University of Tabriz

Journal of Plant Physiology and Breeding

Print ISSN: 2008-5168 Online ISSN: 2821-0174

2025, 15(1): 17-36

Parenchymal motor cells of pulvini base: An ideal explant for efficient direct regeneration in white poplar

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Article Info

Article type:
Research article

Article history:
Received January 19, 2025
Revised February 19, 2025
Accepted March 19, 2025
Published online June 30, 2025

Keywords:
Direct organogenesis,
Microscopy analysis,
Populus alba,
Pulvinus,
Thin cell layer,
Tissue maturity culture,
tTCL

Abstract

Objective: Poplars (*Populus* sp.) are the most industrially important woody plants, garnering significant global attention for the introduction of new traits using genetic engineering and gene/genome editing approaches. However, the efficacy of genetic transformation and regeneration response of plant explants remains challenging among *Populus* species due to high variation in their genetic background. This work aimed to identify an optimal explant source to enhance high-frequency genetic transformation and genome editing capabilities in white poplar.

Methods: Transverse and longitudinal thin cell layer (t/lTCL) explants from *Populus alba* L. pulvinus and stem tissues were cultured on the shoot induction media with varying concentrations of BAP (0, 0.25, 0.5, 0.75, and 1.0 mg/L) in combination with IBA/NAA (0, 0.25, 0.5, and 0.75 mg/L) to evaluate their regeneration potential. The effect of node position on pulvini-tTCL regeneration was assessed, and histological analysis was performed on highly responsive explants. The root induction was examined using media with graded levels of IBA (0, 0.1, 0.2, 0.3 mg/L) and NAA (0, 0.05, 0.1, 0.15, and 0.2 mg/L).

Results: We obtained the fast and highly responsive tTCL-mediated shoot regeneration from the parenchyma cell layers (400-500 µm thickness) of the pulvinus basis of the 4th node cultured on shoot induction medium containing BAP 0.75 mg/L and IBA 0.5 mg/L, with a mean of 43.59 ± 0.22 shoots per explant. The regenerated shoots were well-rooted in root induction medium containing IBA 0.2 mg/L and NAA 0.1 mg/L. The well-hardened plants were transferred to greenhouse conditions.

Conclusion: The superior capacity of pulvini-tTCL tissue provides an opportunity to achieve high-frequency transgenic white poplar in genetic engineering and genome editing approaches.

Cite this article: Nayeri S, Baghban Kohnehrouz B. 2025. Parenchymal motor cells of pulvini base: An ideal explant for efficient direct regeneration in white poplar. J Plant Physiol Breed. 15(1): 17-36.

Introduction

White poplar (*Populus alba* L.) from the willow/poplar family (*Salicaceae*) is widely distributed through a vast variety of climates around the globe (FAO 1979; Hamzeh and Dayanandan 2004; Yousefzadeh *et al.* 2019). The unique properties of white poplar, including vegetative propagation ability, fast growth rate, and high biomass production (about 299 oven-dry t ha^{-1} $year^{-1}$), satisfy an increasing worldwide demand for wood, timber, paper and pulp, biofuel, and cellulose-based future industries. Moreover, it is widely cultivated to manage reforestation and afforestation of lowlands as well as phytoremediation approaches (Balatinecz *et al.* 2001; Confalonieri *et al.* 2003; Norris *et al.* 2008; Chen *et al.* 2009; Porth and El-Kassaby 2015). Furthermore, poplar is used for genome editing, genomics, metabolomics, and forest biotechnology approaches due to its whole sequenced small genome, efficient potential in genetic transformation and regeneration (Confalonieri *et al.* 2003; Fan *et al.* 2015; Nayeri and Tohidfar 2017; Muhr *et al.* 2018; Nayeri *et al.* 2019; Movahedi *et al.* 2021; Nayeri *et al.* 2022a,b; Mahna and Nayeri 2024).

Several previous reports have emphasized the undeniable impacts of genetic background variation, and tissue and explant type, on the regeneration response of selected tissue explants and their genetic transformation efficiency in *Populus* species (Confalonieri *et al.* 2000; Han *et al.* 2000; Confalonieri *et al.* 2003). In several reports, the plant tissues such as lateral buds, leaf, internodal stem, and petiole were used as a regenerative explant for micropropagation and (in)direct regeneration in white poplar (*P. alba* L.) and its hybrid clones (Park and Son 1988; Son and Hall 1990; Son *et al.* 1993; Qiao *et al.* 1998; Jafari Mofidabadi and Modir-Rahmati 2000; Kang *et al.* 2000; Pintarić 2008; Wang *et al.* 2008; Bae *et al.* 2011; Wang *et al.* 2011; Tavassoli Asgari *et al.* 2013; Žiauka and Kuusienė 2014; Khosravan *et al.* 2017; Zeng *et al.* 2019; Li *et al.* 2023). Russin and Evert (1984) reported swollen tissues known as pulvinus at the base of petioles of *Populus tremula* using LM microscopy analysis, which is probably a species-specific tissue among *Populus* species. According to literature reviews, no report covers the regeneration response, the effects of explant type and position, and tissue maturity of pulvini tissue at the base of the petiole under different auxin and cytokinin plant growth regulators (PGRs) treatments.

