Title: *In vitro* regeneration for two *Populus* clones. Cell wall components as markers of the cell processes of shoot organogenesis induction

Authors: Penélope García-Angulo (García-Angulo P¹), Irene Villar (Villar I²), Laura Giner-Robles (Giner-Robles L³), María Luz Centeno (Centeno ML^{1*})

* **Corresponding autor:** Centeno ML (mlcenm@unileon.es), phone number: +34 987 291481

Affiliation and address of authors:

1, Dpto. Ingeniería y Ciencias Agrarias, Universidad de León, Facultad de Ciencias Biológicas y Ambientales, Campus de Vegazana, 24071 León, Spain.

2, Estación Experimental de Aula Dei (CSIC), Campus de Aula Dei, Avda. Montañana 1005, 50059 Zaragoza, Spain.

3, Instituto de Biomedicina y Biotecnología de Cantabria, Calle Albert Einstein, 39011 Santander, Spain.

ORCID of authors: García-Angulo P (0000-0001-5517-2238), Villar I (0000-0003-4552-5655), Giner-Robles L (0000-0001-6878-783X), Centeno ML (0000-0002-2443-9275)

Abstract

An efficient plant regeneration protocol has been established for two hybrid clones of *Populus deltoides*, MC (*P. x euramericana*) and UNAL (*P. x interamericana*). The culture of internode segments on MS medium with 0.5 μ M α -Naphthalene acetic acid (NAA) and 4 μ M N⁶-Benzyladenine for 7 weeks –2 in absence of activated charcoal and 5 in its presence—gave the highest frequency of shoot regeneration (100% for MC and 82% for UNAL). Good results also were achieved using leaves as explants, but only if they remained 5 weeks in the first medium. The 100% of regenerated shoots longer than 2 cm were rooted on ½ MS medium, independently of the addition of 0.1 μ M NAA. Nevertheless, shoots developed better-formed roots in NAA-free medium, which had a positive effect in the acclimatization of plants.

To know the cellular processes underlying *in vitro* shoot organogenesis, a histological study was made in UNAL internodes. 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 lead to 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 against several pectin-polysaccharides of the cell wall. Results allow us to point homogalactouronan (HG) with high degree of methyl-esterification and α -1,5-L-

arabinan side chains of ramnogalactouronan-I as markers for cell division and growth, and HG with low degree of methyl-esterification as a marker for bud primordia initiation.

Keywords: poplar, shoot organogenesis, micropropagation, cell wall, immunolocation.

Key message: This work describes regeneration systems for two commercial clones of *Populus*. It also proves that some pectin-polysaccharides of the cell wall mark the initiation of shoot-bud primordia in cultured tissues.

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Author contribution statement: PGA and MLC conceived and designed the experiments, and wrote the manuscript. IV and LG conducted experiments. All authors analyzed data, read and approved the manuscript.

Introduction

Some *Populus* species are known for their remarkable significance among the commercially propagated trees such as eucalyptus and radiate pine. They are highly valued by several industries because of their suitable wood properties, fast growth, ease of vegetative propagation, short time to maturity (3-6 years in most species), and interspecific cross ability. Breeding programs took advantage of these features to obtain a huge number of *Populus* hybrids by controlled crossing (Confalonieri 2003; Gaur et al. 2015), among which the high-performance cultivars are being selected with regard to growth rate, adaptability, yield in terms of fiber quality, or biotic and abiotic tolerance (Noël et al. 2002). The selected cultivars have been propagated as clones by rooting of stem cuttings. In Europe, the commercial clones of *Populus deltoides* (cottonwood) and their interspecific F1 hybrids *P. x euramericana* (*P. deltoides* x *P. nigra* L) and *P. x interamericana* (*P. deltoides* x *P. trichocarpa*) represent the bulk of intensive poplar cultivation.

In vitro culture techniques have implemented the conventional breeding of cottonwood and have allowed some of its limitations to be overcome. Specifically, *in vitro* regeneration represented fast and reliable methods for mass cloning of elite cultivars and could play an important role in germplasm conservation. Moreover, it is a prerequisite for some biotechnological applications such as the improvement through transformation or gene editing. Nevertheless, woody trees are difficult to regenerate under *in vitro* conditions (Giri et al. 2004). Among all the hybrids of *Populus*, *P. x euramericana* and *P. x 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 (Cui et al. 2014; Kwon et al. 2015; Noël et al. 2002). These researchers have used different organogenic pathways, explants and cytokinins to obtain the best results on adventitious shoot induction and growth. Mingozi et al. (2008) and Kwon et al. (2015) even demonstrated that environmental conditions, such as light quality, photon flux and CO₂ concentration, may also control bud formation. 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 accompany the process in cultured tissues from a hybrid clone of *P. tremula* x *P. alba* and compared them to results from *Arabidopsis*. They found that the major changes took place during the first of the three characteristic phases of organogenesis, namely the cellular dedifferentiation, which precedes the re-entry of the undifferentiated cells into cell cycle and the determination of cell fate to form bud

primordia (second phase or induction). Among the up-regulated genes putatively identified using *Arabidopsis* matches, those related with the cell wall were preponderant.

