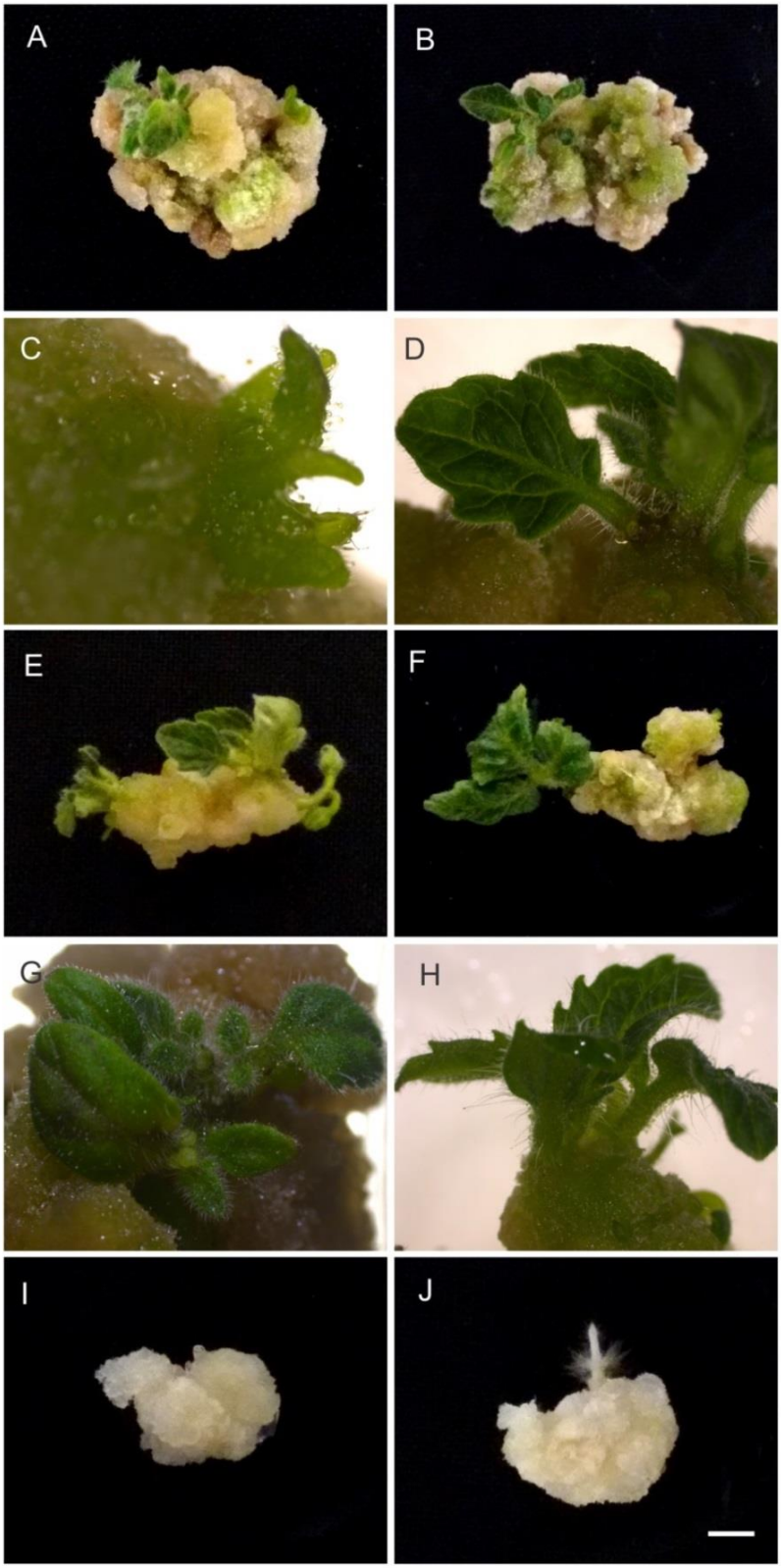

Figure 4. *In vitro* regeneration of different root explants pre-incubated on CIM for four days, followed by SIM, according to the culture medium 2. Explants consisted of root tip (A-D) and medium section (E-H) of seedlings eight days after sowing, and root tip of 21 days (after sowing) seedlings (I-J). Bar= 1 cm (A-B, E-F, I-J), 2mm (C-D, G-H).

