

Optimized protocol for *in vitro* indirect organogenesis and shoot regeneration of *Platycladus orientalis*

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Abstract

Background: *Platycladus orientalis* L. is a drought-tolerant conifer valued for its ornamental and medicinal properties. However, efficient regeneration systems for this species remain limited, hindering its propagation and conservation.

Aims: The aim of this study was to develop a reliable protocol for indirect organogenesis of *Platycladus orientalis* under *in vitro* conditions, evaluating the influence of explant type, culture medium, light exposure, and pretreatment on regeneration efficiency.

Methods: Cotyledon, hypocotyl, and radicle explants were cultured on different media formulations. The effects of light and darkness during callus induction and shoot elongation were compared. Seeds underwent or avoided vernalization and scarification treatments to assess their influence on germination and callus formation.

Results: Cotyledon explants achieved the highest callus induction rate, reaching 74.06%, particularly under dark conditions. Exposure to light during elongation significantly enhanced callus proliferation and shoots differentiation. Quoirin and LePoivre medium promoted the greatest number of adventitious shoots, with an average of 7.9 shoots per explant, while other media tested showed lower effectiveness. Germination was higher in non-vernalized and non-scarified seeds cultured in Quoirin and LePoivre medium.

Conclusions: The established protocol enables efficient indirect organogenesis and shoot regeneration of *Platycladus orientalis* using cotyledon explants and Quoirin and LePoivre medium. The finding provides a valuable tool for clonal propagation and conservation of this species, supporting both ornamental cultivation and the preservation of its genetic resources.

Keywords: callus induction, culture medium, indirect organogenesis, *in vitro* regeneration, *Platycladus orientalis*, vernalization.

Introduction

Platycladus orientalis (L.), commonly known as Chinese Arborvitae or Oriental Thuja, is a distinctive and highly valued member of the Cupressaceae family. This evergreen conifer is the sole species in the genus *Platycladus*. It is native to northeastern Asia – particularly China, Korea, and parts of Russia – where it has been cultivated and revered for thousands of years (Li et al., 2016). The foliage consists of scale-like leaves arranged in flattened sprays, giving the tree a characteristically feathery appearance.

These leaves are bright green, turning bronze or brownish in winter. The seed cones are small (1.5 - 2.5 cm long), with thick, woody scales that transition from bluish-green to brown as they mature.

One of the most notable traits of *P. orientalis* is its adaptability. It is highly drought-tolerant and capable of thriving in poor soils, making it a resilient choice for diverse landscapes. It is frequently planted in urban environments due to its tolerance of pollution, compacted soils, and other stressful conditions (Yao et al., 2025). The seeds have traditionally been used for their purported

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BAP - benzylaminopurine; DCR - Douglas Fir Cotyledon Revised; LP - Quoirin and LePoivre; MS - Murashige and Skoog.

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medicinal benefits, including cardiovascular support and calming effects.

In horticulture, *P. orientalis* is propagated by seeds or cuttings; however, propagation remains challenging due to the tree's slow growth and specific germination requirements (Dong et al., 2023). Consequently, this species is of particular interest to botanists and conservationists. Understanding its propagation through both conventional and *in vitro* techniques is essential, particularly in regions with limited genetic diversity.

Conifer reforestation efforts have traditionally relied on natural or direct seeding and, more recently, the planting of nursery-grown stock. Only in the past decade have rooted cuttings been used on a commercial scale (Reddy et al., 2022). These propagation techniques are often employed for rare or valuable genotypes (Celestino et al., 2005). However, such methods frequently fail to meet the demand or deliver consistent, high-quality plant material due to species-specific requirements (Barthwal et al., 2025).

In this context, *in vitro* culture technologies – based on the cultivation of organs, tissues, cells, or protoplasts – offer a viable alternative to conventional vegetative propagation, especially for species with germination challenges (Pais, 2019). Juvenile explants, such as those derived from seeds or seedlings, are generally the most suitable for *in vitro* culture. Vegetative propagation includes shoot culture, which promotes axillary bud development, and callus culture, which enables regeneration through shoots or embryoids (Mehubub et al., 2022). This approach has been successfully applied to numerous woody plant species, including fruit, forest, and ornamental trees. However, organogenesis as a regeneration pathway remains understudied in conifers (Krasnoperova and Bukharina, 2020). Each species requires specifically tailored culture conditions and protocols (Barthwal et al., 2025).