Plant cell wall is an extracellular matrix that surrounds plant cells, whose two main components are the pectin-Ca²⁺ matrix and the cellulose/xyloglucan network, both connected between them. Changes in cell differentiation stage, volume and/or shape occurring during shoot organogenesis require chemical modifications of the polymeric network—on their polysaccharide components and on the assembly between them—which are highly modulated by a plethora of loosening and stiffening agents (Chebli and Geitmann 2017). Therefore, certain cell wall chemical and structural status could be associated to tissues undergoing shoot organogenesis, and therefore some cell wall components and their localization could be used as markers of the organogenic process.

The present work reports findings from two separate sets of experiments. The first one was designed to develop a successful system for plant regeneration of the hybrid clones MC (*P. x euramericana*) and UNAL (*P. x interamericana*). Both were selected mainly because of their rusticity and adaptability to changing environmental conditions, including dryness in the case of UNAL. The influence of explants type, growth regulators and Kolliphor-P188 on regenerative capacity was investigated. The goal of the second set of experiments was to find possible markers of the cellular changes that lead to adventitious shoot organogenesis among some of the cell wall components. For this, we made a histological study in tissues that showed bud primordia formation and in those which did not, in order to characterise the cellular modifications accompanying the process. Furthermore, some cell wall components were immunolocated using specific antibodies in both types of tissues.

Materials and Methods

Plant material

Stem cuttings collected from adult trees of one *Populus deltoides* x *P. nigra* L. (*P. x euramericana*) hybrid clone (clone MC) and one *P. deltoides* x *P. trichocarpa* (*P. x interamericana*) hybrid clone (clone UNAL) were supplied from the National Centre of Forest Genetic Resources (“El Serranillo”, Guadalajara, Spain). Cuttings with 4-5 dormant buds were washed and treated with dicopper chloride trihydroxide (16% w/v, 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 and photoperiodic conditions (16h light/day). Dormant buds started to sprout after one week, and some of developing shoots have five fully expanded leaves after 3-4 weeks. These sprouted shoots represented the material to initiate the *in vitro* culture.

Sterilization procedures and in vitro culture conditions

Growing leaves and stems were excised from young shoots, washed and surface sterilized with 70% ethanol v/v (1 min), followed by 0.4% sodium hypochlorite w/v with 0.01% Tween-20 v/v for 10

and 15 min, for leaves and stems respectively. After being rinsed four times with sterile distilled water, leaves pieces (1 x 0.5-1 cm) and internodal segments (1 cm in size) were prepared, and some cuts were made. All handlings were performed under sterile conditions and placing the explants on filter paper imbibed in a 0.1 g/L ascorbic acid and 0.15 g/L citric acid solution to avoid the oxidation of external tissues.

Leaf and internode explants were cultured in media adjusted to pH 5.8 and supplemented with 0.9% w/v agar prior to autoclaving. The cultures were sealed and set in a growth room at 25±2°C under a 16h photoperiod conditions provided by cool white fluorescent light (Grolux F36/GRO lamps, Sylvania) at 2.000 lx. All experiments were performed under these conditions unless otherwise noted.

Shoot regeneration

For induction of adventitious shoot buds in leaf and internode segments, three different N⁶-Benzyladenine (BA) concentrations with a constant concentration α-Napthalene acetic acid (NAA) were assessed. Explants were cultured in Petri dishes containing 20 mL of induction media, consisting on salt mineral nutrients and vitamins of Murashige and Skoog (MS) (1962), 3% w/v sucrose, 0.05% w/v Kolliphor P-188 —a wetting agent with surfactant properties—, 0.5 μM NAA and 4, 16 or 40 μM BA. After 2 weeks of culture, the explants were transferred to culture vessels with the same media but without Kolliphor P-188 and supplemented with 0.5% w/v activated charcoal (AC). Then, cultures were maintained in growing conditions during 5 weeks more for buds differentiation and shoot development.

In a second set of experiments, internode segments were inoculated on induction media with a lower NAA concentration (0.1 μM) than earlier, and two BA concentrations (1 and 4 μM). On the other hand, to test the effect of Kolliphor P-188 on organogenic response, internode explants were also cultured in both media but devoid of the surfactant agent. The whole regeneration period newly consisted in 2 weeks of culture in the described media and 5 weeks in the same media with 0.5% w/v AC and without Kolliphor P-188.

In order to improve bud formation on leaf explants, leaf pieces of the two hybrid clones (MC and UNAL) were cultured on induction medium with 0.5 μM NAA and 4 μM BA during 5 weeks, followed by another 5 weeks of incubation in the same medium with 0.5% w/v AC. So that, the whole shoot regeneration period in this third experiment was 10 weeks.

In the three experiments about shoot regeneration, each treatment comprised seven replicates (plates), each containing four explants. At the end of the regeneration period (7 or 10 weeks), we determined the survival, the percentage of alive forming-buds explants and the mean number of shoots regenerated per responding explant. Tissues showing shoot buds in any developmental stage were considered to count budding explants. Nevertheless, only shoot buds higher than 2 cm were took into account to calculate the mean number of shoots per explants. These two variables defined

the shoot regeneration ability of explants. Data obtained from all experiments were presented as the means $\pm$ ES of the seven replicates.