2.3.4. The environment of culture affects regeneration frequency and shoot morphology

Additionally to the media composition and explant related factors, the environment of culture can also affect the success of *in vitro* regeneration (Bathia et al., 2004). The requirement of sterile conditions and avoidance of dehydration impose the necessity of sealed containers for plant tissue culture. Since all previous assays were successfully conducted in plates sealed with a gas-permeable tape (3M), the influence of gas exchange on organogenesis efficiency was evaluated culturing the same type of explant (root tip isolated from 8-days old seedlings) in plates sealed with both the non-gas permeable Parafilm and the gas permeable 3M tape. The material used for sealing the Petri dishes, and consequently the gas-exchange conditions, did not affect the regeneration capacity of root explants (Tab. 4). Cultures sealed by Parafilm and 3M tapes generated 35% of explants recovering shoots each and similar number of shoots per explant (Tab. 4). However, plates sealed with Parafilm developed shoots with morphological anomalies, such as the hyperhydricity, succulence and glassiness (Fig. 5).

Table 4. Effect of gas-exchange conditions imposed by different sealing materials on shoot organogenesis of root explants incubated on culture medium 2. Root tip explants were isolated from seedlings 8-days (after sowing) and cultured on CIM for four days, followed by SIM. Plates were sealed with gas-permeable 3M tape and non-gas-permeable Parafilm. After 60 days in culture, the percentage of explants producing shoots and mean number of shoots per explant (± SE) were recorded (n = 5 Petri dishes with 10 explants each). Different letters indicate significant differences at $P \leq 0.05$ (Tukey's test).

Sealing material	% explants with shoots	Mean nº of shoots per explant
3M tape	35 A	0.8 ± 0.2 A
Parafilm	35 A	0.4 ± 0.1 A

It is relevant to emphasize that shoot organogenesis parameters obtained in sections 3.1 and 3.2 (Fig. 1A; 2A; Tab.2) were superior to those reached in sections 3.3 and 3.4 (Tab. 3). This is probably because experiments described in the sections 3.1 and 3.2 were incubated in growth room maintained at 22 ± 1°C, while those related to items 3.3 and 3.4 were conducted at 25 ± 1°C. Under warmer conditions, explants went into a stressful environment, developing tissue browning and presenting oxidation (Fig. 4; 5). The data confirm that temperature in culture room is also important for successful shoot regeneration from tomato root explants.