In conifers, organogenic regeneration typically involves the induction of adventitious buds following callus formation, especially in genera such as *Pinus*, *Picea*, and *Pseudotsuga* (Long et al., 2022; Zhu et al., 2022; Stojičić et al., 2024). Callus induction facilitates tissue regeneration and variability. During this process, both adventitious buds and embryoid tissues may develop (Guo and Jeong, 2021).

Previous studies have proposed various protocols for callus induction, shoot bud differentiation, or regeneration in *Pinus taeda* (Tang et al., 2001), *Pinus strobus* (Tang and Newton, 2005), *Picea abies* (Guo et al., 2024), *Abies koreana* (Guo and Jeong, 2021), *Picea pungens* (Krasnoperova and Bukharina, 2020), *Cupressus sempervirens* (Khamushi et al., 2019) and *Larix* species (Shmakov and Konstantinov, 2020). Precedents also exist for *in vitro* cultivation of *P. orientalis* using different culture media aimed at establishing cryopreservation systems for clonal propagation. Notably, no significant differences were found between seedlings derived from cryopreserved and non-cryopreserved cells (Ahn and Choi, 2017).

The leaves of *P. orientalis* have been traditionally used to treat productive cough, chronic bronchitis, and asthma. Some of its extracts have been chemically characterized, and specific secondary metabolites were validated for

their traditional medicinal uses in China (Fan et al., 2012). Additional studies have investigated its anti-inflammatory properties and underlying mechanisms, reinforcing its use in the treatment of respiratory inflammatory diseases (Fan et al., 2012). *In vitro* assessments of anti-inflammatory activity using solvent-partitioned extracts have also been reported (Ho et al., 2022), with the chemical composition of key secondary metabolites determined.

More recently, researchers have examined how the age of the donor plant affects growth performance, stress resilience, and clonal propagation success. These studies included donors ranging from 6 to 3 000 years old (Dong et al., 2023). It appears that basal stem diameter and plantlet height decreased with increasing donor age. Further analyses revealed that older plants accumulate secondary metabolites that inhibit rooting of seedlings and cuttings (Chang et al., 2023). Specifically, the concentrations of phenylpropanoid biosynthesis intermediates (e.g., caffeic acid, coniferyl alcohol) and flavonoids (e.g., cinnamoyl-CoA, isoliquiritigenin) increased significantly in cuttings from older donors, likely impeding rooting and promoting callus lignification.

Despite the existing literature, no comprehensive studies have yet described the optimal conditions and parameters for developing a reliable organogenic regeneration protocol for *P. orientalis*.

In this work, we investigated the effects of explant type, culture medium, photoperiod, and pre-germination treatments – scarification and vernalization – on indirect organogenesis in *P. orientalis*. The impact of these variables on callus induction rates, callus size, and shoot formation was analyzed to establish an optimized protocol for efficient regeneration. The resulting protocol represents a valuable tool for the large-scale propagation and potential biotechnological applications of this important conifer species.

Materials and methods

Plant material: Initial explants of *Platyclusus orientalis* were obtained from seeds collected from trees grown in 'Las Lagunillas' Campus, in Jaen (Andalusia, Spain) (UTM coordinates: 37.785981, -3.774653) between June and September 2020. The cones were allowed to dry under sunlight for one week to facilitate their opening and seed release. Half of the seeds underwent artificial vernalization by being stored at 5°C in a refrigerator for two months to simulate the natural cold conditions they would experience in the wild. Scarification was performed by making a longitudinal incision on the seed surface using a sterilized scalpel.

Culture media: Three culture media were used: Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), Quoirin and LePoivre (LP) medium (Aitken-Christie et al., 1988), and Douglas Fir Cotyledon Revised (DCR) medium (Gupta and Durzan, 1985). For both the MS and LP media, macronutrients were used at half strength, hence referred to as MS/2 and LP/2. In all cases, the pH was adjusted to 5.8.