Rooting and acclimatization

Regenerated shoots longer than 2 cm were separated from explants and cultured on half-strength MS medium supplemented with 1.5% w/v sucrose and 0.1 μ M NAA or without the synthetic auxin for inducing roots. All rooting cultures, consisting on one shoot per culture vessel, were kept in the culture room. Once shoots were rooted and reached a minimum length of 7 cm, plantlets were acclimatized. Before that, the rooting percentage, the mean number of roots per shoot and the mean 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 internal humidity and they were placed in the growth room. Cultures were watered first with 30 mL of mineral solution (Hoagland and Arnon 1950) and thereafter with 10 mL of water every 2 days. After the acclimatization by progressively opening the plastic bags during 3 weeks, cultures were transferred to room conditions. The percentage of acclimatized plants was evaluated separately for plants rooted on media with and without NAA after one month.

Statistical analysis

Data were analysed for Normal distribution and for homogeneity of variance using the Kolmogorov-Smirnov and the Levene test respectively. When both tests were positive and data were grouped in k sets, they were evaluated applying the analysis of variance (ANOVA, F), and the groups that showed variance were then subjected to Tukey range test to determine the significance of differences. T-Student test (t) was used with the same objective for data grouped in 2 sets. When the K-S and/or Levene tests were negative, data were analysed employing the non-parametric Kruskal-Wallis test (H) for k groups and the Mann-Whitney test (U) for 2 groups. The significance level (p -value) was 0.05 for all analysis and statistical tests were performed using IBM-SPSS Statistic (version 21) software.

Histological study and immunolocalization of cell wall components

Tissues (*in vivo* and *in vitro* internode) were fixed in 2% w/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 resin (LR White, 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 days. One micrometre-thick sections (1 μ m width) were obtained in a Ultracut microtome LKB 2088 (Reichert-Jung, 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% w/v blue toluidine in 1% w/v sodium borate solution and then observed on an Olympus BX61 microscope.

In order to immunolocate cell wall components, serial sections were incubated for 2 h with a 1/10 dilution of primary monoclonal antibodies JIM5, JIM7, LM5 or LM6 in phosphate-buffered saline (PBS: 0.14 M NaCl, 2.7 mM KCl, 7.8 mM Na₂HPO₄ 12H₂O, 1.5 mM KH₂PO₄, pH 7.2) containing 4% w/v fat-free milk powder (MPBS). These antibodies specifically recognised the pectin-polysaccharides of the cell wall shown in Table 1. After washing exhaustively with PBS, sections were incubated in darkness for 2 h with a 1/100 dilution of an antirat immunoglobulin G linked to fluorescein isothiocyanate (FITC, Sigma) in MPBS at room temperature. Finally, sections were newly washed with PBS and mounted in a glycerol/PBS-based antifade solution (Citifluor AF1; Agar Scientific, London, UK). Observations were performed with the Olympus BX61 microscope equipped with epifluorescence irradiation and using filters 450-490 nm FT-510-LP-520, proper to observe green emission of FITC.

Results and Discussion

In vitro regeneration for the MC and UNAL hybrid clones.

In the first experiment of this study, two sources of explants (leaf and internode segments) and three BA concentrations in combination with 0.5 µM NAA were used for *in vitro* shoot regeneration from MC and UNAL, two clones of *P. x euramericana* and *P. x interamericana* hybrids respectively. Few results were obtained from leaf explants because most of them suffered browning and became completely necrotic after 4-5 weeks of culture (Fig. 1a). In fact, only 30.95% ± 3.98 of leaf explants survived vs. 76.19% ± 4.34 of internode explants, this difference being significant (U= 233.5, *p*= 0.000). Even the surviving leaf explants showed a very low ability to form adventitious shoots (Table 2). Browning and necrosis often affect woody tissue cultures, especially in angiosperm and particularly in *Populus* spp. (Confalonieri 2003). Browning is mainly caused by polyphenolic compounds exuded from the cut edges of explants, which can inhibit the organogenesis and provoke necrosis. Researchers have partially overcome this problem in *Populus* tissue by frequent sub-culturing of explants (Maheshwari and Kovalchuk 2011) and/or by adding adsorptive materials such as AC (Kang et al. 2009) or polyvinylpyrrolidone (Thakur et al. 2012) into the culture medium. In the present work, tissue treatment with an antioxidant solution, plus explants transference to a medium with AC two weeks after the start of culture, were enough to avoid necrosis in almost all internode segments but not in leaf explants.