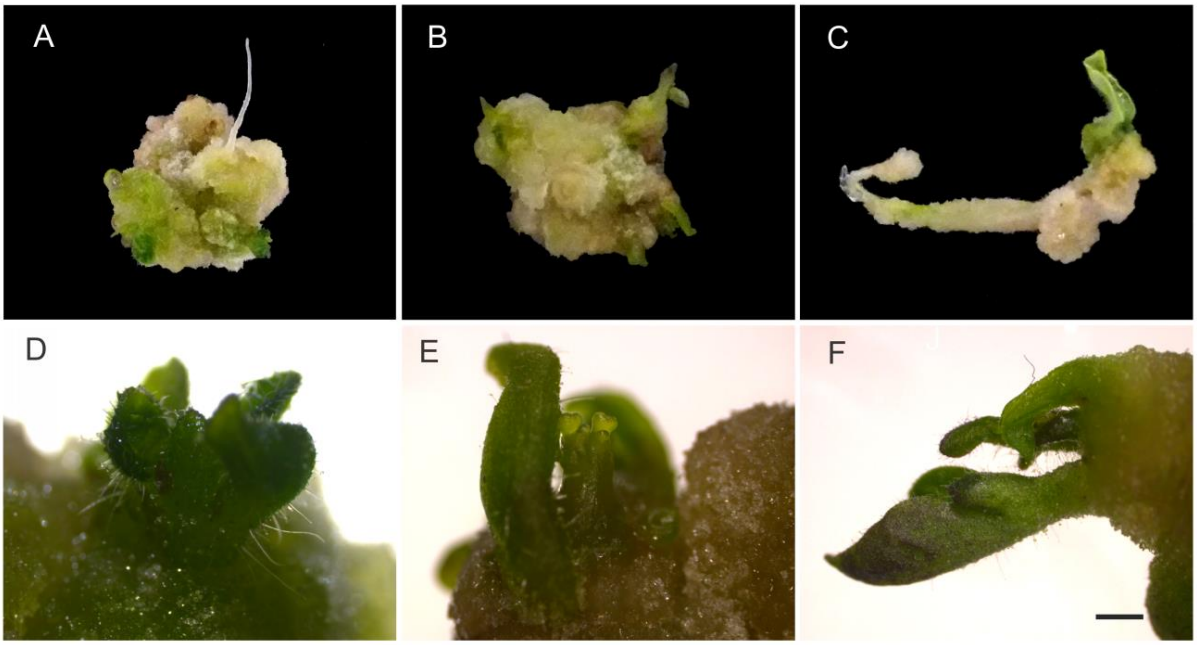

Figure 5. Morphological aspects of the *in vitro* regeneration of root tip explants pre-incubated on CIM for four days, followed by SIM, according to the culture medium 2. Explants were isolated from 8-days old (after sowing) seedlings and cultured in Petri dishes sealed with Parafilm. Root explants individualized (A-C) and details of hyperhydric (D-E) and glassy (F) shoots regenerated at 60 days after explant inoculation are shown. Bar= 1 cm (A-C), 2mm (D-F).

2.3.5. Acclimatization and *ex vitro* phenotype

Shoots regenerated from root explants were isolated from callus tissue and maintained on basal medium for development and elongation. Root formation were induced by incubation on RIM. Plants rooted *in vitro* (Fig. 6A) were acclimatized and transferred to the greenhouse. After 60 days, root-originated plants developed usual morphology and fruit development (Fig. 6B). All fruits collected exhibited seeds (Fig. 6C).