The thin cell layer (TCL) culture method is an efficient and effective technique in plant tissue culture that has been introduced to enhance in vitro regeneration and micropropagation of a high number of plantlets in various plant species, including both crops and woody plants. The TCL explants with 0.5-1 mm in size are divided into two types, including transversally (tTCL) or longitudinally (lTCL) sliced explants from the tissue of interest (Klimaszewska and Keller 1985;

Detrez *et al.* 1988; Heylen and Vendrig 1988; Carimi *et al.* 1999; de Carvalho *et al.* 2000; Nhut *et al.* 2000; Trân Thanh Vân and Van Lê 2000; Nhut *et al.* 2001; Zhao *et al.* 2007; Scherwinski-Pereira *et al.* 2010). Only Lee-Stadelmann *et al.* (1989) showed that the transverse thin slices (100-500µm in size) of leaf mid-vein explants from poplar have been regenerated with low efficiency.

Here, we focused on the investigation of a high-capacity explant from pulvinus tissue at the base of the petiole of *P. alba* L. using the TCL culture technique for *in vitro* shoot regeneration without callus induction.

Materials and Methods

Plant material, surface sterilization, and TCL explant type

The young branches (45-50 cm in length and 0.2-0.5 cm in diameter) with fresh internodal stems and pulvini of petioles were isolated from a >10 years-old white poplar tree as the field-grown donor plant (Supplementary Figure 1A). The plant material was obtained from the Botanical Garden of the University of Tabriz, Tabriz, Iran (38° 03' 30.4" N latitude and 46° 20' 07.8" E longitude with an average rainfall of 330 mm). The cut branches (45-50 cm long and 0.2-0.5 cm diameter) were surface sterilized as described in Supplementary Method 1. The sampling was performed during mid-spring to early summer (May-August). The sterilized stem segments were divided into two tissue sources of explants: 1 cm pulvini bases of 1^{st}-7^{th} petioles (Supplementary Figure 1B), and the adjacent tissues of internodal stem segments close to the axillary buds (1 cm in length) (Supplementary Figure 1C). Each of the internodal stem and pulvini explants was split into slices longitudinally (with 0.5-1 mm thickness) and transversely (250-500 µm thickness) to be used as lTCL (Supplementary Figure 1D) and tTCL explants (Supplementary Figure 1E), respectively.

The effect of TCL explant type and PGR combination on shoot regeneration

Due to the unmanageably large-scale experiments, the four TCL explant types were examined in separate experiments using benzyl-amino-purine (BAP) in combinations with indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA) for shoot regeneration (Supplementary Tables 1 and 2). The shoot induction medium (SIM) was Murashige and Skoog (MS) (Murashige and Skoog 1962) supplemented with BAP at five concentrations (0, 0.25, 0.5, 0.75, and 1.0 mg/L) and IBA or NAA at four concentration each (0, 0.25, 0.5, and 0.75 mg/L), 3% sucrose, solidified by 0.6% agar (extra pure Agar CAS 9002-18-0, Merck, Germany) and autoclaved at 121 °C for 20 min. Finally, four TCL explants were compared under the best PGR combination (BAP, 0.75 mg/L; IBA, 0.5 mg/L) to obtain the high-capacity shoot regeneration on SIM. All experiments were carried out through eight factorial

treatments based on a completely randomized design (CRD) with three replicates. Each experimental unit (plate) contained either 100 tTCL or 40 lTCL explants. The cultures were maintained in the growth chamber at 26 °C and 16/8 hrs light/dark photoperiod. The light condition was set up at 4500 Lux light intensity using a cool white and yellow fluorescent lamp. The explants were subcultured every two weeks. The frequency of total shooting explants and the number of shoots per explant were determined after eight weeks and used for statistical analysis.

The effect of node position and pulvini tissue maturity on shoot regeneration capacity of pulvini-tTCL explant

To investigate whether node position affects the shoot regeneration capacity of pulvini-tTCL explants from *P. alba* L., the pulvini-tTCL explants from the bases of petioles 1st - 7th close to the apical bud were cultured on the SIM supplemented with BAP 0.75 mg/L and IBA 0.5 mg/L. The effect of pulvini maturity on the shoot regeneration capacity of the tTCL explant was evaluated by dividing pulvinus tissue (1 cm long) into five equal sections each ~2 mm long, including A-E sections (Supplementary Figure 1C). Each section was sliced into five pulvini-tTCL explants with ~400-500 µm thickness and cultured on the SIM supplemented by BAP 0.75 mg/L and IBA 0.5 mg/L. All experiments were carried out based on a CRD with three replicates. Each experimental unit (plate) for node position and pulvini maturity analysis contained 30 and 20 tTCL explants, respectively. The *in vitro* culture conditions were mentioned in the previous section.