Culture of internode segments from MC and UNAL cultivars in the presence of BA and NAA stimulated adventitious buds regeneration at the cut ends within 2 weeks (Fig. 1b). The sub-culture of explants onto the same medium with AC and without Kolliphor P-188 allowed for the

development of shoots and accentuated shoot regeneration (Fig. 1e, f). Statistical analysis determined that MC showed greater efficiency to produce shoots than UNAL ($t= 2.461, p= 0.015$). The strong genotype dependence for *in vitro* shoot organogenesis was earlier observed in four *P. x interamericana* and two *P. x euramericana* cultivars by Noël et al. (2002), who pointed out the convenience of adjusting the concentration of some medium components to each cultivar. In this way, the number of shoots-forming stem discs in a medium with zeatin (Z) was influenced by the genotype of the explants belonging to five different clones of *P. x euramericana* (Cui et al. 2014). Among the various BA concentrations tried in bud induction medium, it was found that 4 μM was more effective than 16 and 40 μM (Table 2), since the number of shoots per internode explant was significantly higher in the medium containing 4 μM BA for both clones ($F= 12.871, p= 0.000$ for MC, and $F= 8.744, p= 0.003$ for UNAL). The maximum value of the percentage of responding explants was also observed in the presence of 4 μM BA (Table 2). Moreover, the increase of cytokinin concentration caused callus formation around the cut ends of explants to the detriment of shoot organogenesis (Fig. 1c, d). BA has been successfully used as inductor of shoot organogenesis in calli and in explants from leaf, petiole, node and internode segments of *P. deltoides* and its hybrids (Chaturvedi et al. 2004; Mingozi et al. 2008; Maheshwari and Kovalchuk 2011; Thakur et al. 2012; Cui et al. 2014; Kwon et al. 2015). However, side effects of too high concentrations of BA, such as decline in shoot bud formation and increase in associated callusing, were observed by Chaturvedi et al. (2004), Cui et al. (2014) and in the present work. Similar negative effects have been described not only for BA but also for kinetin (Yadav et al. 2009), Z (Cui et al. 2014) and thidiazuron (TDZ) (Noël et al. 2002), which even caused hyperhydricity and morphological abnormalities of regenerated shoots.

Taking into account our results, we proposed a second experiment to verify if BA and NAA concentrations lower than 4 and 0.5 μM could enhance the organogenic response of internodes of MC and UNAL clones. Thus, explants were cultured in a new medium containing less NAA (0.1 μM), and in other with lower concentrations of both (1 μM BA and 0.1 μM NAA). For the MC clone, the results obtained for the two variables defining shoot regeneration ability of explants were worse than the previous ones (Tables 3 & 2). The response of UNAL explants in the medium with the lowest BA and NAA concentrations was close to the best values that were observed earlier (Tables 3 & 2). In the second experiment, we also evaluated the efficiency of Kolliphor P-188 as a differentiation-stimulating agent, culturing MC and UNAL internode explants in the described media but in the absence of this compound. Kolliphor P-188 is equivalent to Pluronic F-68, the non-ionic co-polymer surfactant successfully used by Iordan-Costache et al. (1995) and by Noël et al. (2002) to enhance shoot regeneration induced by BA from leaf explants of a *P. tremula x P. tremuloides* hybrid clone and by TDZ from calli of five *P. x deltoides* hybrid clones respectively. In our work, the presence of 0.05% w/v Kolliphor P-188 in culture media neither had a significant effect on the number of shoots formed per explant ($H= 6.122, p= 0.106$ for MC, and $F= 0.686, p= 0.572$ for UNAL)

(Table 3), nor on the percentage of bud-forming explants for MC clone ($H = 0.938$, $p = 0.816$) (Table 3). The lack of the expected positive influence of Kolliphor P-188 could be related with the fact that we only tested one concentration, whereas Iordan-Costache et al. (1995) and Noël et al. (2002) checked concentrations of Pluronic F-68 ranging from 0.001 to 0.1% w/v. In agreement with this idea, Noël et al. (2002) obtained the best results when they modulated the Pluronic F-68 concentration according to the cultivar.

In an attempt to improve shoot regeneration from leaf segments, explants were cultured in induction medium during 5 weeks instead of 2 before sub-culturing, according to the protocol of Mingozzi et al. (2008). Considering our previous results, the medium in this third experiment contained 4 μM BA and 0.5 μM NAA and lacked Kolliphor P-188. As shown in Table 4, we observed that the extension of the culture period increased the survival of leaf explants from 30.95% to 50-53.57%. The enhancement of shoot regeneration was even more noticeable, so the percentage of responding tissues increased by around 20% (Tables 2 & 4), and the mean number of shoots formed per explant reached 4.28 for MC and 3.5 for UNAL (Table 4) (Fig. 1g). An explanation for the results may be that leaf explants required an initial culture period longer than 2 weeks to adapt to the *in vitro* conditions. Therefore, the interruption of this period by sub-culturing might have reduced the survival of tissues and hampered the process of bud induction and development already initiated. This hypothesis is supported by the following: problems of necrosis and low regeneration potential of *P. deltoides* and *P. x deltoides* tissues were irrelevant when leaf explants derived from previously *in vitro* grown plants and/or shoots (Chaturvedi et al 2004; Yadav et al. 2009), being pre-adapted to these conditions. However, these problems appear if cultured tissues come directly from plants grown in a greenhouse or from adult trees (Maheshwari and Kovalchuk 2011; Thakur et al. 2012; present work)