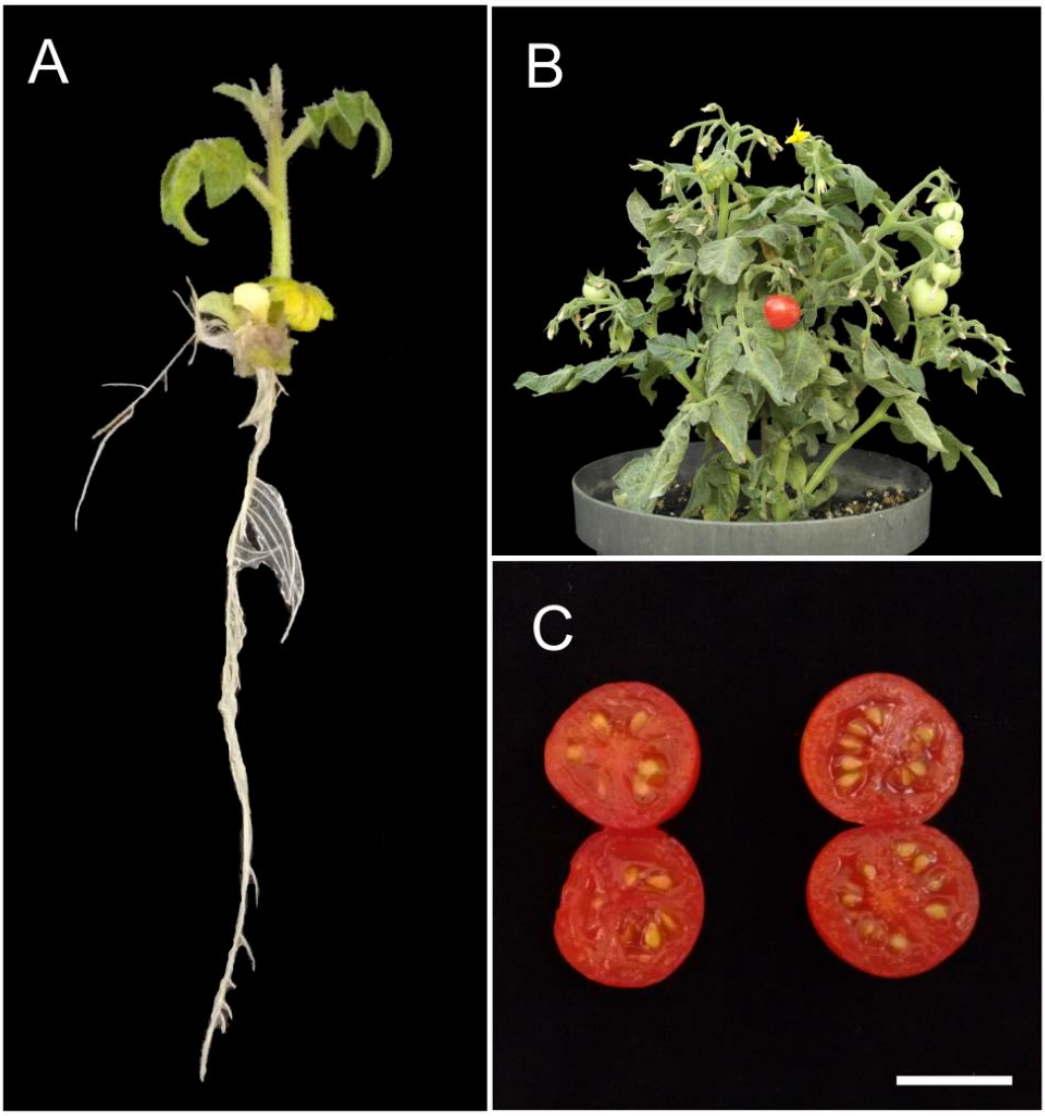

Figure 6. Morphology of *Solanum lycopersicum* cv. Micro-Tom plants (A-B) and fruits (C) originated from root explants. (A) *In vitro* rooted plant; (B) *ex vitro* plant and its fruits (C) at 60 days after acclimatization. Bars= 2 cm (A and C), 5 cm (B).

2.4. DISCUSSION

2.4.1. The cultivated tomato shoot regeneration capacity is influenced, but not dependent, on type of explant and culture conditions

In this work, we show that explant characteristics and culture conditions influence the organogenesis capacity and the morphology of regenerated shoots (Tab. 3, Fig. 4; 5). The optimization of these factors might be responsible for our success in regenerate shoots from cultivated tomato root explants. According to Bathia et al. (2004), age is one of the main factors that influence the explant regeneration ability. Young and soft tissues are generally more competent to regenerate new organs than old tissues. In fact, the tissue recalcitrance, which

results in non-responsive explants, has higher occurrence with the advance in the age of explants (Brar and Jain, 1998). Previous investigations showed a decrease in regeneration capacity with the increase of cotyledon explants age in MT (Pino et al., 2010), but no similar investigative study was conducted using root explants until this report. We also showed that explant age dramatically reduce the regenerative capacity of tomato root tip tissues. Root tip explants from old plants (21 days after sowing) exclusively developed in friable callus unable to regenerate shoots (Tab. 3; Fig. 4I-J). The plant regenerative capacity declines with age, due to the age-related microRNA, miR156, which regulates shoot regenerative capacity (Zhang et al., 2015). As a plant ages, the gradual increase in miR156-targeted SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors leads to the progressive decline in shoot regenerative capacity. In old plants, SPL reduces shoot regenerative capacity by attenuating the cytokinin response through binding with the B-type ARABIDOPSIS RESPONSE REGULATORs, which encode the transcriptional activators in the cytokinin signaling pathway (Zhang et al., 2015).

The root section isolated as explants had a little influence in the regeneration (Tab. 3, Fig. 4A-H). In Arabidopsis, which has root as the most usual explant source (Valvekens et al., 1988), tissues derived from root apical region (the tip part) form callus, and later develop shoots, more efficiently than those derived from the basal (hypocotyl) region of the root (Sugimoto and Meyerowitz, 2013). Surprisingly, our data indicate that the root region used as explant did not influence shoot recovery in tomato (Tab. 3). Although root tip tissues contain the primary root meristem plenty of embryonic meristematic cells, differentiated root regions maintained adult stem cells confined at the pericycle and procambium (Atta et al., 2009; Sugimoto et al., 2010). However, the dependence on auxin pre-incubation (*e.g* the 2,4D present in CIM) for tomato shoot regeneration from root explants suggests that the limiting factor is not necessarily the preexistence of meristematic tissues but the presence of cell able to be reprogramed for further shoot regeneration. Such cells are likely to be present in both kinds of tomato root regions from 8-days old plants but absent in root tips from 21-days old plants (Tab. 3).