Seed germination: Seeds were disinfected by immersing them in a 20% sodium hypochlorite (commercial bleach) solution for 10 min. Under aseptic conditions, approximately half of the seeds were incised in the seed coat, while the remainder were left intact, resulting in four distinct groups: vernalized, non-vernalized, incised, and non-incised. All seeds were sown in the three culture media described above (MS/2, LP/2, and DCR) without growth regulators. The culture tubes were kept in darkness for seven days, after which they were transferred to a growth chamber (*Phytotron Model 600PHL-LED, ARALAB*, Oeiras, Portugal) for 60 days. Environmental conditions were controlled: a 16/8 h light/dark photoperiod, a temperature of 22°C, and 60% relative humidity. After 60 days, the following parameters were measured for each treatment: germination percentage, root length, shoot length, total plant length, and number of leaves.

Organogenesis induction: Organogenesis was induced in three types of explants: cotyledon, hypocotyl, and radicle. These were dissected from seedlings obtained after seed germination. Initially, seeds were disinfected by immersion in a 20% sodium hypochlorite solution for 10 min. Germination was performed by placing the seeds on sterile filter paper in Petri dishes. The papers were moistened with sterile distilled water, and the dishes were incubated at 21°C in darkness for 21 d. After this period, the resulting seedlings were dissected under aseptic conditions to obtain the three explant types.

Callus induction: To induce callus formation, all three explant types were cultured in Petri dishes containing the three culture media tested in the experiment, each supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg/L benzylaminopurine (BAP). To assess the effect of light conditions on callus induction, the Petri dishes were divided into two groups: one incubated in darkness and the other under light conditions. Both groups were maintained under controlled conditions (21°C, 60% relative humidity, and a 16/8 h light/dark photoperiod for the light group) for 6 weeks. After this period, the percentage of callus induction and callus size were recorded.

Callus cell proliferation and elongation of organogenic structures: The regeneration process continued by transferring the induced calli to elongation medium. This medium consisted of the same three culture media as previously described, supplemented with 0.4 mg/L 2,4-D and 0.5 mg/L BAP. The calli were incubated under the same conditions as before for an additional 6 weeks. After this period, callus size and the number of buds/shoots were recorded.

Statistical analyses: Statistical analyses were performed using *STATGRAPHICS Centurion XIX* software (version 19.1.2) for Windows. Specifically, parametric Analysis of Variance (ANOVA) tests and Fisher's least significant difference (LSD) procedure were applied.

Results

This study provides a comprehensive analysis of the factors affecting the *in vitro* development of new seedlings of the ornamental and medicinal species *Platycladus orientalis*. Experiments were carried out using three types of explants for callus induction, which were subsequently cultured in three different media under both light and dark conditions. Half of the seeds used to obtain the explants were subjected to a two-month vernalization treatment prior to germination. Additionally, some seeds underwent a scarification process. The outcomes of these experimental conditions are detailed below.

The effectiveness of mechanical scarification as a pre-germination treatment varies significantly and is highly dependent on the specific seed species, regardless of its taxonomic family. Mechanical scarification of *Platycladus orientalis* seeds markedly reduced the germination rate to 8.33%, compared to 64.72% in seeds with intact seed coats. Similar results were reported in *Pinus lambertiana*, where scarification resulted in a germination rate of only 5% (Shen and Cho, 2021).

It has been demonstrated that certain pre-germinative treatments – such as physical scarification, hydration, or acid immersion – can negatively affect germination in some forest species, including the palm *Lepidocaryum tenue* (Huapaya, 2015) and *Hymenaea courbaril* L. (Orozco-Cardona et al., 2010). The morphology, consistency, and presence of germination inhibitors in seed coats vary considerably among coniferous species. For instance, it has been reported that seed coat acts as a mechanical barrier between embryo and growth medium, limiting germination in *Pinus cubensis* Griseb. (Ardebol et al., 2006). Conversely, scarification of *Iliamna rivularis* (Malvaceae) seeds increased the germination rate up to 49% (Himanen et al., 2012).

Regarding vernalization in seeds, it is a crucial process that involves exposing seeds to prolonged periods of cold temperatures to break dormancy and promote germination. This cold treatment simulates the natural winter conditions that seeds would typically experience in their native habitats, thereby ensuring germination occurs at the appropriate time in spring when environmental conditions are favorable for seedling establishment (Bonner and Brand, 2004).

Vernalization is particularly important for certain species within the Cupressaceae family, such as cypresses and junipers, as it helps synchronize germination with seasonal changes, enhancing seedling survival and growth in temperate climates (Ma et al., 2022). The mechanism of vernalization involves inducing epigenetic changes such as histone modifications that alter gene expression and facilitate the transition from vegetative to reproductive growth (Akter et al., 2018). This process is essential for species adapted to temperate environments, where exposure to low temperatures signals the onset of spring and supports optimal seedling development.

