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In vitro regeneration of two *Populus* hybrid clones. The role of pectin domains in cell processes underlying shoot organogenesis induction

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Abstract

An efficient plant regeneration protocol has been established for two commercial *Populus* hybrid clones, MC (*Populus* × *euramericana*) and UNAL (*Populus* × *interamericana*). The culture of internode segments on Murashige and Skoog (MS) medium with 0.5 μM α-naphthalene acetic acid (NAA) and 4 μM N^6-benzyladenine for 7 weeks (2 weeks in absence of activated charcoal and 5 weeks in its presence) resulted in the highest frequency of shoot regeneration (100 % for MC and 82 % for UNAL). All regenerated shoots longer than 2 cm rooted on half-strength MS medium, independent of the addition of 0.1 μM NAA. Nevertheless, shoots developed better-formed roots in NAA-free medium, which had a positive effect on the acclimatization of plants. In order to know the cellular processes underlying *in vitro* shoot organogenesis, a histological study was made in UNAL internode-explants. Results revealed that *in vitro* culture caused swelling around the cut-off zones in all explants, but only those undergoing organogenesis formed proliferation centers under subepidermal cells, which led to formation of bud primordia. Moreover, *in vivo* tissues and explants with different *in vitro* response showed different immunolabelling patterns when they were treated with fluorescent-monoclonal antibodies directed to several pectin-polysaccharides of the cell wall. Results allow us to assign a predominant role of homogalacturonan with a low degree of methyl-esterification in the initiation of bud primordia, a role of β-1,4-D-galactan side chains of rhamnogalacturonan-I in the cellular differentiation, ra ole of α-1,5-L-arabinan side chains of rhamnogalacturonan-I and of homogalacturonan with a high degree of methyl-esterification in cell division and growth.

Additional key words: cell wall, homogalacturonan, immunohistochemistry, micropropagation, poplar, rhamnogalacturonan-I.

Introduction

Some *Populus* species are highly valued by several industries because of their suitable wood properties, fast growth, ease of vegetative propagation, short time to maturity, and interspecific cross ability. Breeding programs took advantage of these features to obtain a huge number of *Populus* hybrids by controlled crossing, among which the high-performance cultivars are being selected with regard to growth rate, adaptability, yield, or biotic and abiotic tolerance (Confalonieri *et al.* 2003, Gaur *et al.* 2016). The selected cultivars have been propagated as clones by rooting of stem cuttings. In Europe, *Populus deltoides* [Marshall] W. Bartram (cottonwood) and their interspecific F1 hybrids *Populus* × *euramericana* (*P. deltoides* × *Populus nigra* L.) and *Populus* × *interamericana* (*P. deltoides* × *Populus trichocarpa* [Hook] Torr. & A. Gray) are widely used for commercial plantations (Noël *et al.* 2002).

In vitro culture techniques have complemented the conventional breeding of cottonwood to overcome some its limitations. Specifically, *in vitro* regeneration represents fast and reliable methods for mass cloning of elite cultivars. Moreover, it is a prerequisite for some bio-

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Abbreviations: AC - activated charcoal; BA - N^6-benzyladenine; HG - homogalacturonan; NAA - α-naphthalene acetic acid; PBS - phosphate-buffered saline; PFD - photon flux density; PGR - plant growth regulator; RG-I - rhamnogalacturonan-I.
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technological applications such as transformation or gene editing. Nevertheless, woody trees are difficult to regenerate under *in vitro* conditions (Giri *et al.* 2004). Among *Populus* hybrids, *P.* × *euramericana* and *P.* × *interamericana* are known to be particularly recalcitrant to shoot organogenesis (mainly if stock plants are adult individuals) and their *in vitro* morphogenesis ability depends largely on the genotype. In fact, several authors have proved that efficient methods of plant regeneration should be optimized for each commercial clone (Noël *et al.* 2002, Cui *et al.* 2014, Jiang *et al.* 2015, Kwon *et al.* 2015). These researchers have used different organogenic pathways, explants, and cytokinins to obtain the best results on adventitious shoot induction and growth. In addition, regenerated shoots must be properly rooted so that the plants are acclimated successfully.

In vitro regeneration also provides a useful experimental system for studying the cellular processes taking place during shoot organogenesis, as well as their regulatory mechanism (Zhao *et al.* 2008). There are extensive studies about this subject in *Arabidopsis thaliana* (see Duclercq *et al.* 2011 and Motte *et al.* 2014 for review). However, a minor number of works have been driven in *Populus*, the genus designated as a model system for tree biology (Janson and Douglas 2007). Regarding *de novo* shoot formation, Bao *et al.* (2009) characterized the changes in gene expression that accompanies the process in cultured tissues from a hybrid clone of *P. tremula* × *P. alba* and compared them to results from *A. thaliana*. They found that the major changes took place during the first of the three characteristic phases of organogenesis (Christianson and Warnick 1985), namely the cellular dedifferentiation, which precedes the re-entry of the undifferentiated cells into the cell cycle and the determination of cell fate to form bud primordia (the second phase or induction). Among the up-regulated genes putatively identified using *A. thaliana* matches, those related to the cell wall were preponderant.

Two main components of plant cell wall are the cellulose/xyloglucan network and the pectin matrix. Changes in cell differentiation stage, volume, and/or shape occurring during shoot organogenesis require chemical modifications of the cell wall involving a plethora of loosening and stiffening agents (Chebli and Geitmann 2017). The orientation in the deposition of cellulose microfibrils into the cell wall and their binding with xyloglucan chains are well-known processes. Recent studies indicated that changes in pectin polymers can precede organogenesis even before cellulose orientation changes occur (Bidhendi and Geitmann 2016). Pectins have three main domains, homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II, which can be covalently linked to each other (Ridley *et al.* 2001, Caffall and Mohnen 2009). Pectins are synthesized in Golgi apparatus and then transported and secreted into the cell wall. The HG domains (homopolymer of 1,4-linked α-D-galacturonic acid) are synthetized with a high degree of methyl esterification in Golgi and once inside the cell wall, different pectin methylesterases produce their de-esterification in a blockwise or non-blockwise manner (Mohnen 2008). The pattern and degree of methylation determine the interaction among the HG domain (only de-esterified domains of the HG are able to cross-link by means of calcium ions) and other cell wall polysaccharides and proteins. As a consequence of these interactions, several mechanical properties of the cell wall such as porosity, hydration, stiffness, and ion exchange could be altered (Bidhendi and Geitmann 2016). On the other hand, RG-I structure (heteropolymer of 1,2-α-L-rhamnose-1,4-α-D-galacturonic acid units) is highly versatile and it may contain galactan, arabinan, and/or arabinogalactan side chains (Caffall and Mohnen 2009). The alteration in the ratio of these side chains affects the mechanical behavior of the cell wall (Bidhendi and Geitmann 2016, Chebli and Geitmann 2017) due to the ability of the RG-I to modify the extensibility of the cell wall cross-linking with the cellulose (Zykwinska *et al.* 2005) and other polysaccharides and proteins (Scheller and Ulvskov 2010) through their side chains. Taking all this into account, it is plausible to assume that the cell wall changes required for shoot organogenesis depend, at least in part, on the chemical, structural, and mechanical modifications of the pectin matrix.