In addition to the explant related factors, the success in tomato regeneration is known to be related to other culture conditions, such as the physical factors (gas exchange and temperature) and plant growth regulators supplemented on medium culture (Bathia et al., 2004). The requirement for both sterility and avoidance of dehydration in plant tissue cultures impose sealing requirements that result in severe limitation for the diffusive flux of gases, such as ethylene, O_2 and CO_2 (Jackson, 2003). Among them, ethylene stands out due to its influence in developmental processes (Jackson, 2003) and its relation with auxin-induced callus formation in tomato (Lima et al., 2009). Furthermore, the absence of gas exchange in and out of Petri dishes

induces explant morphological anomalies due to excessive humidity in culture, such as the hyperhydricity, translucency, succulence and glassiness (Ziv, 1991; Hazarika, 2006). Gas permeable tapes have been successfully used in Arabidopsis organogenesis protocols (Valvekens et al., 1988; Sugimoto and Meyerowitz, 2013), preventing the accumulation of moisture and ethylene inside the Petri dish (Lindsey and Wei, 2000). In our study, root tissues were cultured in Petri dishes sealed with gas permeable tape (3M) and Parafilm, which does not allow gas exchange. Both conditions allowed the formation of the same percentage of regenerating explants and similar number of shoots per explant. However, a decrease in the quality of shoots formed was described in explants from the plates wrapped with Parafilm (Fig. 5).

Besides the sealing requirements, the temperature normally adopted for *in vitro* culture of *Solanum* (section *Lycopersicon*) species, which ranges from 19º to 25ºC (Gubis et al., 2004; Lima et al., 2004; Steinitz et al., 2006; Cruz-Mendivil et al., 2011), seems to affect the regeneration process (Yildiz, 2012). According to Reynolds et al. (1982), regeneration potential of tomato stem explants reduced from cooler (19ºC) to warmer temperatures (28ºC). The adoption of distinct temperature of incubation in our assays, due to distinct growth room used, showed the influence of temperature in the regeneration success. Under 25ºC, shoot recovering frequency decreased considerably in comparison with cultures incubated at 22ºC. Additionally, the explants developed tissue browning and oxidation under the warmer temperature (Fig. 4; 5).

Among plant growth regulators affecting morphogenic responses, a wide variety of cytokinins has been used either separately or in combination with auxins to induce tomato shoot organogenesis (Bathia et al., 2004). These authors also concluded that zeatin and 6-benzylaminopurine (BAP) have been the most used cytokinins when aiming tomato adventitious shoot regeneration. A comparison between trans-zeatin (Culture medium 2; Tab. 1) and BAP (Culture medium 3; Tab. 1) shoot induction capacity from root explants was also conducted. We showed that although both cytokinins triggered shoot regeneration, incubation on shoot-inducing medium (SIM) supplemented with trans-zeatin resulted in a higher regeneration frequency compared to BAP (Tab. 2). Most of regeneration studies have shown that a higher regeneration response in tomato is achieved with zeatin (or its derivatives) in comparison with BAP, regardless of the explant used (Ichimura and Oda, 1995; Gubis et al., 2004; Pino et al., 2010). The superiority of trans-zeatin could be explained by its natural occurrence, whereas BAP is a synthetic molecule (Cruz-Mendivil et al., 2011). Furthermore, Arabidopsis cytokinin receptors Histidine Kinase2 (AHK2), AHK3 and AHK4 have a higher affinity for trans-zeatin than BAP (Stolz et al., 2011; Lomin et al., 2012).

In summary, the failure in regenerating shoots from cultivated tomato root explants described in the literature (Garcia-Reina and Luque, 1988; Koornneef et al., 1993; Peres et al., 2001; Lima et al., 2004; Zhang et al., 2012; Trujillo-Moya et al., 2014) seems not to be related with known limiting factors in plant tissue culture, such as culture conditions and plant growth regulators. Hence, most of these factors were taken into account in the mentioned studies and, as shown above, although they influenced shoot regeneration, their variation do not preclude shoot formation on root explants. Indeed, the limiting factor for shoot formation from tomato root explants seems to be the requirement of pre-incubation on callus-inducing medium (CIM), as discussed below.