However, the effectiveness of vernalization can vary considerably depending on species and environmental conditions. Table 1 summarizes the effects of vernalization

Table 1. Effect of vernalization on germination and growth parameters in *Platyclusus orientalis*. Mean \pm SD, $n = 30$. Different letters indicate statistically significant differences according to Fisher's least significant difference (LSD) test ($P \leq 0.05$).

Parameters	Treatment	
	Vernalized	Non-vernalized
<i>In vitro</i> germination (%)	44.047 ^a \pm 2.259	71.111 ^b \pm 2.259
Root length (cm)	8.909 ^a \pm 1.582	5.870 ^a \pm 1.199
Shoot length (cm)	3.964 ^a \pm 0.309	3.702 ^a \pm 0.239
Total length (cm)	12.759 ^a \pm 1.724	9.869 ^a \pm 1.335
Number of leaves	13.531 ^a \pm 2.915	13.369 ^a \pm 2.256

Table 2. Effect of culture media on germination and growth parameters in *Platyclusus orientalis*. Mean \pm SD, $n = 30$. Different letters indicate statistically significant differences according to Fisher's least significant difference (LSD) test ($P \leq 0.05$).

Parameters	Culture media		
	MS/2	LP/2	DCR
<i>In vitro</i> germination (%)	16.667 ^a \pm 2.767	87.500 ^b \pm 2.767	68.571 ^c \pm 2.767
Root length (cm)	13.319 ^a \pm 3.152	3.737 ^b \pm 1.014	5.112 ^b \pm 1.082
Shoot length (cm)	4.630 ^a \pm 0.615	3.595 ^a \pm 0.214	3.275 ^a \pm 0.211
Total length (cm)	17.745 ^a \pm 3.434	7.811 ^b \pm 1.195	8.387 ^b \pm 1.176
Number of leaves	15.080 ^a \pm 5.805	11.645 ^a \pm 2.021	13.625 ^a \pm 1.989

on the germination rate and growth parameters of *Platyclusus orientalis*. Vernalized seeds showed a reduced germination rate (44.05%) compared to non-vernalized seeds (71.11%).

Temperature requirements for the production of mature seeds differ among conifer species; for example, *Picea abies* has lower temperature needs than *Pinus sylvestris* (Almqvist et al., 1998). While extreme temperatures can cause seed mortality, it has been reported that low temperatures result in lower seed mortality than high temperatures in *Eucalyptus globulus* (Rix et al., 2011). Therefore, low temperature may represent a limiting factor in the germination of *Platyclusus orientalis* seeds.

Similarly, seed germination rate was also influenced by the culture medium. LP/2 medium resulted in the highest germination percentage (87.5%) compared to DCR (68.57%) and MS/2 (16.67%). As shown in Table 2, the culture medium also affected plant growth, with MS/2 medium promoting greater root length (13.32 cm) and total seedling length (17.75 cm) compared to LP/2 medium (3.74 cm and 7.81 cm, respectively) and DCR (5.11 cm and 8.39 cm, respectively).

It is noteworthy that seedlings grown in MS/2 medium, in addition to being significantly taller, produced a greater number of leaves than those grown in the other two media, although this last difference was not statistically significant. In consonance, it has been reported an enhanced leaf production in *Moringa oleifera* seedlings cultured in MS/2 medium (Ledea-Rodríguez et al., 2020).

Organogenesis – callus induction: Fig. 1 illustrates the different conditions studied in this work and their effects on the percentage of callus formation and its size. The mean callus induction rate was 61.73%, and the

average size of the induced callus was 0.26 cm. As shown in Fig. 1A, the type of explant significantly influenced callus formation, with cotyledons achieving a higher induction rate (74.06%) compared to hypocotyls (60.05%) and radicles (41.7%). Numerous previous studies have successfully achieved callus induction from cotyledonary explants in various plant species (Furmanek and Banas, 2011; Konate et al., 2013; Yaroshko et al., 2023), and some authors have identified the cotyledon as the most suitable explant for callogenesis (Roca and Mroginski, 1991). However, it has been reported better callus formation results from hypocotyl explants in *Arctium lappa* L. (He et al., 2006).