The present work shows findings from two separate sets of experiments. The first one was designed to develop a successful system for plant regeneration of the MC clone of *P.* × *euramericana* and of the UNAL clone of *P.*× *interamericana*. Both hybrid genotypes were selected mainly because of their adaptability to changing environmental conditions. In the second set of experiments, monoclonal antibodies directed towards different pectin domains combined with microscopy techniques were used in order to determine the putative role of these pectin domains during the induction of the shoot organogenesis.

Materials and methods

Plant materials: Stem cuttings collected from adult trees of one *P. deltoides* × *P. nigra* (*P.* × *euramericana*) hybrid clone (MC) and one *P. deltoides* × *P. trichocarpa* (*P.* × *interamericana*) hybrid clone (UNAL) were supplied by the National Centre of Forest Genetic Resources (El Serranillo, Guadalajara, Spain). Cuttings with 4 to 5 dormant buds were washed and treated with 16 % (m/v) dicopper chloride trihydroxide (*Cu Key-S*). Then, in order to promote the outbreak of buds, cuttings were put in containers with water and they were kept in a growth chamber at 25 ± 2 °C with a 16-h photoperiod under a photon flux density (PFD) of 45 ± 5 μmol m^{-2} s^{-1}

provided by daylight fluorescent tubes (*TLD 36W/830*, *Philips*, Eindhoven, the Netherlands). Dormant buds started to sprout after one week.

When developing shoots had five fully expanded leaves, stems were excised, washed and surface sterilized with 70 % (v/v) ethanol (1 min), followed by 0.4 % (m/v) sodium hypochlorite with 0.01 % (v/v) *Tween-20* (15 min). After being rinsed four times with sterile distilled water, internode segments were prepared (1 to 1.5 cm in size), and longitudinal cuts were made in their ends. These handlings were performed under sterile conditions always placing the tissues on filter paper imbibed in a 0.01 % (m/v) ascorbic acid and 0.015 % (m/v) citric acid solution to avoid the oxidation of external tissues.

Culture conditions and culture medium for shoot induction and development: The bud-induction medium consisted of mineral nutrients and vitamins of Murashige and Skoog (1962, MS), 3 % (m/v) sucrose, and 0.05 % (m/v) *Kolliphor P-188* (*Sigma-Aldrich*, Madrid, Spain). This last compound is a wetting agent with surfactant properties equivalent to *Pluronic F-68*, the non-ionic copolymer successfully used by Iordan-Costache *et al.* (1995) and by Noël *et al.* (2002) to enhance shoot regeneration in *Populus*. To study the effect of the concentration of plant growth regulators (PGRs) on regeneration, the medium was supplemented with different combinations of α-naphthalene acetic acid (NAA) (0.1 or 0.5 μM) and N^6-benzyladenine (BA) (1, 4, 16, or 40 μM). Moreover, *Kolliphor P-188* was not added in some cultures in order to test the effect of its absence on the organogenic induction. The pH of the medium was adjusted to 5.8 and 0.9 % (m/v) of agar-agar was added as gelling agent prior to autoclaving at 120 °C for 20 min.

Explants, consisting of the prepared internode segments, were cultured in plastic Petri dishes containing 20 cm^3 of bud-induction medium. For each genotype-PGRs combination, seven dishes (independent replications) with four explants in each one were evaluated. Cultures were sealed and set in a growth chamber for two weeks at 25 ± 2 °C, a 16-h photoperiod, and a PFD of 32 ± 2 μmol m^{-2} s^{-1} provided by cool daylight fluorescent tubes (*OSRAM L30W/865*, *Lumilux*, Seelze, Germany).

For shoot development from induced buds, internode explants were transferred to 150-cm^3 glass jars, each containing 20 cm^3 of the medium described earlier but supplemented with 0.5 % (m/v) activated charcoal (AC) and without *Kolliphor P-188*. After five weeks, we determined the survival, the percentage of surviving bud-forming explants, and the number of shoots regenerated per responding explant. Tissues showing buds in any developmental stage were considered as budding explants. Nevertheless, only shoots higher than 2 cm were taken into account to calculate the number of shoots per explant. These two variables defined the shoot regeneration ability of explants.

Rooting and acclimatization: Regenerated shoots longer than 2 cm were separated from primary explants and cultured on half-strength MS medium supplemented with 1.5 % (m/v) sucrose and in presence/absence of 0.1 μM NAA. All cultures (one shoot per 350-cm^3 glass jar), were kept in the culture room under the growing conditions described above. For each NAA treatment, 15 shoots (independent replications) were evaluated. Once shoots were rooted and root reached a minimum length of 7 cm, plantlets were acclimatized. Before that, the rooting percentage, the number of roots per rooted shoot, and the length of the longest root were evaluated.

For acclimatization, plantlets were transferred to plastic pots containing autoclaved substrate (peat moss: *Vermiculite*, 4:1). Pots were covered with polyethylene bags to maintain the high air humidity, and they were placed in the growth room. Cultures were watered first with 30 cm^3 of mineral solution (Hoagland and Arnon 1938) and thereafter with 10 cm^3 of water every 2 d. After the acclimatization by progressively opening the plastic bags during 3 weeks, adapted cultures were transferred to open space. The percentage of acclimatized plants was evaluated separately for plants rooted on media with and without NAA after one month.

Statistical analysis: All the *in vitro* experiments were conducted using a randomised complete block design. Data were presented as means ± standard errors (SEs). The statistical analysis of frequencies (percentages of explants forming buds, shoots forming roots, and acclimatized plants) were carried out with the χ^2 test for overall and pairwise comparisons. Quantitative data were evaluated by Student *t*-test or by analysis of variance (*ANOVA*) and Duncan´s multiple range test, depending on whether data were grouped in 2 or *k* sets. The *P*-value was 0.05 for all analyses and statistical tests were performed using *IBM-SPSS Statistic* (*v. 21*) software.