Histological analysis

The two-week-old tTCL explants from region A of pulvini of the 4th petiole cultured on SIM were used for light microscopy analysis. The uncultured tTCL explant from region A of the pulvinus tissue of the 4th petiole was used as a control sample. The samples for light microscopy were fixed and sectioned as described in the Supplementary Method 2. The sections were dried for observation under a light microscope (BX41, Olympus, Tokyo, Japan) at ×100 and ×400 magnifications.

The root induction of the shoots

The regenerated shoots were cultured on shoot elongation medium containing MS medium supplemented with 0.15 mg/L BAP and 0.1 mg/L IBA for two weeks. The elongated shoots with 1-2 cm in length were transferred to the root induction medium (RIM) containing MS medium supplemented with IBA at four concentrations (0, 0.1, 0.2, and 0.3 mg/L) in combination with NAA at five concentrations (0, 0.05, 0.1, 0.15, and 0.2 mg/L). The experiment was carried out as a factorial

arrangement based on a CRD with three replicates. The samples were maintained in the growth chamber as described in previous sections. Each replicate contained five plantlets per 450-ml jar. The explants were sub-cultured every two weeks and the rooting rate (% of rooted plantlets per experimental unit), the number of roots per shoot, and the mean of root length per shoot were determined after eight weeks.

Acclimatization and hardening

The regenerated plantlets were gently washed under running tap water and acclimatized by transplanting into the pots. They were filled with a mixture of sterile sand: peat moss: perlite (3:2:1) and maintained in the growth chamber. The pots were covered with plastic bags to maintain a stable relative humidity of 70-85% for three weeks. Then, the hardened plants were planted into the pots with garden soil and transferred into a greenhouse.

Statistical analysis

The data normality was confirmed using the Kolmogorov-Smirnov test. After analysis of variance, means were compared by Tukey's test at $p \leq 0.05$. The statistical analysis was performed using IBM SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Effect of tissue source and TCL explant type on shoot regeneration

The shoot regeneration efficiencies of the TCL explants from both pulvini and internodal stem tissues were examined by culturing on the SIM. As a result, the pulvini at the base of the petioles showed a better regeneration response than those from the internodal stem. Furthermore, a significant increase in the size of the TCL explants following 10-12 days, and protrusions were observed within 15–20 days of culture of both internodal stems and pulvini of petioles. The tTCL explants from the internodal stem and pulvini of petioles further developed into shoots within 50–60 days, while the shoot development of lTCL explants from both tissues occurred within 70-80 days. Therefore, the results indicate that the source and type of explants could significantly affect the shoot regeneration capability in *P. alba* L.

The effect of PGRs combinations and concentrations on shoot regeneration of the t/lTCL explants

The shoot regeneration results related to the four explant types with two different PGR combinations were summarized in Supplementary Tables 1-4. Increasing BAP concentration (0.25-1.0 mg/L)

resulted in 100% shooting frequency in samples, regardless of the IBA concentration. However, the same and higher concentrations of IBA compared with BAP showed a callus induction without any shoot regeneration in all samples (Supplementary Table 1). The highest number of shoots was observed for pulvini-tTCL (26.3 ± 0.064), pulvini-lTCL (24.90 ± 0.071), internodal stem-tTCL (21.52 ± 0.065), and internodal stem-lTCL (14.77 ± 0.07) explants cultured on SIM containing 0.75mg/L BAP and 0.5mg/L IBA (Supplementary Table 2). However, in the presence of NAA in combination with BAP, the highest shooting frequency of 100% was obtained from SIM containing 0.5-1.0 mg/L BAP for all explant types (Supplementary Table 3). The highest number of shoots per explant was observed for pulvini-tTCL (7.8 ± 0.022), pulvini-lTCL (8.1 ± 0.023), internodal-stem tTCL (7.9 ± 0.022), and internodal stem-lTCL (7.88 ± 0.022) explants cultured on SIM containing 0.75mg/L BAP (Supplementary Table 4).

The mean comparison of TCL explants cultured on the best responsive PGR combination of BAP 0.75 mg/L and IBA 0.5 mg/L showed the highest shoot regeneration in the pulvinus-tTCL explant with the mean ± SE of 28.54 ± 0.006 shoots per explant with 2.49 ± 0.17 cm in length (Table 1).

The step-by-step shoot regeneration of pulvini-tTCL explant cultured on SIM during eight weeks was illustrated in Figures 1A-1D. The shortened procedure in regeneration from the pulvinus-derived tTCL explants on this medium resulted in the formation of multiple shoots without callus induction (Figure 1D).

Table 1. Comparing the shoot regeneration of internodal stems and pulvini in the t/lTCL explants, cultured on the shoot induction medium with 0.75mg/L BAP and 0.5mg/L IBA.

	Internodal stem		**Pulvinus of the petiole**	
	Transverse TCL	**Longitudinal TCL**	**Transverse TCL**	**Longitudinal TCL**
No. of shoots per explant	21.57 ± 0.07^{c}	15.04 ± 0.07^{d}	**28.54 ± 0.006^{a}**	24.75 ± 0.01^{b}

Means ± SE; Means with different letters are statistically significant at $p \leq 0.05$ based on Tuckey's test; t/lTCL: Transverse and longitudinal thin cell layer; Measurements were taken after eight weeks of culture.