In order to complete the protocol for *in vitro* plant regeneration for MC and UNAL hybrid clones, adventitious shoots longer than 2 cm (Fig. 1h) were transferred to rooting medium. 100% of shoots easily rooted without callus intervening, and elongated up to 7 cm within 3 to 5 weeks. The presence of NAA did not affect the mean number of roots formed per shoot ($t = -1.865$, $p = 0.073$) (Table 5), but influenced the length of the longest root ($t = 4.532$, $p = 0.000$) and the morphology of the root system. In its absence, shoots formed long and well-developed roots with fine lateral roots (Fig. 1i), while shoots cultured on medium with NAA regenerated shorter and thicker roots with anomalous appearance (Fig. 1j). It is well known that high concentration of auxin inhibits the elongation of adventitious roots (Li et al. 2009), so the mentioned disorders could be caused by an excess of auxin activity produced as consequence of the accumulation of endogenous auxin and exogenous NAA in the basal cut 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 root system determined the success of the further acclimatization of regenerated plants (Fig. 1k) which was significantly higher when the rooting process took place in the NAA-free medium ($U= 60.000$, $p= 0.029$) (Table 5). It was possible that fine, branched and long roots ensured water uptake from deep layers of substrate during acclimatization, whereas shoots with shorter and shallower roots induced in presence of NAA could not be able to have access to that water reservoir. Then, these last plants could have suffered brief chapters of water deficit between watering periods leading to more acclimatization faults. The importance of the regeneration of an efficient adventitious root system with proper morphology and length, which ensures water uptake, has been previously revealed for *Populus* hardwood cuttings employed in short rotation forestry systems (Zalesny and Zalesny 2009). The major factor limiting the success of these plantations is the delay in root length growth, together with an increasing number of precipitation-free periods during the phase of establishment in field, when developing plants do not have access to groundwater and they do not withstand drought periods (Krabel et al. 2015). Thus, to induce rhizogenesis from *in vitro* regenerated shoots of MC and UNAL we recommend the use of a hormone-free medium, as well as Noël et al. (2002) and Mingozi et al. (2008) suggested for several cultivars of *Populus*.

Immunolocalization of some cell wall components during adventitious bud induction

The plant cell wall is a dynamic structure surrounding the protoplasm of the cell with important functions in organ morphogenesis. Specifically, *in vitro* shoot organogenesis is initiated by changes in the gene expression pattern that occurred mainly during cell dedifferentiation (Che et al. 2006; Bao et al. 2009), and that results sequentially in cell division and in structural and biochemical specialization of the new determined cells to form bud primordia. All these cellular processes are regulated by the chemical structure and mechanical properties of the cell wall (Chebli and Geitman 2017), and the correct deposition and assembly of cell wall materials is critical for coordinating them (Ikeuchi et al. 2013). The microscopic techniques are a powerful tool to understand better the cellular processes that underlie adventitious organogenesis in *Populus*. Moreover, the microscopic analysis may be combined with immunohistological studies to locate some cell wall components in tissues that suffered shoot organogenesis, in order to make a comparison with the not organogenic ones. If differences were found, the corresponding cell wall components could be used as markers of shoot bud initiation. For this last purpose, we used monoclonal antibodies towards several cell wall polysaccharides. The studies were developed in internode segments of UNAL clone after 5 weeks of culture in the medium with 4 μ M BA and 0.5 μ M NAA that shown adventitious organogenesis (Fig. 2.3). In addition, to establish the possible polysaccharides-markers properly, two types of controls were made. The first one consists in an internode segment never cultured *in vitro* (Fig. 2.1: *ex vitro* control) and the other was a piece of internode tissue cultured *in vitro* but without organogenic response (Fig. 2.2: *in vitro* control). Besides, a piece of tissue with dedifferentiation processes (Fig. 2.4: callogenesis) was taken.

The microscopy slice of the internode segment of poplar never cultured *in vitro* (*ex vitro* control) (Fig. 2a) was composed 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 –the ground tissue in plants—was between epidermis and the vascular cambium and it was constituted of live cells with thin primary cell walls that could become meristematic at the maturity state if they were properly stimulated. Under the parenchyma tissue, the vascular cambium was observed. Vascular cambium is a secondary meristem, which produces the two transport tissues: phloem outside and xylem inside. The most internal parenchyma tissue was the pith tissue. However, tissue organization in plants is not clear and some cells shown transition shapes between contiguous tissues (Álvarez 2002).

Under *in vitro* culture, tissues showed a thickening and an increase in size on the section zone (Fig. 2.2). Nevertheless, on *in vitro* control only minor disorganization on vascular tissue was shown although the parenchyma cells were swollen allowing more aerial spaces among cells (Fig. 2b). This increase in size in the tissues, primarily in the cut off zone, was promoted by the plant growth regulators present in the culture media as well as the dedifferentiation of the cells due to the wound (Lup et al. 2016). During adventitious organogenesis swelling around the cut off zone was observed which corresponds with putatively adventitious primordia (Fig. 2.3, c). In those primordia, the parenchymatic cells just under uniseriate epidermal cells were smaller, isodiametric and more compactly connected to each other, whereas the aerial spaces into the inner parenchyma tissue were larger than on the *in vitro* control. When adventitious primordia were induced using leaf explants of MC poplar clone (Giner 2013) or *Populus euphratica* Oliv explants (Ferreira et al. 2009) this kind of cellular organization was also observed. On the other hand, callogenesis induced a complete structural disorganization in all tissues and as a result, cells were irregular and utterly different in size (Fig 2.4, d).