2.4.2. The fine-tuning between acquisition of competence and minimum callus proliferation is probably the key factor for shoot regeneration from root explants in cultivated tomato

A wide range of types and concentrations of cytokinins in combination with auxins are used to induce callus (Bhatia et al., 2004; Shah et al., 2015). Generally speaking, an intermediate ratio between auxin and cytokinin promotes callus induction (Skoog and Miller, 1957). These two plant growth regulators are thought to induce the reprogramming of somatic cells from a previous determined state to a dedifferentiated stated able to assume a new developmental pathway (Gaba, 2004). An investigation on the effect of different concentrations of the auxins 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) and the cytokinin BAP on callus induction in Micro-Tom cotyledons pointed that 1.0 μM 2,4D in combination with 0.5 μM BAP comprehend the optimal medium composition to induce callus in this genotype (data not published).

Although 2,4-D is not a naturally occurring auxin, it has been described as the most effective auxin for callus induction (Valvekens et al., 1988; Sugimoto et al., 2010; He et al., 2011). The efficient 2,4-D differs from other common auxins, such as NAA and IAA, due to its nature, chemical structure, metabolism and transport characteristics (Ender and Straders, 2015). It is suggested that the interaction of 2,4-D with particular auxin receptors differs from other auxins (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). In addition, active influx carriers mediate a strong 2,4-D import (Enders and Strader, 2015). Once 2,4-D has entered the cell, the molecule is hardly metabolized due to its artificial chlorinated nature (Gaba, 2004). Consequently, relatively high concentration of 2,4-D accumulate inside the explant cells since there is no carrier system to facilitate its efflux (Petersen et al., 2016). In this way, explant incubation on medium

supplemented with 2,4-D probably ensures the establishment of the endogenous auxin maxima necessary to initiate cell proliferation and pluripotency (Atta et al., 2009).

Tomato explants are usually directly cultured on SIM aiming the induction of callus formation and shoot regeneration (Bhatia et al., 2004; Zhang et al., 2012; Khaliluev et al., 2014). This method of culture is applied due to the recalcitrance of cultivated tomato shoot-derived explants (cotyledon, hypocotyl, and leaf) in regenerate shoot from callus induced by CIM (Chapter 1; Lombardi-Crestana et al., 2012; Shah et al., 2015). No evidence of CIM pre-treatment previous to SIM aiming shoot induction from root explants was verified in the literature until this work. This absence of information could be associated with the ability of tomato wild related species in regenerate shoots from root explants directly cultured on zeatin-rich medium (Koornneef et al., 1993; Peres et al., 2001; Trujillo-Moya et al., 2014).

During *de novo* organogenesis process, shoot induction is a cytokinin-dependent step while the acquisition of competence phase occurs under different media incubation (Christianson and Warnick, 1985; Lombardi-Crestana et al., 2012). Thus, the time at which CIM or RIM can substitute SIM without decreases the process efficiency and do not induce roots (in the case of RIM pre-incubation) is the time at which the tissue achieves shoot competence (Pinto et al., 2017). The acquisition of competence seems to be triggered by auxin maxima and the molecular events related to this process have been elucidated in the last years mostly based in studies with the model plant *Arabidopsis thaliana* (Kareem et al., 2016; Ikeuchi et al., 2016).

Some shoot regeneration in tomato root explants cultured on SIM, without previous CIM pre-incubation, was reported in our study exclusively in explants incubated to culture medium 1 (CM1) (Fig. 1A). This shoot regeneration, although low, might be related to the combination of high zeatin and low indole-3-butyric acid (IBA) concentrations on SIM described in CM1 (Tab. 1). This plant growth regulator proportion has been described to induce both callus formation and shoot regeneration in tomato shoot-derived explants (Bathia et al., 2004). Conversely, direct incubation on SIM contained exclusively the cytokinin zeatin (CM2) or BAP (CM3) in lower concentration than CM1 (Tab 1), was not able to induce shoots in cultivated tomato roots (Fig. 2A, 3A). Under this condition, the non-regenerating explant only developed friable calli (Figure 2N-O, 3N-O) that ultimately oxidized and died, a similar pattern to that described by Peres et al. (2001). This developmental pattern indicates that cell proliferation for callus formation is uncoupled to acquisition of competence for shoot regeneration. Following CM2 and CM3 regeneration systems, at least two days CIM pre-treatment is required to induce shoots from root explants (Tab. 2). Explants cultured on CM2 and CM3 achieved optimal regeneration using four days CIM incubation followed by a further SIM culture (Tab. 2). However, incubation in the

auxin-rich CIM longer than four days substantially decreased the regeneration capacity (Tab. 2). Hence, CIM incubation longer than 10 days completely blocked shoot formation in the subsequent transference to SIM. These data suggest that four days incubation on CIM is the time required to reach competence for shoot organogenesis in tomato root explants. In addition, the acquisition of competence was strongly linked to the hormone auxin.