High shoot regeneration response in pulvini-tTCL explants from intermediate node position

In the previous experiment, we observed a significantly high number of regenerated micro-shoots above the mean in some pulvini-tTCL explants cultured on SIM supplemented with BAP 0.75 mg/L and IBA 0.5 mg/L. With regards to the result, this hypothesis has arisen as to whether the regeneration response of the pulvini-tTCL explant is influenced by the pulvinus tissue maturity associated with the pulvinus of the petiole from related node positions along the shoot axis and the explant region in the pulvinus tissue. Therefore, we first designed an independent experiment to evaluate the regeneration

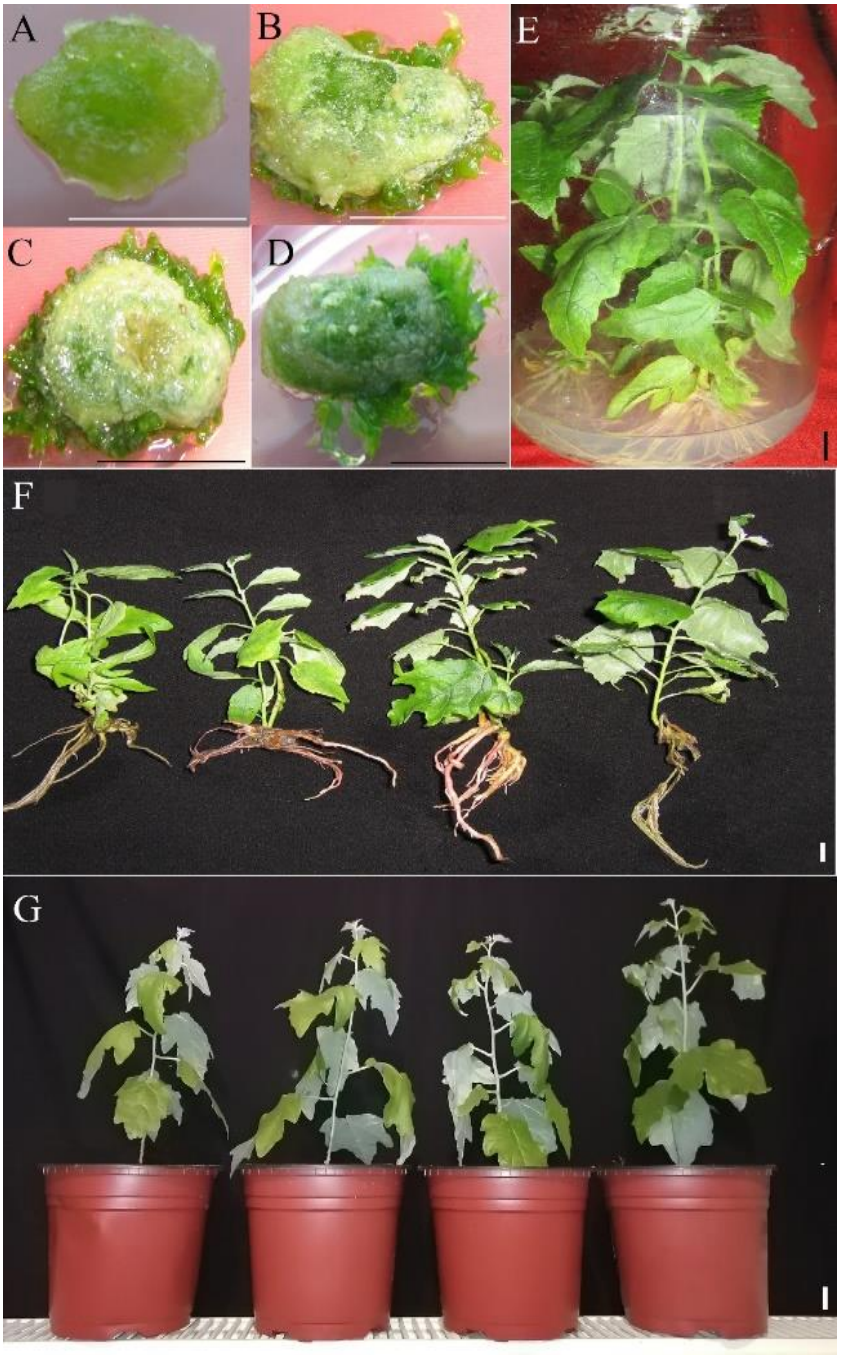

Figure 1. Plant regeneration from the highly responsive pulvinus-tTCL explants of white poplar (*Populus alba* L.) without callus induction. A-D) Shoot induction and regeneration after 2 (A), 4 (B), 6 (C), and 8 (D) weeks from the tTCL explants of pulvini basis at node 4 of one-year-old shoots of *P. alba* L. cultured on the shoot induction medium with BAP (0.75 mg/L), IBA (0.5 mg/L), sucrose (3%), and agar (0.6%) at day 25; E) Root development in the root induction medium with IBA (0.2 mg/L), NAA (0.1 mg/L), sucrose (2%), and agar (0.6%) after four months; F) Plantlets with well-established roots after 5 months; G) A hardened *P. alba* plant growing in soil mixture (sand, peat moss, and perlite in the 3:2:1 ratio) after 10 months. Bars indicate 1 cm; tTCL: Transverse thin cell layer.