As it was earlier mentioned, the plant cell wall is a semi rigid structure that limits and orients the cellular growth, and it has a main role in the morphology and differentiation of cell types. Pectins are cell wall acid polysaccharides implicated in extensibility and rigidity of the cell wall, and both properties are crucial points during cell division and development (Wolf et al. 2012; Levesque-Tremblay et al. 2015). One of these acid polysaccharides present in the cell walls is the homogalacturonan (HG) which is a homopolymer of 1,4-linked α -D-galacturonic acid with different degree and pattern of methyl-esterification (Ridley et al. 2001). In fact, it has been demonstrated that the degree of methyl-esterification of the pectins determines the elasticity of the tissues, which is crucial for cell division (Paucele et al. 2008, 2011). Other pectins like rhamnogalacturonan I (RG-I) have the ability to modify the extensibility of the cell wall crosslinking with the cellulose (Zykwinska 2005) and other polysaccharides and proteins (Scheller and Ulvskov 2010) through their side chains. In the present work, different antibodies towards HG with low (JIM5) and high (JIM7) degree of

methyl-esterification, β -1,4-D-galactan (LM5) and α -1,5-L-arabinan (LM6) side chains of RG-I, were used as possible organogenetic markers.

Internode segments extracted from bud sprouting (Fig. 2.1: *ex vitro* control) shown an homogenous labelling with JIM7 and JIM5 antibodies although the JIM7 signal was more intense in all tissues (Fig. 2e, i). Similarly, in these internode sections the immunofluorescence of arabinan side chains of RG-I labelling with LM6 was homogenous and intense especially in the meristematic cells of the cambium (Fig. 2q). These results were in accordance with other works that point to a high presence of epitopes recognized by JIM7 (Bosch and Hepler 2005) as well as epitopes recognized by LM6 (Willats et al. 1999) in tissues growing actively, like the tissues present in *ex vitro* controls. However, the epitope recognized by LM5 had been associated with differentiated cells (Serpe et al. 2001; Bush et al. 2001). 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. 2m).

When internode segments were cultured *in vitro* (Fig 2.2: *in vitro* control) all the antibodies assayed shown less labelling compared with *ex vitro* control. This reduction in the signal was more appreciable on JIM7 (Fig. 2f) and LM6 (Fig. 2r) labelling which could be in accordance with the lack of growth and cell division in these tissues.

The explants with adventitious organogenesis response (Fig 2.3: caulogenesis) shown an increase of labelling with JIM5 (Fig. 2g) in the subepidermal zone. These results were in agreement with the organogenesis processes in leaf of MC poplar clon (Giner 2013). During organogenesis performed in *Arabidopsis*, Peaucelle et al. (2008, 2011) demonstrated that the homogalacturonan with low degree of methyl esterification (recognized by JIM5) temporarily increase in subepidermal zone before a lack of stiffness and an increase of elasticity take place in the cell walls. No changes in LM5 and LM6 (Fig. 2o, s) were appreciated during organogenesis compared with *in vitro* control.

Tissues with cell dedifferentiation processes (Fig 2.4: callogenesis) shown an intense immunofluorescence when they were labelled with JIM5 and especially with JIM7 antibodies (Fig. 2h, i). This highly methylated homogalacturonan increase into cell wall is proper to cells which are growing and/or dividing actively. Although in dividing carrot cell suspension an increase in arabinan side chain of RG-I (detected by LM6) was described (Willats et al. 1999), our calluses shown less LM6 epitopes compared with controls (Fig. 2t). Nevertheless, it must be considered that the methyl esterification degree of HG and the presence or not of side chain in RG-I and their bonding to other cell wall components change depending on the species, development stage, organ, tissue or cellular type (Wolf et al. 2009, 2012; Levesque-Tremblay et al. 2015).

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Fig. 1 *In vitro* plant regeneration in *P. x euramericana* clone MC and in *P. x interamericana* clone UNAL from internode and leaf explants. **a** Necrotic aspect of leaf explant cultured for 2 weeks in a first induction medium and other 5 weeks in the same medium without Kolliphor P-188. **b** Shoot organogenesis from internode explant of MC clone after 2 weeks of culture in a medium with 4 μ M BA and 0.5 μ M NAA. **c & d** Callus development from internode explants of MC clone cultured 7 weeks on media with 0.5 μ M NAA and 16 (c) or 40 μ M BA (d). **e & f** Shoot regeneration from internode explants of MC (e) and UNAL (f) clones cultured for 7 weeks on medium with 4 μ M BA and 0.5 μ M NAA. **g** Shoot regeneration from leaf explants of UNAL clone cultured on medium with 4 μ M BA and 0.5 μ M NAA for 10 weeks instead of 7. **h** Aspect of regenerated shoot of UNAL clone before being excised from explant and transferred to rooting medium. **i & j** Root systems developed from shoots of MC clone after culture in a free-hormone medium (i) or in a medium containing 0.1 μ M NAA. **k** Acclimatized plant of *P. x euramericana* clone MC