Histological study and immunolocation of pectin domains: These studies were made on internode segments of UNAL collected after five weeks of culture on the medium with 0.5 μM NAA and 4 μM BA that showed adventitious organogenesis (see Fig. 1 Suppl.). To establish the possible role of pectin-polysaccharides, different types of controls were made. The first one consisted of an *in vivo* internode segment (named *ex vitro* control), the second and third control were internode tissues cultured *in vitro* on the same medium and did not show organogenesis (*in vitro* control), or showing dedifferentiation (callogenesis).

Tissues were fixed in 2 % (m/v) paraformaldehyde in 0.1 M phosphate buffer pH 7.5 at 4 °C overnight. After washing twice with phosphate buffer, tissues were dehydrated in an increasing ethanol series [10, 20, 30, 50, 70, 90, and 96 % (v/v)] prior to embedding in *LR White* resin (*London Resin*, Reading, UK). After that, tissue pieces were placed in gelatin capsules containing the resin and allowed to polymerize at 37 °C for 5 d. Sections (1 μm thick) were obtained by an ultracut microtome

LKB 2088 (*Reichert-Jung*, Vienna, Austria) and applied to multi-well slides (*ICN Biomedicals*, Cleveland, OH, USA) coated with VecTabond reagent (*Vector Laboratories*, Burlingame, CA, USA). For histological study, some sections were stained with 0.4 % (m/v) toluidine blue in 1 % (m/v) sodium borate solution and then observed on a *BX61* (*Olympus,* Tokyo, Japan) microscope.

In order to locate cell wall components, serial sections were incubated in a diluted (1:10) primary monoclonal antibodies JIM5, JIM7, LM5, or LM6 in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 7.8 mM $Na_2HPO_4 . 12\ H_2O$, 1.5 mM KH_2PO_4, pH 7.2) containing 4 % (m/v) fat-free milk powder for 2 h. These antibodies specifically recognized the pectin-polysaccharides of the cell wall (see Table 1 Suppl.). After washing exhaustively with PBS, sections were incubated in a 1:100 diluted anti-rat immunoglobulin G linked to fluorescein isothiocyanate in milk powder/PBS at room temperature in darkness for 2 h. Finally, sections were newly washed with PBS and mounted in a glycerol/PBS-based anti-fade solution (*Citifluor AF1*, *Agar Scientific*, London UK). Observations were performed with an *Olympus BX61* microscope equipped with epifluorescence observation by using filters *FT-510-LP-520* (450 - 490 nm) necessary to observe green emission of fluorescein isothiocyanate.

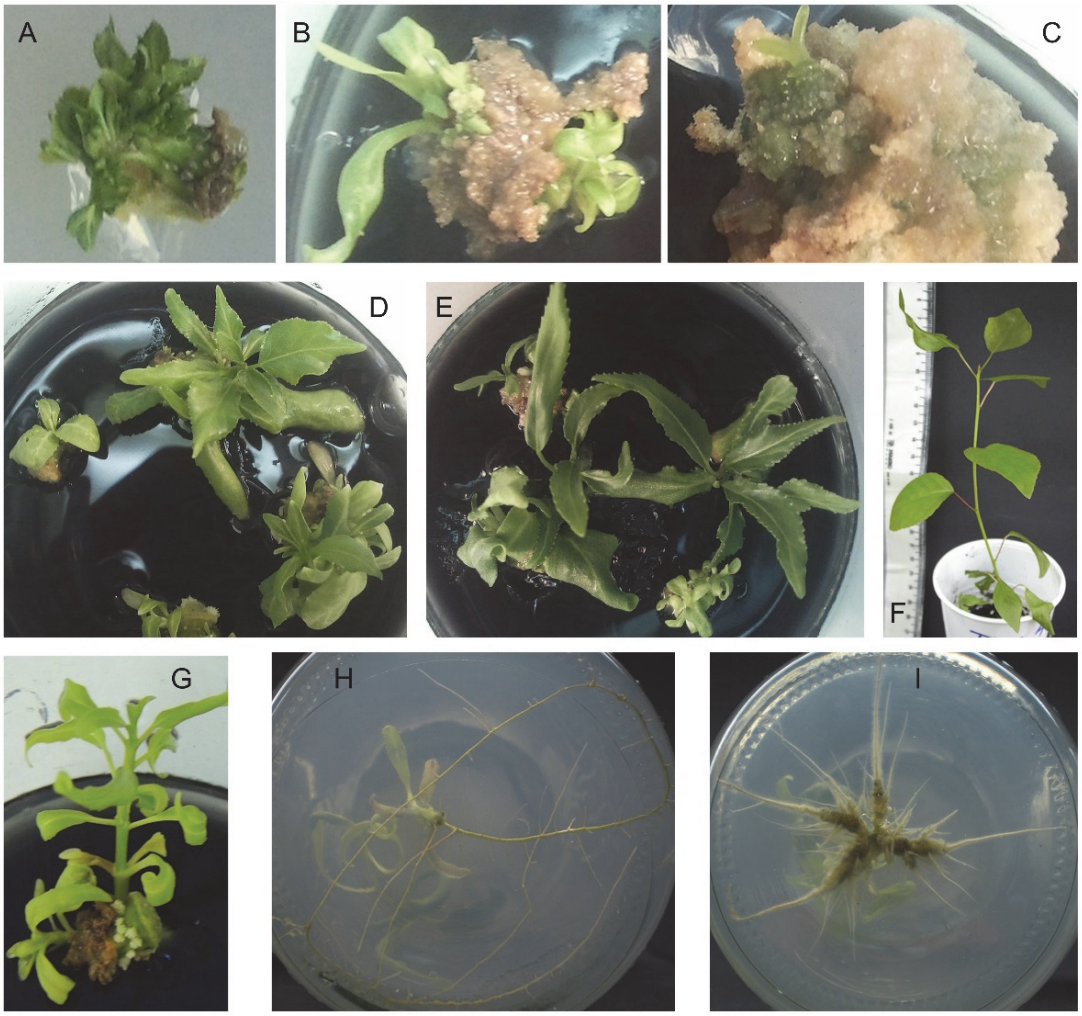

Fig. 1. *In vitro* plant regeneration of *Populus* × *euramericana* (MC) and in *P.* × *interamericana* (UNAL) from internode explants. *A* - Shoot organogenesis from internode explants of MC after 2 weeks of culture on a medium with 4 µM BA and 0.5 µM NAA. *B, C* - Callus development from internode explants of MC cultured 7 weeks on media with 0.5 µM NAA and 16 (*B*) or 40 (*C*) µM BA. *D*, *E* - Shoot regeneration from internode explants of MC (*D*) and UNAL (*E*) cultured for 7 weeks on medium with 4 µM BA and 0.5 µM NAA. *F* - Acclimatized plant of MC. *G* - The regenerated shoot of UNAL before being excised from the explant and transferred to rooting medium. *H*, *I* - Root systems developed from shoots of MC after culture in a free-hormone medium (*H*) or in a medium containing 0.1 µM NAA (*I*).