Regarding callus size, cotyledons also produced the largest calli (0.37 cm; Fig. 1D), followed by hypocotyls (0.21 cm) and radicles (0.15 cm). These findings highlight the high potential of juvenile or cotyledonary leaf explants for successful callogenesis, as previously described in other conifer species such as *Fitzroya cupressoides* (Cob-Uicab et al., 2011).

On the other hand, callus induction was significantly enhanced under dark conditions (63.6%) compared to light conditions (53.6%) (Fig. 1B), in line with other results obtained in *Abies guatemalensis*, in which it has been demonstrated that darkness promoted a higher callus formation rate (Ramírez Rodas, 2006). It is well established that darkness favors callus induction, and many researchers employ dark incubation to induce callogenesis in gymnosperms, such as *Pinus koraiensis* (Gao et al., 2021; 2023), *Abies pindrow* (Bhat et al., 2014), and *Pinus halepensis* (Tavares, 2019).

Despite this, the largest callus size was obtained under light conditions (0.3 cm; Fig. 1E), compared to 0.19 cm in darkness. Thus, while darkness promotes a higher callus induction rate, light conditions support greater cell

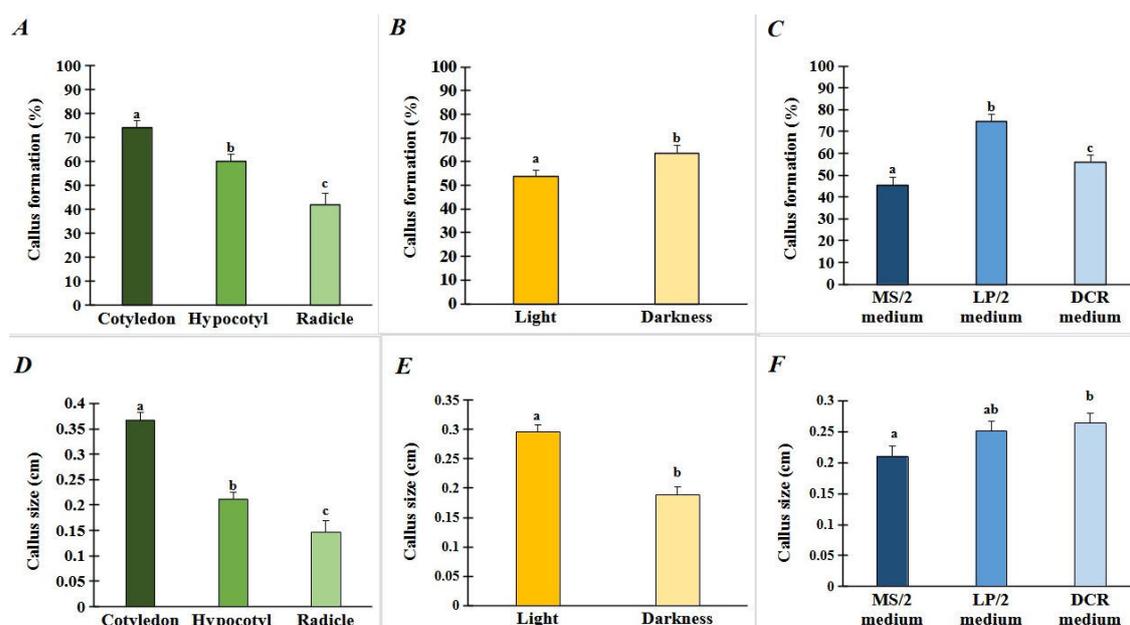


Fig. 1. Effect of the different factors on the percentage of callus formation (A - C) and on the callus size (D - F). Percentage of callus formation for: A - three types of explants used (cotyledon, hypocotyl, and radicle), B - light/dark conditions to which cultures were exposed, C - type of culture media used. Callus size using: D - three types of explants, E - light/dark conditions, F - three types of culture media. Different letters indicate statistically significant differences according to Fisher's least significant difference (LSD) test ($P \leq 0.05$). Mean \pm SD, $n = 40$.

proliferation and expansion during callus development in *Platycladus orientalis*.