pulvinus tissue. Therefore, we first designed an independent experiment to evaluate the regeneration capacity of pulvini tissues along the shoot axis based on node positions from the apical bud

(Supplementary Figure 1A). The mean comparison of pulvini-tTCL explants among node positions showed the highest shoot regeneration in the pulvinus tissue of the petiole at the 4th node with a mean ± SE of 37.95 ± 0.098 shoots per explant (Table 2). There was a strong association between node position and the regeneration response of the pulvinus tissue. The results indicated that the regeneration response of the pulvinus tissues from the intermediate position of shoots is higher than that of young nodes (Nodes 1-3) or old nodes (5-7), which is highly dependent on tissue maturity.

Table 2. The effect of node position on shoot regeneration of pulvini-tTCL explants cultured on the shoot induction medium with 0.75mg/L BAP and 0.5mg/L IBA.

Node position	No. of shoots per explant
Node 1	19.54 ± 0.056^{e}
Node 2	26.95 ± 0.077^{c}
Node 3	31.74 ± 0.091^{b}
Node 4	**37.95 ± 0.098^{a}**
Node 5	26.58 ± 0.076^{c}
Node 6	24.23 ± 0.069^{d}
Node 7	17.15 ± 0.049^{f}

Means ± SE; Means with different letters are statistically significant at $p \leq 0.05$ based on Tuckey's test; tTCL: Transverse thin cell layer; Measurements were taken after eight weeks of culture.

The high-capacity regenerative cell layers from the base of the pulvini tissue

To examine the regeneration response of different cell layers in the pulvinus tissue from the petiole of the fourth node, we defined five sections in the pulvinus tissue (1 cm long), including sections of (A), (B), (C), (D), and (E) with equal size of about 2 mm. Each section contained five pulvini tTCL explants with ~400-500 µm thickness cultured on the SIM with BAP 0.75 mg/L and IBA 0.5 mg/L. The result showed a significantly high shoot regeneration response in the section (A) from the pulvinus tissue of the petiole at the 4th node with a mean ± SE of 43.59 ± 0.22 shoots per explant (Table 3). Thus the cell layers close to the base of the pulvinus from mature intermediate nodes (Node 4) of the shoots are the most highly regenerative cell layers in *P. alba* L.

Histological analysis of the morphogenesis in the pulvini-tTCL explants

The light microscopy analysis of the methylene blue-stained 8-µm cross-sections from the base of pulvini tissue (region A) of the fourth petiole (Figure 2A) showed the arrangement of cell layers, including a layer of epidermal, collenchyma, parenchyma, and vascular cambium cells. The thickness and diameter of the tTCL explants were significantly increased after two weeks of culturing on the

SIM (Figure 2B). Followed by swelling around the cut-off area, the formation of putative adventitious bud primordia was observed from the pulvini-tTCL explant with compact and smaller parenchyma cells (Figure 2C), which are known as motor cells in the pulvinus tissue (Moran 2007; Russin and Evert 1984).

Table 3. The comparison of shoot regeneration in different regions of tTCL explants from the pulvini tissue at the base of the fourth petiole close to the apical bud, which was cultured on the shoot induction medium with 0.75mg/L BAP and 0.5mg/L IBA.

Regions in the pulvinus tissue	No. of shoots per explant
Region A	**43.59±0.22[a]**
Region B	38.741±0.097[b]
Region C	36.57±0.183[c]
Region D	35.56±0.178[d]
Region E	35.12±0.176[d]

Means ± SE; Means with different letters are statistically significant at $p \leq 0.05$ based on Tuckey's test; tTCL: Transverse thin cell layer; Measurements were taken after eight weeks of culture.

Root induction and acclimatization

The highest rooting efficiency was achieved for the combination of IBA (0.2 mg/L) and NAA (0.1 mg/L) at the rooting frequency of 100% (Supplementary Table 5) with 25.80 ± 0.134 roots/shoot (Table 4; Figure 1E) and well-established roots with 26.14 ± 0.501 cm root length (Table 4; Figure 2F). However, a significant increase in the concentration of the IBA/NAA combination led to callus induction and a significant decrease in rooting efficiency. The well-rooted plantlets successfully hardened and acclimated with a 100% survival rate (Figure 1G).

Discussion

The poplar species are considered cellulose-rich plant resources with commercial uses in paper and pulp, biofuel, and nano-fibrillated cellulose industries. They are known for their multi-purpose functional traits, including massive lignocellulosic material production, rapid growth, and easy vegetative propagation (Balatinecz *et al.* 2001; Chen *et al.* 2009; Bae *et al.* 2011; Yoon *et al.* 2014; Porth and El- Kassaby 2015). *Populus* species are highly recalcitrant in terms of *in vitro* shoot regeneration capacity and are greatly dependent on their extremely diverse genetic background (Confalonieri *et al.* 2000; Han *et al.* 2000; Confalonieri *et al.* 2003).