Results and discussion

Leaves, petioles, and internode segments from herbaceous stems have been used as explants for adventitious shoot formation for several clones of *P. deltoides* and its hybrids (Chaturvedi *et al*. 2004, Yadav *et al.* 2009, Takur *et al.* 2012, Cui *et al.* 2014, Jiang *et al.* 2015, Kwon *et al*. 2015). To achieve the same goal for MC and UNAL hybrid genotypes, internode segments were chosen in the present work according to Noël *et al.* (2002), Chaturvedi *et al.* (2004), Yadav *et al.* (2009) and Cui *et al.* (2014). These latter authors developed a protocol for five *P.* × *euramericana* clones based on the culture of stem discs with only a 1 - 2 mm thick cross section, showing the ability of this kind of explants to form shoots even when their thickness was extremely reduced.

Most of MC and UNAL explants survived after seven weeks under *in vitro* conditions (76.19 % ± 4.34), whereas the rest turned brown and were necrotic after two weeks, probably due to the oxidation of polyphenolic compounds exuded from the cut edges of the explants. Taking into account that this problem often affects woody tissue cultures, particularly in *Populus* spp. (Confalonieri *et al.* 2003), it could be considered that the treatment of MC and UNAL tissues with the anti-oxidant solution used in this work sufficiently prevented the death of explants. Another way to partially overcome browning and necrosis is by adding adsorptive materials such AC into the medium, as Kang *et al.* (2009) did to micropropagate the Nisqually-1 clone of *P. trichocarpa*. In the current study, AC was not incorporated into bud induction medium because the absorption of PGRs by the explants could be compromised. However, it was added to the shoot development medium.

The presence of BA and NAA in the induction medium stimulated the adventitious buds regeneration at the cut ends of internode-explants within the first two weeks of culture (Fig. 1*A*). The subculture of explants

Table 1. Effect of NAA and BA concentrations and the presence/absence of *Kolliphor P-188* on the shoot regeneration ability of internode explants of *P.* × *euramericana* (MC) and *P.* × *interamericana* (UNAL). The internode explants were cultured on the induction medium for 2 weeks, and then transferred to the same medium but with activated charcoal (0.5 %) and without *Kolliphor P-188*. Data were recorded after 5 weeks on this last medium. Means ± SEs, n = 7 replicates, each replicate consisting of four explants. The means followed by the same letters within each clone are not significantly different ($P \geq 0.05$) according to χ^2 test (%) and Duncan´s multiple range test (number of shoots). For counting only survived explants and only responding explants were included.

Clone	NAA [μM]	BA [μM]	Kolliphor P-188	Explants forming buds [%]	Number of shoots ≥ 2 cm [explant^{-1}]
MC	0.5	40	+	75.00 ± 10.12 ab	0.33 ± 0.28 a
	0.5	16	+	91.67 ± 5.05 a	1.94 ± 0.51 b
	0.5	4	+	100.00 ± 0.00 a	3.85 ± 0.46 c
	0.1	4	+	33.33 ± 16.50 b	1.83 ± 0.32 b
	0.1	1	+	52.38 ± 14.84 b	1.00 ± 0.00 ab
	0.1	4	-	53.33 ± 17.09 b	1.00 ± 0.00 ab
	0.1	1	-	57.14 ± 18.70 b	1.25 ± 0.12 ab
UNAL	0.5	40	+	62.50 ± 17.03 ab	0.08 ± 0.05 a
	0.5	16	+	77.78 ± 9.39 ab	0.42 ± 0.17 a
	0.5	4	+	82.14 ± 9.73 a	1.73 ± 0.34 b
	0.1	4	+	51.20 ± 14.91 ab	1.70 ± 0.15 b
	0.1	1	+	78.57 ± 7.87 ab	2.07 ± 0.26 b
	0.1	4	-	30.95 ± 11.51 b	1.83 ± 0.19 b
	0.1	1	-	78.57 ± 10.63 ab	1.56 ± 0.28 b

Table 2. Effect of the NAA concentration on the rooting of regenerated shoots and on the acclimatization of plantlets of *P.* × *euramericana* (MC) and *P.*× *interamericana* (UNAL). Isolated shoots were cultured on rooting medium with 0.1 μM NAA or without it. The rooting response of shoots was evaluated once roots reached a minimum length of 7 cm. The number of acclimatized plants was recorded after 7 weeks of acclimatization. Means of both genotypes ± SEs, n = 15, means followed by the same letters are not significantly different at $P \geq 0.05$ according to χ^2 test (%) and Student t-test (number of roots per shoot and length of the longest root).

NAA [μM]	Rooting response			Acclimatized plants [%]
	Shoots forming roots [%]	Number of roots per shoot	Length of the longest root [cm]	
0.0	100 ± 0.00 a	2.87 ± 0.40 a	5.77 ± 0.59 a	73.33 ± 12.24 a
0.1	100 ± 0.00 a	3.93 ± 0.41 a	2.29 ± 0.34 b	26.67 ± 12.24 b

onto the same medium with AC and without *Kolliphor P-188* supported the regeneration of new shoots and allowed the development of shoots already formed (Fig. 1*D,E*).