As shown in Fig. 1C, the LP/2 medium produced the highest callus induction percentage (74.5%) compared to DCR (55.9%) and MS/2 (45.38%). These results are consistent with those reported in *Pinus pinea*, in which it has been found that LP medium supplemented with auxins and cytokinins resulted in the highest proliferation rates (Carreros et al., 2009). It was already known the suitability of LP medium for callus formation in other conifer species, mainly in micropropagation of pine species (Jacoby, 1999). It has also achieved the highest success rates in callus induction using LP medium in *Abies koreana* (Guo and Jeong, 2021).

DCR medium yielded the largest callus size (0.26 cm), closely followed by LP/2 (0.25 cm), with no statistically significant differences between them. In contrast, MS/2 medium produced significantly smaller calli (0.21 cm), as shown in Fig. 1F.

Organogenesis – callus cells proliferation and organogenic structures elongation: After 6 weeks of callus induction in the three selected culture media, the calli were transferred to fresh media supplemented with a cytokinin to promote the development of adventitious shoots from callus cells. During this stage, the callus undergoes an initial adaptation phase to the new culture conditions. Cell division, stimulated by the cytokinin BAP, drives callus growth. After 6 to 8 days, cellular differentiation begins. This process can be observed under a stereomicroscope as the emergence of dense cell clusters (pro-organogenic structures), which subsequently differentiate into shoots.

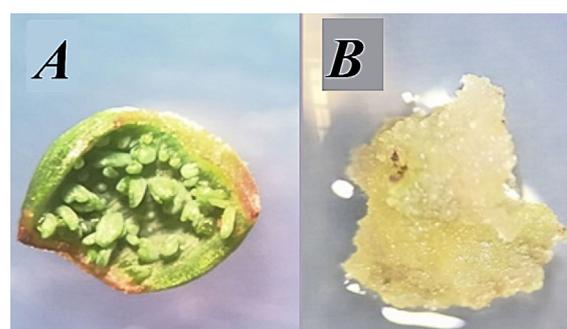


Fig. 2. Callus and adventitious buds formation from cotyledon of *Platycladus orientalis* after 6 weeks on induction medium under lighting conditions. A - adventitious buds induction on callus cultured on LP/2 medium; B - callus induction on explant culture on DCR medium.

Fig. 2A shows numerous adventitious shoots with evident photosynthetic capacity. It has been reported that BAP can directly induce the formation of adventitious shoots in cotyledon explants derived from mature embryos of *Pinus pinea* (Sarmast, 2018), which is consistent with our findings. Fig. 2B depicts the appearance of callus tissue formed on the induction medium, characterized by an amorphous mass of non-photosynthetic cells exhibiting rapid and disorganized growth. Within this mass, denser structures begin to emerge, indicating the onset of differentiation into adventitious buds or root primordia.

Fig. 3 illustrates the effects of the three factors analyzed in this study on callus proliferation (Fig. 3A-C) and bud differentiation (Fig. 3D-F): explant type, light conditions, and culture media. Cotyledon and hypocotyl explants