Fig. 2 Internode segments of UNAL clone before (**1**: *ex vitro* control) and after 5 weeks of culture without morphogenesis response (**2**: *in vitro* control), with organogenesis (**3**: caulogenesis) or callogenesis (**4**) responses. The culture medium contained in all cases 4 μ M BA and 0.5 μ M NAA. Toluidine blue-stained section of the internodal segments (**a-d**) and immunofluorescence due to JIM7 (**e-h**), JIM5 (**i-l**), LM5 (**m-p**) and LM6 (**q-t**) antibodies

Table 1 Monoclonal antibodies (mAb) used in the cell wall studies, as well as the antigen and epitope they recognize. HG: homogalactouronan; RG-I: ramnogalatocuronan-I

mAb	Antigen / epitope	References
JIM5	HG with low degree of methyl-esterification	Knox et al. (1990), Willats et al. (2000)
JIM7	HG with high degree of methyl-esterification	Knox et al. (1990), Willats et al. (2000)
LM5	β -1,4-D-galactan side chains of RM-I	Jones et al. (1997), Willats et al. (1999)
LM7	α -1,5-L-arabinan side chains of RM-I	Willats et al. (1998)

Table 2 Effect of BA concentration on shoot regeneration ability of explants of two *Populus x.* clones. Internode and leaf explants were cultured on induction medium containing BA, 0.5 μ M NAA and 0.05% w/v Kolliphor P-188 during 2 weeks, and then transferred to the same medium without Kolliphor P-188 and in the presence of activated charcoal. Data were recorded after 5 weeks in this last medium. Values are means $\pm$ ES for n= 7 replicates (4 explants per replica)

Explant	Clone	BA (μ M)	Shoot regeneration ability	
			Explants ¹ forming buds (%)	Mean number of shoots $\geq$ 2 cm per explant ²
Internode segment	MC	40	75.00 $\pm$ 13.12	0.33 $\pm$ 0.37 a
		16	91.67 $\pm$ 5.89	1.94 $\pm$ 0.59 a
		4	100	3.85 $\pm$ 0.54 b
	UNAL	40	62.50 $\pm$ 22.08 a	0.08 $\pm$ 0.09 a
		16	77.78 $\pm$ 12.17 a	0.42 $\pm$ 0.22 a
		4	82.14 $\pm$ 11.35 a	1.73 $\pm$ 0.40 b
Leaf segment	MC	40	0	-
		16	16.68 $\pm$ 11.11	0.33 $\pm$ 0.41 a
		4	66.67 $\pm$ 40.83	0 a
	UNAL	40	8.33 $\pm$ 9.13	0 a
		16	16.68 $\pm$ 18.26	-
		4	19.05 $\pm$ 15.43	1.00 $\pm$ 0.71 a

Means followed by the same letters for each variable, explant and clone are not significantly different at $p \leq 0.05$ according to parametric tests ANOVA and Tukey or T-Student. Data that did not adjust to a Normal distribution were analysed using Kruskal-Wallis non-parametric test and the means do not appear followed by letters

¹ Only survival explants were included

² Only responding explants were included

Table 3 Effect of BA concentration and of the presence/absence of Kolliphor P-188 (0.05% w/v) on shoot regeneration ability of internode segments of two *Populus x.* clones. Explants were cultured on induction medium containing BA, 0.1 μ M NAA and with or without Kolliphor P-188 during 2 weeks, and then transferred to the same medium without Kolliphor P-188 and with activated charcoal. Data were recorded after 5 weeks in this last medium. Values are means $\pm$ ES for n= 7 replicates (4 explants per replica)

Clone	BA (μ M)	Kolliphor P-188	Shoot regeneration ability	
			Explants ¹ forming buds (%)	Mean number of shoots $\geq$ 2 cm per explant ²
MC	4	+	33.33 $\pm$ 19.24	1.83 $\pm$ 0.74
	1	+	52.38 $\pm$ 17.31	1.00 $\pm$ 0.00
	4	-	53.33 $\pm$ 25.28	1.00 $\pm$ 0.00
	1	-	57.14 $\pm$ 21.82	1.25 $\pm$ 0.20
UNAL	4	+	51.20 $\pm$ 17.40 ab	1.70 $\pm$ 0.22 a
	1	+	78.57 $\pm$ 9.18 a	2.07 $\pm$ 0.31 a
	4	-	30.95 $\pm$ 13.42 b	1.83 $\pm$ 0.33 a
	1	-	78.57 $\pm$ 12.40 a	1.56 $\pm$ 0.33 a

Means followed by the same letters for each variable and clone are not significantly different at $p \leq 0.05$ according to parametric tests ANOVA and Tukey. Data that did not adjust to a Normal distribution were analysed using Kruskal-Wallis non-parametric tests and so, the means do not appear followed by letters