Among the five combinations of PGR concentrations tested, the most effective for MC was 0.5 µM NAA and 4 µM BA, since 100 % of explants formed adventitious shoots and the number of shoots per internode explant was the highest (3.85 ± 0.46, Table 1). The reduction of the concentration of NAA only, or the concentration of both PGRs had negative effects on shoot regeneration ability of MC genotype (Table 1). On the other hand, the increase of BA concentration up to 16 or 40 µM significantly reduced the number of shoots higher than 2 cm and caused callus formation around their cut ends (Fig. 1*B,C*). These last results were also observed in explants from UNAL (Table 1). The BA has been successfully used as inductor of shoot organogenesis in calli and explants of *P. deltoides* and its hybrids (Chaturvedi *et al.* 2004, Mingozzi *et al.* 2008, Maheshwari and Kovalchuk 2011, Thakur *et al.* 2012, Cui *et al.* 2014, Jiang *et al.* 2015, Kwon *et al.* 2015). However, side effects resulting from too high concentrations of BA, such as a decline in bud formation and the increase in associated callusing, were observed as well by Chaturvedi *et al.* (2004) and Cui *et al.* (2014). In genotype UNAL, the maximum percentage of responding explants (82.14 % ± 14.91) and one of the highest number of shoots per explants (1.73 ± 0.34) was also on medium with 0.5 µM NAA and 4 µM BA. However, the medium supplemented with 0.1 µM NAA and 1 µM BA led to similar results. Therefore, the decrease of both PGR concentrations did not have a significant negative effect on the organogenic response of UNAL internode segments (Table 1). The genotype dependence for *in vitro* shoot organogenesis had previously been observed by Noël *et al.* (2002) and Cui *et al.* (2014).

Besides the effect of PGRs, we evaluated the

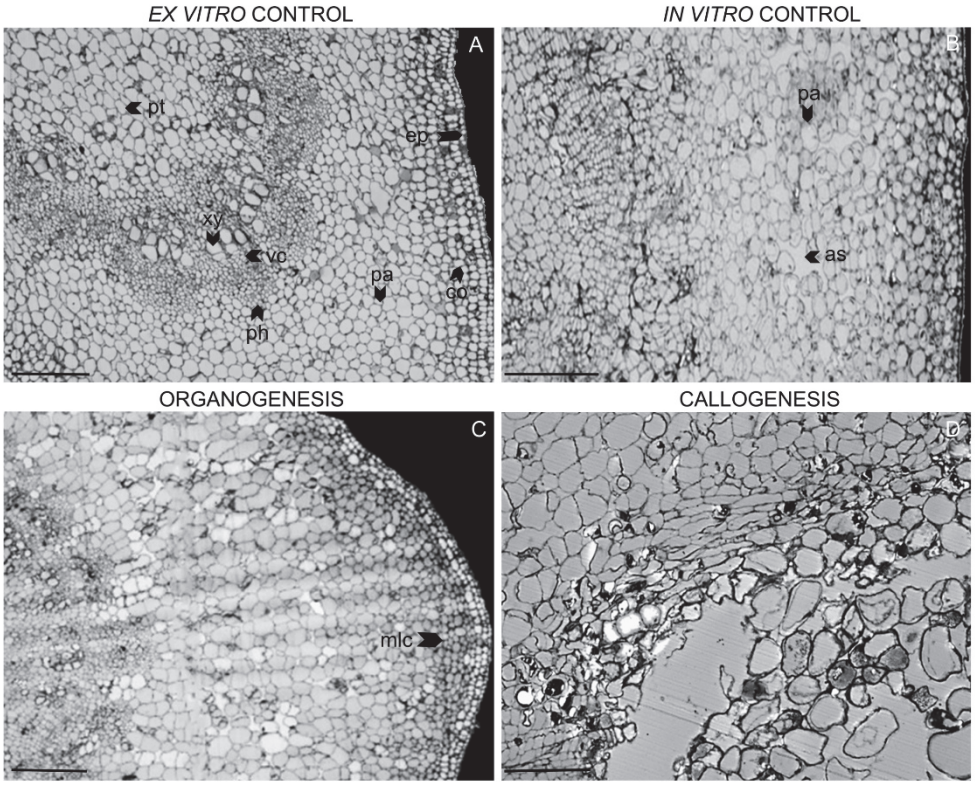

Fig. 2. Toluidine blue-stained section of internode segments of *P.* × *interamericana* UNAL before culture (*A*, *ex vitro* control), segments do not showing morphogenesis after five weeks of culture (*B, in vitro* control), segments showing organogenesis (*C*) or callogenesis (*D*). The culture medium was the same and contained 4 µM BA and 0.5 µM NAA. The *arrows* point to ep - epidermal cells, co - collenchyma tissue, pa - parenchyma, vc - vascular cambium, ph - phloem, xy - xylem, pt - pith tissue, as - aerial spaces, mlc - meristematic-like cells. *Scale bars* = 200 µm in *A, B, C* and 100 µm in *D*.

efficiency of *Kolliphor P-188* as a differentiation-stimulating agent in poplar (Iordan-Costache *et al.* 1995, Noël *et al.* 2002). However, the absence of this surfactant agent did not have a significant effect either on the percentage of bud-forming explants or on the number of shoots formed per explant in any of the four genotype-PGR combinations tested (Table 1). The lack of the expected positive influence of the compound upon shoot formation could be related to the fact that we only tested one concentration (0.05 %), whereas Iordan-Costache *et al.* (1995) and Noël *et al.* (2002) checked concentrations ranging from 0.001 to 0.1 % (m/v). In

agreement with this idea, Noël *et al*. (2002) obtained the best results from calli of six *Populus* genotypes when they modulated the concentration of *Pluronic F-68*, equivalent to *Kolliphor P-188*, according to each cultivar.

In order to complete the protocol for *in vitro* plant regeneration for MC and UNAL, adventitious shoots longer than 2 cm (Fig. 1*G*) were transferred to rooting medium. The 100 % of shoots easily rooted without callus formation and elongated up to 7 cm within 2 - 5 weeks. The presence of NAA did not affect the average number of roots formed per shoot, but significantly influenced the length of the longest root (Table 2) and the morphology of the root system. In its absence, shoots formed long and well-developed roots with thin lateral roots (Fig. 1*H*), whereas shoots cultured on medium with NAA produced shorter and thicker roots with anomalous appearance (Fig. 1*I*). Jiang *et al*. (2015) found similar effects of NAA in the development of adventitious roots in shoots of *P.* × *euramericana* Neva. It is well known that high concentration of auxin inhibits the elongation of adventitious roots (Li *et al.* 2009), so this evidence could be caused by an excess of auxin activity produced as a consequence of the accumulation of endogenous auxin and exogenous NAA in the basal area of the shoots. In this sense, Dong *et al.* (2012) showed the accumulation of indole-3-acetic acid in the basal regions of petiole explants, just where poplar leaves formed adventitious roots when they were cultured in a hormone-free medium.