Among different factors, shoot regeneration is highly influenced by the source of plant tissue and the explant type (Han *et al.* 2000; Confalonieri *et al.* 2003; Han *et al.* 2013). The callus-mediated

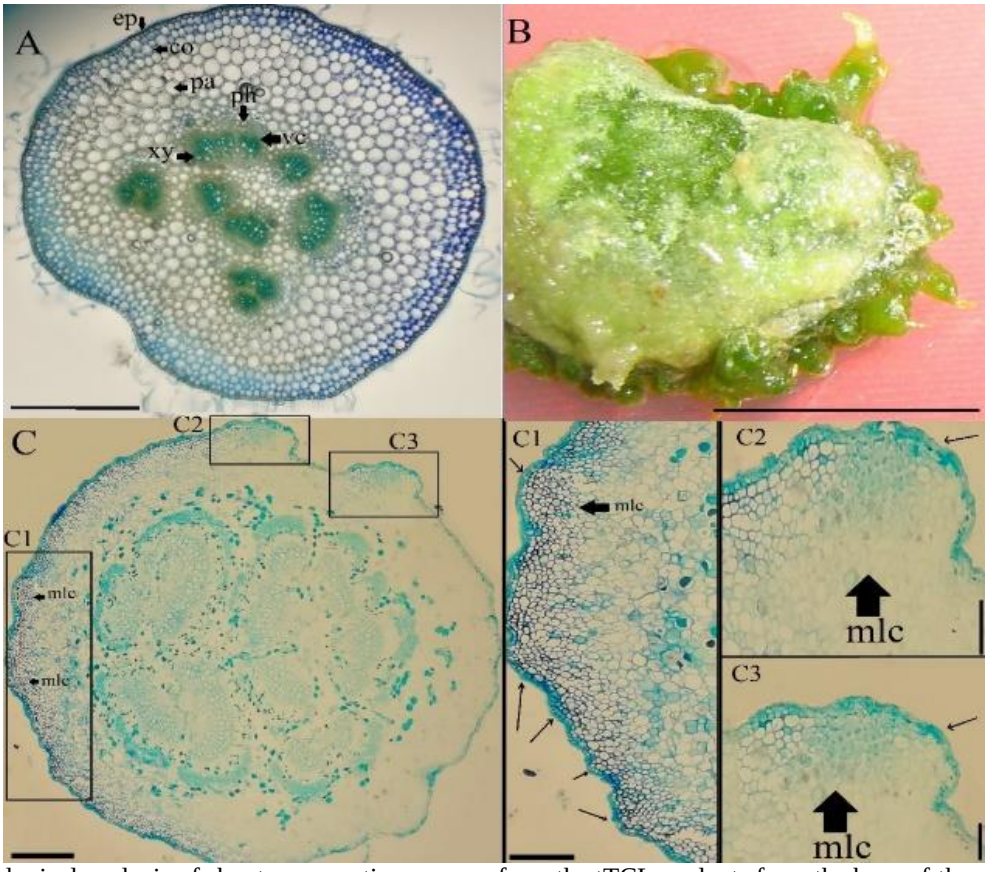

Figure 2. Histological analysis of shoot regeneration process from the tTCL explants from the base of the pulvinus tissue (region A) at the basis of 4th petiole from field-grown *P. alba* L.; A) A methylene blue-stained section before tTCL culture; B) Pulvini-tTCL explant from region A at 4th petiole showing morphogenesis after two weeks of TCL culture in the shoot induction medium containing BAP (0.75 mg/L) and IBA (0.5 mg/L), sucrose (3%), and agar (0.6%), pH 5.8; C) The segment showing organogenesis (black rectangular). Figures C1, C2, and C3 are a close-up view of the highly active organogenesis regions in the section. The arrows indicate an active morphogenesis region. co: Collenchyma; ep: Epidermal cells; mlc: Meristematic-like cells; pa: Parenchyma; ph: Phloem; vc: Vascular cambium; xy: Xylem. Scale bars = 400 µm in A, 5 mm in B, 800 µm in C, and 200 µm in C1, C2, and C3; tTCL: Transverse thin cell layer.

regeneration of poplar using *in vitro* culture of either leaf (2-3 cm^2 in size) or internodal stem (1 cm long) explants from *in vitro*-grown *Populus spp*. on SIM preceded by culturing on callus induction medium (CIM) has frequently been reported (Confalonieri *et al.* 1997; Confalonieri *et al.* 1998; Confalonieri *et al.* 2000; Delledonne *et al.* 2001; Confalonieri *et al.* 2003; Han *et al.* 2013; Movahedi *et al.* 2014; Maheshwari and Kovalchuk 2016; Liu *et al.* 2018). Compared with many of the other woody plants, some *Populus* species and their hybrid clones show a high callus-mediated regeneration capacity from the leaf, stem internode, petiole, and root segments, even without extreme applied PGRs (Žiauka and Kuusienė 2014). In several reports, the plant tissues such as lateral buds, leaf, internodal stem, and petiole were used as a regenerative explant for micropropagation and (in)direct

Table 4. Effect of IBA and NAA combinations on rooting of the regenerated shoots in *Populus alba* L. after eight weeks in culture.