An auxin-rich root-inducing medium (RIM) pre-incubation during the acquisition of competence phase, although not an essential procedure, also improves the shoot regeneration in Micro-Tom cotyledon explants (Chapter 1; Pino et al., 2010). Similarly to tomato, Arabidopsis root explants seems to require at least two days of CIM incubation to gain competence to produce shoots (Che et al., 2007; Motte et al., 2011), showing optimal response under four days of CIM incubation (Valvekens et al., 1988). After seven days of treatment, the regeneration efficiency significantly decreases, and after 14 days shoot regeneration no longer occurs (Valvekens et al., 1988). Arabidopsis shoot organogenesis from root explants can be directly achieved by SIM incubation, but incubation on auxin-rich CIM for the early four days is a widely applied strategy to improve shoot regeneration in this species (Valvekens et al., 1988; Sugimoto and Meyerowitz, 2013).

It is interesting to note that the four days CIM pre-incubation required for optimum shoot formation from tomato root explants is longer than the two days described for acquisition of competence in tomato cotyledon explants (Chapter 1; Pinto et al., 2017). This could reflect that these two explant sources have different times for acquisition of competence. Alternatively, the four days CIM pre-incubation necessary for optimum shoot regeneration from root explants might reflect the sum of two processes: the cell reprograming and then the acquisition of competence itself. Sugimoto et al. (2010) demonstrated that the molecular and morphological events coordinating Arabidopsis shoot regeneration from different tissues are similar and that callus resembles the root primordia in explants isolated from roots, cotyledons, and petals. On the other hand, the sum of factors studied here indicates that although exogenous auxin supplementation improved tomato shoot regeneration from both cotyledon and root explants, shoot organogenesis does not strictly follow the same timing and molecular regulation in this two explants.

2.4. CONCLUDING REMARKS AND FUTURE PROSPECTS

The cultivated tomato (*Solanum lycopersicum* L), in special the miniature cultivar Micro-Tom (MT), has been considered an alternative model system for genetics (Meissner et al., 1997),

functional genomics (Dan et al., 2006; Sun et al., 2006) and plant development studies (Lima et al., 2009; Vicente et al., 2015). Although numerous tomato regeneration protocols using cotyledons and shoot-derived explants have been published in the last decades (Gubis et al., 2004; Pino et al., 2010; Sun et al., 2015, Gupta and Van Eck, 2016), the capacity of cultivated tomato in regenerate shoots from roots explants remained elusive until this work (Garcia-Reina and Luque, 1988; Peres et al., 2001; Lima et al., 2004; Zhang et al., 2012). We also described a successful regeneration system from tomato root explants using the cv. Micro-Tom. The shoot organogenesis from tomato roots was achieved by a fine-tuning of the high exogenous auxin offered on CIM incubation exclusively during the early four days in culture spanning the period required to acquisition of competence for shoot formation. Moreover, the adoption of optimal explant features (age and root tissue portion excised as explant) and proper culture conditions (plant growth regulators, temperature and use of gas-permeable tapes) also guaranteed the regenerative success.

Tomato wild-related genotypes with higher organogenic capacity have similar ability to regenerate shoots from primary and hairy roots (Peres et al., 2001). Also, the transformation with *Agrobacterium rhizogenes* generates large numbers of transformed hairy root in about 70–80% of hypocotyls inoculated (Shahin et al., 1986; Hashimoto et al., 1999; Peres et al., 2001). Tomato transformation based on co-cultivation with *A. tumefaciens* has a low efficiency due to the recalcitrance of most of the transformed cotyledon and leaf explants (Chapter 1; Sun et al., 2006; Dan et al., 2006; Pino et al., 2010). Therefore, the regeneration system from root tissues described in this work could allow the achievement of transgenic domesticated tomato plants from hairy roots through a simple and rapid procedure. This new approach extends the possibility of using tomato hairy roots, which can be now transformed into transgenic plants for agronomic and genetic (Moghaieb et al., 2004; Ron et al., 2014) applications, instead to be only used for secondary metabolites production (Hu and Du, 2006).

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