The architecture of the root system determined the success of the further acclimatization of regenerated plants (Fig. 1*F*) which was significantly higher when the rooting took place in the NAA-free medium (Table 2). It is possible that thin, branched, and long roots may ensure water uptake from the deep layers of the substrate during acclimatization, whereas shoots with shorter and shallower roots induced in the presence of NAA could not be able to access to that water reservoir. Therefore, these plants could suffer from water deficit between watering periods. Thus, to induce rhizogenesis from *in vitro* regenerated shoots of MC and UNAL, we recommend the use of PGR-free medium.

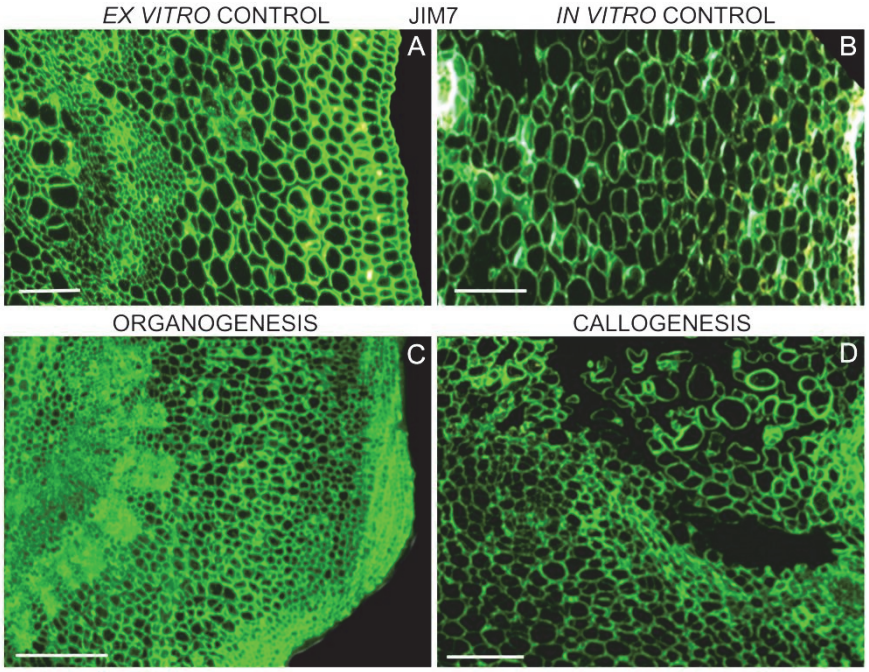

Fig. 3. Immunolocation of homogalacturonan with a high degree of methyl esterification (JIM7) in internodal segments of *P.* × *interamericana* UNAL before (*A, ex vitro* control) and segments after five weeks of culture without morphogenesis (*B, in vitro* control), segments showing organogenesis (*C*) or callogenesis (*D*). The culture medium contained in all cases 4 µM BA and 0.5 µM NAA. *Scale bars* = 200 µm in *C, D* and 100 µm in *A, B.*

Once the protocol of plant regeneration for MC and UNAL was developed, we used explants from UNAL to determine the cellular processes that underlie adventitious shoot organogenesis. A good way to achieve the proposed goal was to study histological changes undergone by *in vitro* cultured tissues that formed bud primordia (organogenesis), those that did not (*in vitro* control), and tissues which only showed dedifferentiation (callogenesis), in order to make a comparison among them. Further, an internode segment of UNAL never cultured *in vitro* was also included in the study (see Fig. 1 Suppl.).

The sections of the *ex vitro* control (Fig. 2*A*) consisted of a layer of epidermal cells under which it was possible to observe the collenchyma tissue. This was composed of cells with thicker cell walls that provide structural support in woody species. Parenchyma was between the epidermis and the vascular cambium and it was constituted by live cells with thin primary cell walls.

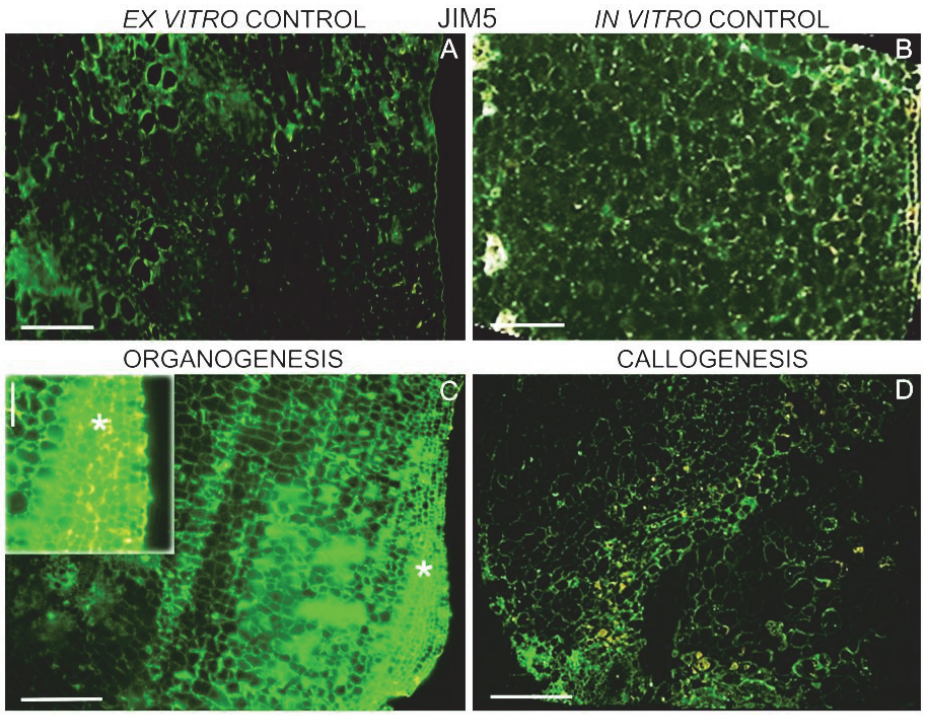

Fig. 4. Immunolocation of homogalacturonan with a low degree of methyl esterification (JIM5) in internodal segments of *P.× interamericana* UNAL before (*A, ex vitro* control) and after five weeks of culture without morphogenetic response (*B, in vitro* control), with organogenesis (*C*) or callogenesis (*D*). The MS medium contained in all cases 4 µM BA and 0.5 µM NAA. *Scale bars* = 200 µm in *C, D,* 100 µm in *A, B* and 50 µm in *inset.*