¹ Only survival explants were included

² Only responding explants were included

Table 4 Survival and shoot regeneration ability of leaf segments of two *Populus x.* clones cultured on induction medium with 0.5 μ M NAA and 4 μ M BA during 5 weeks, and then transferred to the same medium with activated charcoal. Data were recorded after 5 weeks in this last medium (10 weeks of whole culture period). Values are means $\pm$ ES for n=7 replicates (4 explants per replica)

<i>P.x</i> clone	Survival (%)	Shoot regeneration ability	
		Explants ¹ forming buds (%)	Mean number of shoots $\geq$ 2 cm per explant ²
MC	50.00 $\pm$ 8.83 a	85.71 $\pm$ 15.43	4.28 $\pm$ 0.88 a
UNAL	53.57 $\pm$ 11.88 a	42.86 $\pm$ 21.82	3.50 $\pm$ 1.06 a

Means followed by the same letters for each variable are not significantly different at $p \leq 0.05$ according to T-Student test. Data that did not adjust to a Normal distribution were analysed using Mann-Whitney non-parametric test and so, means do not appear followed by letters

¹ Only survival explants were included

² Only responding explants were included

Table 5 Effect of NAA concentration in the rooting response of regenerated shoots and in the acclimatization of plantlets of *Populus x*. Isolated shoots were cultured on rooting medium with 0.1 μ M NAA or without the auxin regulator. Rooting response of shoots was evaluated once shoots reached a minimum length of 7 cm. Number of acclimatized plants was recorded after seven weeks of acclimatization period. Values are means $\pm$ ES for n=15 replicates (1 shoot or plantlet per replica)

NAA (μ M)	Rooting response			Acclimatized plants (%)
	Shoots forming roots (%)	Mean number of roots per shoot	Mean length of the longest root (cm)	
0	100	2.87 $\pm$ 0.41 a	5.77 $\pm$ 0.59 a	73.33 $\pm$ 12.24
0.1	100	3.93 $\pm$ 0.42 a	2.29 $\pm$ 0.34 b	26.67 $\pm$ 12.24

Means followed by the same letters for each variable are not significantly different at $p \leq 0.05$ according to T-Student test. Data sets that did not adjust to a Normal distribution were analysed using Mann-Whitney non-parametric test and so, means do not appear followed by letters

Fig 2. Toluidine blue-stained section of internode segments of *P. x interamericana* 'UNAL' before (A, *ex vitro* control) and after five weeks of culture without morphogenesis response (B, *in vitro* control), with organogenesis (C) or callogenesis (D) responses. The culture medium contained in all cases 4 μ M BA and 0.5 μ M 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 μ m in A, B, C and 100 μ m in D.

275x206mm (300 x 300 DPI)

Fig 3. Immunolocalization of HG with a high degree of methyl esterification (JIM7) in internodal segments of *P. x interamericana* 'UNAL' before (A, *ex vitro* control) and after five weeks of culture without morphogenesis response (B, *in vitro* control), with organogenesis (C) or callogenesis (D) responses. The culture medium contained in all cases 4 μ M BA and 0.5 μ M NAA. Scale bars = 200 μ m in C, D and 100 μ m in A, B.

190x142mm (300 x 300 DPI)

Fig 4. Immunolocalization of HG with a low degree of methyl esterification (JIM5) in internodal segments of *P. x interamericana* 'UNAL' before (A, *ex vitro* control) and after five weeks of culture without morphogenesis 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, D, 100 µm in A, B and 50 µm in C*.

190x142mm (300 x 300 DPI)

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

190x142mm (300 x 300 DPI)

Fig 6. Immunolocalization of α -1,5-L-arabinan side chains of RG-I (LM6) in internodal segments of *P. x interamericana* 'UNAL' before (A, *ex vitro* control) and after five weeks of culture without morphogenesis response (B, *in vitro* control), with organogenesis (C) or callogenesis (D) responses. C# is an image of the vascular tissues of the C section. The culture medium contained in all cases 4 μ M BA and 0.5 μ M NAA. Scale bars = 50 μ m in C# and 100 μ m in all others.

190x142mm (300 x 300 DPI)

1 **Supplementary Material**

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3 Table. Monoclonal antibodies (mAb) used in the cell wall studies, as well as the antigen and
4 epitope they recognize. HG - homogalacturonan; RG-I - **rhamnogalatocuronan-I**.

mAb	Antigen / epitope	References
JIM5	HG with a low degree of methyl-esterification	Knox <i>et al.</i> (1990), Willats <i>et al.</i> (2000)
JIM7	HG with a high degree of methyl-esterification	Knox <i>et al.</i> (1990), Willats <i>et al.</i> (2000)
LM5	β -1,4-D-galactan side chains of RG-I	Jones <i>et al.</i> (1997), Willats <i>et al.</i> (1999)
LM6	α -1,5-L-arabinan side chains of RG-I	Willats <i>et al.</i> (1998)

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12 Fig. Plant material from *P. x interamericana* 'UNAL' used in the histological study and
13 immunolocation of pectin domains. Internode segments before (*A*, *ex vitro* control) and after five
14 weeks of culture without morphogenesis response (*B*, *in vitro* control), with organogenesis (*C*) or
15 callogenesis (*D*) responses.

16