IBA	NAA	No. of roots per shoot	Root length
0	0	0.7 ± 0.065^{q}	1.734 ± 0.194^{p}
0.1	0	1.38 ± 0.189^{p}	4.86 ± 0.567^{k}
0.2	0	1.62 ± 0.171^{o}	4.32 ± 0.562^{m}
0.3	0	2.04 ± 0.246^{n}	6.2 ± 0.76^{h}
0	0.05	4.7 ± 0.501^{k}	6.1 ± 0.374^{i}
0	0.1	0.54 ± 0.07^{r}	2.16 ± 0.285^{o}
0	0.15	3.68 ± 0.054^{m}	4.38 ± 0.324^{m}
0	0.2	9.94 ± 0.175^{i}	8.4 ± 0.13^{f}
0.1	0.05	8.14 ± 0.358^{j}	14.58 ± 0.422^{d}
0.2	0.05	21.70 ± 0.144^{d}	15.7 ± 0.144^{c}
0.3	0.05	4.44 ± 0.204^{l}	6.44 ± 0.271^{h}
0.1	0.1	20.58 ± 0.103^{e}	15.22 ± 0.096^{cd}
0.2	**0.1**	**25.80 ± 0.134^{a}**	**26.14 ± 0.501^{a}**
0.3	0.1	18.88 ± 0.39^{g}	17.58 ± 0.07^{b}
0.1	0.15	23.0 ± 0.25^{1}	4.0 ± 0.117^{n}
0.2	0.15	20.52 ± 0.52^{e}	4.5 ± 0.128^{l}
0.3	0.15	20.16 ± 0.542^{f}	5.6 ± 0.43^{j}
0.1	0.2	25.0 ± 0.151^{b}	6.5 ± 0.357^{g}
0.2	0.2	22.82 ± 0.492^{c}	10.8 ± 0.178^{e}
0.3	0.2	10.16 ± 0.542^{h}	7.6 ± 0.7^{fg}

Means ± SE; Means with different letters are statistically significant at $p \leq 0.05$ based on Tuckey's test; Measurements were taken after eight weeks of culture.

regeneration in white poplar (*P. alba* L.) and its hybrid clones (Park and Son 1988; Son and Hall 1990; Qiao *et al.* 1998; Jafari Mofidabadi and Modir-Rahmati 2000; Pintarić 2008; Wang *et al.* 2008; Wang *et al.* 2011; Tavassoli Asgari *et al.* 2013; Žiauka and Kuusienė 2014; Khosravan *et al.* 2017; Zeng *et al.* 2019). However, among these tissues from *P. alba* clones, only indirect regeneration from internodal stem tissue has been reported so far (Confalonieri *et al.* 2000; Delledonne *et al.* 2001; Confalonieri *et al.* 2003; Žiauka and Kuusienė 2014). Recently, Zheng *et al.* (2019) reported highly regenerative petiole explants from intermediate nodes along with shoots of the triploid clone of *Populus alba* × *P. glandulosa* × *P. tomentosa* and the importance of tissue maturity in regeneration

response of petiole and leaf explants (Zeng *et al.* 2019). Furthermore, in agreement with Russin and Evert (1984), we have realized that there are swollen tissues known as pulvinus at the base of the petioles of *Populus alba* L. using LM microscopy analysis, which is probably a species-specific tissue among *Populus* species. According to literature review, no report covers the regeneration response, the effects of explant type and position, and tissue maturity of pulvini tissue at the base of the petiole under different auxin and cytokinin PGR treatments. Furthermore, efficient protocols for (in)direct regeneration of pulvini tissue have not been reported so far. To cover this gap, we employed the TCL culture technique to obtain the high-capacity pulvini-based cell layers for shoot regeneration without callus induction in *P. alba* L. This technique uses a thin layer excised from different parts of a plant with a small number of cells or tissue (0.5-1.0 mm thickness) (da Silva *et al.* 2015). The TCL-mediated regeneration of poplar was first reported by Lee-Stadlemann *et al*. (1989) using transverse thin slices (100-500µm) only from leaf mid-vein explants in poplar cultured on a medium supplemented with 0.2 mg/L BAP and 0.01 mg/L NAA (Lee-Stadelmann *et al.* 1989). However, the regeneration and transformation capacity of TCL explants from pulvini and internodal stem tissues of poplar remains unclear. Based on our findings, the pulvini-tTCL explants showed a 9-10-fold increase in the frequency of shoot regeneration without callus induction compared with the internodal stem-derived ones (Figure 1A-1D).