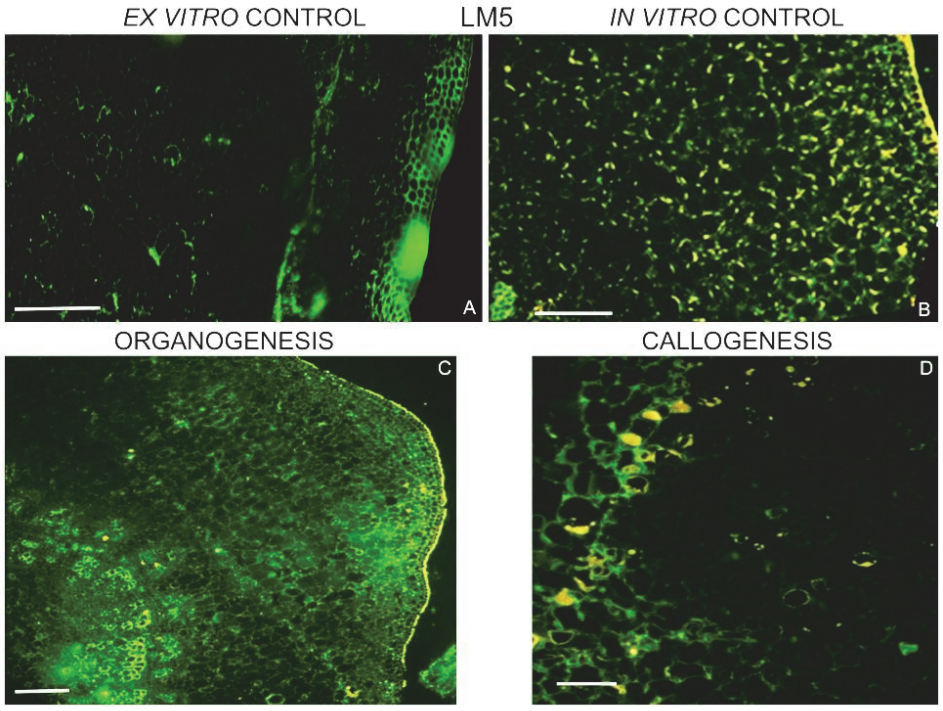

Fig. 5. Immunolocation of β-1,4-D-galactan side chains of RG-I (LM5) in internodal segments of *P.× interamericana* UNAL before (*A, ex vitro* control) and after five weeks of culture without morphogenetic response (*B, in vitro* control), with organogenesis (*C*) or callogenesis (*D*) responses. The culture medium contained in all cases 4 µM BA and 0.5 µM NAA. *Scale bars* = 200 µm in *C* and = 100 µm in all others.

Some parenchyma cells could differentiate to another cell type at the maturity state if they are properly stimulated (Sugimoto *et al.* 2011). Under the parenchyma tissue, the vascular cambium was observed. The vascular cambium is a secondary meristem, which produces phloem outside and xylem inside. The most internal parenchyma tissue was the pith tissue.

Under *in vitro* culture, explants showed a thickening and an increase in size around the cut-off area (Fig. 2*B,C*). Nevertheless, on *in vitro* control, only minor disorganization on the vascular tissue was shown although the parenchyma cells were swollen, thus allowing more aerial spaces among cells (Fig. 2*B*). This increase in size, primarily in the cut-off area, was promoted by the PGRs present in the culture medium together with the dedifferentiation of the cells due to the wound (Lup *et al.* 2016). During adventitious organogenesis, swelling around the cut-off area was observed, which corresponds with putative adventitious bud-primordia formation (Fig. 2*C*). In those primordia, the parenchyma cells just under uniserial epidermal cells were smaller, isodiametric, and more compactly connected to each other, whereas the aerial spaces between the inner parenchyma cells were larger than those in the *in vitro* control. Ferreira *et al.* (2009) described a similar cellular re-organization during adventitious bud-primordia initiation in leaf explants of *P. euphratica.* On the other hand, callogenesis induced a complete structural disorganization in all tissues of explants and as a result, cells were irregular and utterly different in size (Fig 2*D*).

The cellular processes accompanying *in vitro* morphogenic responses, and so the cell changes also described in cultured tissues from UNAL, are regulated by the chemical structure and mechanical properties of the cell wall. In the last years, pectin-mediated cell wall loosening is shown as an important factor in cell and organ morphogenesis (Bidhendi and Geitmann 2016, Chebli and Geitmann 2017). In order to investigate the role of HG and RG-I domains during morphogenesis in UNAL tissues, fluorescent antibodies towards HG with low (JIM5) and high (JIM7) degrees of methyl-esterification, β-1,4-D-galactan (LM5), and α-1,5-L-arabinan (LM6) side chains of RG-I were used (Table 1 Suppl.). These antibodies allow identification of these specific epitopes by means of intense green fluorescence labelling. In some sections, other colours may appear, such as pale green or yellow, which are related to background and self-fluorescence, respectively.

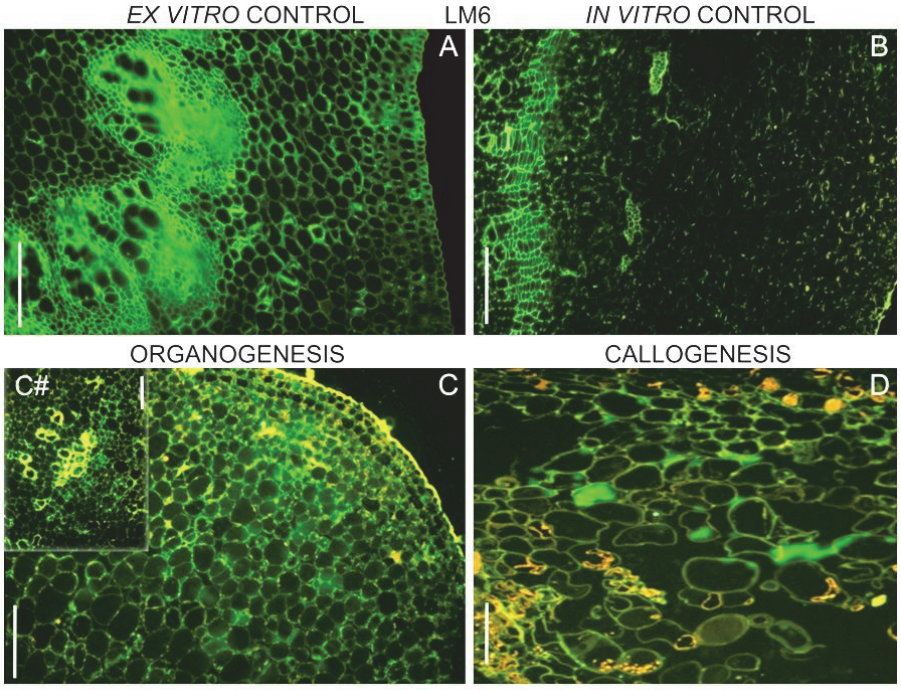

Fig. 6. Immunolocation of α-1,5-L-arabinan side chains of RG-I (LM6) in internodal segments of *P.× interamericana* UNAL before (*A, ex vitro* control) and after five weeks of culture without morphogenetic response (*B, in vitro* control), with organogenesis (*C*) or callogenesis (*D*). C# is an image of the vascular tissues of the *C* section. The culture medium contained in all cases 4 µM BA and 0.5 µM NAA. *Scale bars* = 50 µm in *C#* and 100 µm in all others. *Yellow* areas are due to self-fluorescence.

Internode segments extracted from bud sprouting (*ex vitro* control) showed a homogenous labelling with JIM7 (Fig. 3*A*) and JIM5 (Fig. 4*A*) antibodies although the signal corresponding to JIM7 was more intense in all tissues. Similarly, in these sections, the immuno-fluorescence of arabinan side chains of RG-I labelling with LM6 was homogenous and intense especially in the cambium cells (Fig. 6*A*). These results were in

accordance with other works that point to a high presence of epitopes recognized by JIM7 (Bosch and Hepler 2005) and LM6 (Willats *et al.* 1999) in tissues growing actively, like those present in *ex vitro* controls. During cell growth, HG is secreted in a highly methyl-esterified form (the labelling with JIM7 increased as a result) and subsequently de-esterified by pectin methylesterases. The pattern and degree of esterification determine the formation of calcium-bridges and the action of cell wall remodelling enzymes. Moreover, in poplar shoot sections without *in vitro* culture, LM5 labelling was located both within differentiated cells of the collenchyma under the epidermis and vascular vessels close to the cambium cells (Fig. 5*A*). The epitope recognized by LM5 had been previously associated with differentiated cells (Serpe *et al.* 2001, Bush *et al.* 2001).

When internode segments were cultured *in vitro* (*in vitro* control), all the antibodies assayed presented less labelling compared with *ex vitro* control. This reduction in the signal was more appreciable in JIM7 (Fig. 3*B*) and LM6 (Fig. 6*B*) labelling which could be in accordance with the lack of growth and cell division in these tissues.

In the explants showing organogenesis, an increase in labelling with JIM5 in the subepidermal zone (Fig. 4*C,C#*) was observed. This observation could be surprising because only de-esterified pectins (recognized by JIM5) are able to form calcium-bridges producing stiffer pectin gels in which the cell wall extensibility is expected to be reduced. However, it is necessary to take into account that JIM5 and JIM7 recognize HG with low and high esterification degree, respectively, but the pattern of de-esterification of this HG is unknown. A possible explanation for this could be that if HG is non-blockwise de-esterified, the cross-linking by calcium-bridges is less effective. However, non-blockwise de-esterified HG is more susceptible to degradation by polygalacturonases and, after the action of these enzymes, the cell wall eventually becomes softer (Willats *et al.* 2001, Arancibia and Motsenbocker 2006, Pelloux *et al.* 2007). In order to corroborate this hypothesis, more in-depth studies are necessary. Similarly to our results, Peaucelle *et al.* (2008, 2011) demonstrated that during organogenesis performed in *Arabidopsis,* there is a temporary increase in HG with a low degree of methyl esterification (recognized by JIM5) in the subepidermal zone before a lack of stiffness and an increase of elasticity take place in the cell walls. Moreover, in UNAL explants with organogenesis, all the tissues showed an intense labelling with JIM7 indicating that these cells grew and deposited new pectins in their cell walls (Fig. 3*C*). Once again, the most intense labelling with JIM7 was appreciated in the smaller cells of the subepidermal zone. Nevertheless, no substantial changes in LM5 (Fig. 5*C*) and LM6 (Fig. 6*C,C#*) were appreciated during organogenesis compared to *in vitro* control.

Tissues with cell dedifferentiation (callogenesis) showed an intense immunofluorescence when they were labelled with JIM7 (Fig. 3*D*) and JIM5 antibodies (Fig. 4*D*). These dedifferentiated cells began to grow isodiametrically probably due to the increasing anisotropic orientation of the newly formed cellulose microfibrils, which takes place during the callogenesis. Peaucelle *et al.* (2015) showed that the anisotropic growth is mediated by a selective pectin de-esterification which occurs before the reorientation of the cellulose microfibrils. In this sense, JIM5 labelling increased during callogenesis compared to that of the cells of *in vitro* control (Fig. 4*D vs.* 4*B*) which could indicate the increment of de-esterified pectins in anisotropically growing cells. On the other hand, our calluses showed less LM6 epitopes in comparison with the control (Fig. 6*D*), although in dividing carrot cell suspensions, an increase in arabinan side chains of RG-I (detected by LM6) was described (Willats *et al.* 1999). Nevertheless, it must be considered that the methyl esterification degree of HG and the presence or not of side chains in RG-I and their binding to other cell wall components change depending on the species, development stage, organ, tissue or cell type (Wolf *et al.* 2009, 2012, Levesque-Tremblay *et al.* 2015).

In conclusion, the study of the location of the pectin domains in tissues from *P.* × *interamericana* UNAL allowed us to make the following proposals: *1*) HG with low degree of methyl-esterification could have an important role in the initiation of shoot organogenesis, because JIM5 intensely labelled the putative adventitious bud primordia only present in organogenic tissues; *2*) in the same way, β-1,4-D-galactan side chains of RG-I may be important in cellular differentiation, since the strongest signal produced by LM5 was associated with the most differentiated tissues; *3*) α-1,5-L-arabinan side chains of RG-I (recognized by LM6) and HG with a high degree of methyl-esterification (recognized by JIM7) seemed to have preponderant roles in cell division and growth, although the first was more related to these processes in the *ex vitro* growing tissues, while the latter was more characteristic of *in vitro* cultured tissues suffering cell dedifferentiation.

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