The evaluation of different PGRs and their concentrations on shoot regeneration frequency of the lTCL/tTCL explants showed the best result from the BAP/IBA combination. In many reports, PGRs with an auxin-like effect such as NAA, IAA, and 2,4-D (0.01-0.1mg/L) in combination with BAP (0.2-1.0mg/L) have been applied for shoot induction and regeneration from internodal stem explants in *Populus* species with a shoot regeneration frequency of 30-40% (Park and Son 1988; Lee-Stadelmann *et al.* 1989; Lubrano 1992; Wang *et al.* 2011). Our results revealed that 0.5 mg/L IBA in combination with 0.75 mg/L BAP could cause a significant increase in shoot regeneration frequency per explant, as much as about 8.9 and 9.3-fold higher than BAP-NAA combinations from the internodal stem-derived tTCL and the pulvinus-derived tTCL explants, respectively (Supplementary Table 1). Between two TCL explant sources, not only was the handling of the pulvinus explant so much easier, but also the faster regeneration occurred compared to the internodal stem tissue. The use of any concentration of NAA in this work was not recommended alone or in combination with BAP and resulted in explant necrosis, callus-like tissue, and adventitious rooting.

In woody plants, the *in vitro* regeneration capacity of any type of explant is highly influenced by the maturity of the explant and its position in the tissue (Welander 1988; Mencuccini and Rugini 1993). Lane *et al.* (1998) demonstrated that old explants are highly sensitive to *in vitro* organogenesis

and show the most regeneration response to PGR treatment in *Pyrus pyrifolia.* In contrast, Corredoira *et al.* (2008) and Cuenca *et al.* (2000) showed that young explants are highly responsive to *in vitro* regeneration of *Fagus orientalis* and *Paulownia tomentosa,* respectively. Feng *et al.* (2010) and Zeng *et al.* (2019) reported that young leaf explants from intermediate nodes (node 3) show great capacity for regeneration response with a frequency of 82.25% and 98.33% in *Ziziphus jujuba* 'Huizao' and triploid (*Populus alba* × *P. glandulosa*) × *P. tomentosa* woody plants, respectively. In agreement with previous reports, we obtained the highest shoot regeneration without callus induction from pulvini-tTCL explants at the base of the intermediate petiole (Node 4), which was higher than younger (Node 2, 3) and older (Node 5-7) tissues. Furthermore, our results revealed the significant difference in shoot regeneration response among the pulvini-tTCL explants from different sections of the pulvinus at the base of the 4th petiole. We found that pulvini-tTCL explants (region A) from the cell layers of the pulvinus base at the intersection region of the petiole and stem tissue are very fast regeneration- responsive explants in *Populus alba* L., which have not been evaluated so far. It is concluded that the variations in genetic background among *Populus* species and tissue maturity in plants are the most probable factors that affect the regeneration response of the tissue explant of interest as described by Corredoira *et al.* (2008).

The histological analysis results from the swollen pulvini-tTCL on tissue culture, compared to the control, revealed that the initiation of adventitious buds in the pulvinus-tTCL explants appears from the edges of the tTCL cut-off area with close connections among small parenchymal cells (Figure 2). This phenomenon could be due to the functions of PGRs as described by Lup *et al.* (2016). In agreement with our results, a similar cellular re-organization was reported during adventitious bud-primordia initiation in leaf explants of *P. euphratica* (Ferreira *et al.* 2009). García-Angulo *et al.* (2018) showed that *in vitro* culture of the internodal stem from *P. deltoides* × *P. nigra* causes swelling around the cut-off zones, whereas the organogenesis zones that formed regeneration centers under subepidermal cells formed bud primordia (García-Angulo *et al.* 2018).

The successful rooting of regenerated plantlets and their hardening reveal natural growth and development and propagation of the *in vitro* regenerated plants, as well as field-grown plants. Here, we obtained the maximum root induction frequency of 100% and the highest root number per regenerated plant under RIM containing IBA (0.2 mg/L) and NAA (0.1 mg/L). In agreement with our results, some reports have induced rooting with the frequency of 40-75% in *Populus alba* and its hybrids under MS medium containing IBA (0.2-0.5 mg/L) or in combination with NAA (0.1-0.2 mg/L) (Tavassoli Asgari *et al.* 2013; Khosravan *et al.* 2017).

Conclusion

The availability of a high regeneration responsive tissue is a pivotal factor in employing new technologies like genetic engineering, and genome editing approaches to introduce new traits in poplar (*Populus sp.*). Our results showed that the pulvini-tTCL explants from the cell layers of the pulvinus base close to the intersection region of the petiole and stem at node 4 contain highly totipotent parenchyma cells with high capability in the fast and simple *in vitro* shoot regeneration without callus induction in *P. alba* L. The maximum shoot induction was obtained from this explant type cultured on the SIM with 0.75 mg/L BAP and 0.5 mg/L IBA. The maximum root induction was also achieved in the RIM containing IBA (0.2 mg/L) and NAA (0.1 mg/L). These results reveal high-capacity parenchymal transverse cell layers' explants from the base of pulvinus tissue at intermediate nodes for efficient, fast, trustworthy, stable, and reproducible regeneration and *in vitro* micropropagation of white poplar.

Acknowledgments

This work was performed in the genetic engineering lab at the Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.

Funding

This research was supported by the research grant of the University of Tabriz (Number: ص/472).

Conflict of Interest

The authors declare that they have no conflict and/or competing interests.

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