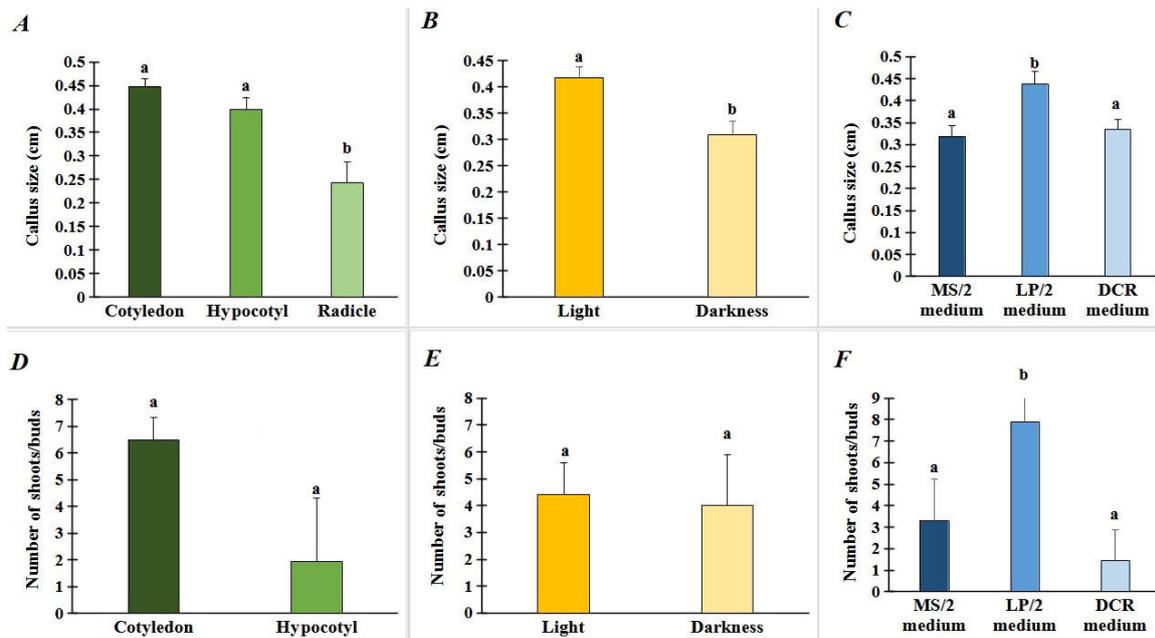


Fig. 3. Effect of different factors on proliferation of callus and buds differentiation. Effect of explant precedence on the size of callus (A) and number of shoots/buds (D). Effect of light/darkness on callus size (B) and number of shoots/buds (E). Effect of culture media on callus size (C) and number of shoots/buds (F). Different letters indicate statistically significant differences according to Fisher's least significant difference (LSD) test ($P \leq 0.01$). Mean \pm SD, $n = 40$.

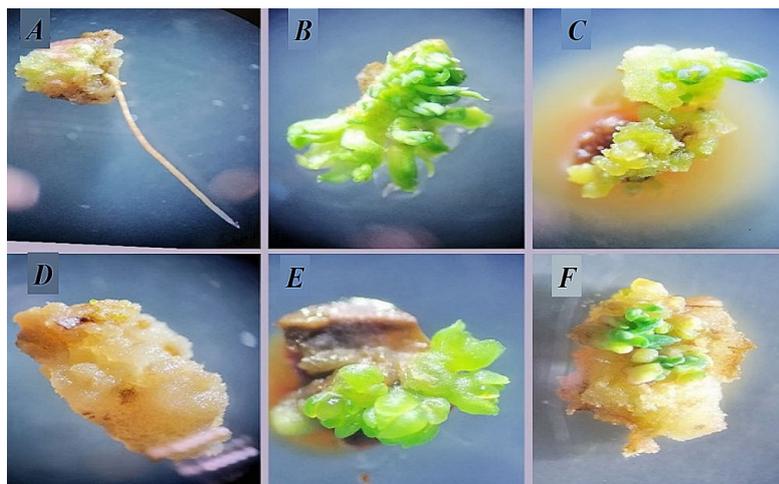


Fig. 4. Callus and organogenesis in different explants of *Platycladus orientalis* after 6 weeks on elongation medium under lighting conditions. A - root induced from hypocotyl in LP/2 medium; B and C - buds and shoots elongation from cotyledon cultured in LP/2 medium; D - callus cells proliferation from cotyledon cultured in MS/2 medium; E and F - buds and shoots differentiation from cotyledon cultured in MS/2 medium.

promoted greater callus growth (0.45 and 0.40 cm, respectively) compared to radicle explants (0.25 cm) (see also Fig. 4A). Similar morphogenetic responses have been observed in cotyledon and hypocotyl explants from *Pseudotsuga menziesii* (Campusano et al., 2019).

In terms of photoperiod, culture under light conditions significantly increased callus growth (0.42 cm) compared to darkness (0.31 cm) (Fig. 3B). Calli cultured in LP/2 medium increased their size by 62.7% compared to the final stage of the induction phase, reaching an average of 0.44 cm. Similar results have been reported in Japanese

pinus species (Maruyama and Hosoi, 2019). In contrast, MS/2 and DCR media did not promote callus development as effectively, resulting in average sizes of 0.32 cm and 0.33 cm, respectively (Fig. 3C).

LP/2 medium also enhanced the differentiation of buds/shoots, with an average of 7.9 buds per explant, compared to 3.30 in MS/2 medium and 1.45 in DCR medium (Fig. 3F). Zygotic embryos of *Pinus ponderosa* cultured in LP medium exhibited the highest percentage of shoot formation (Rojas-Vargas et al., 2023), supporting the use of LP medium as an effective formulation for

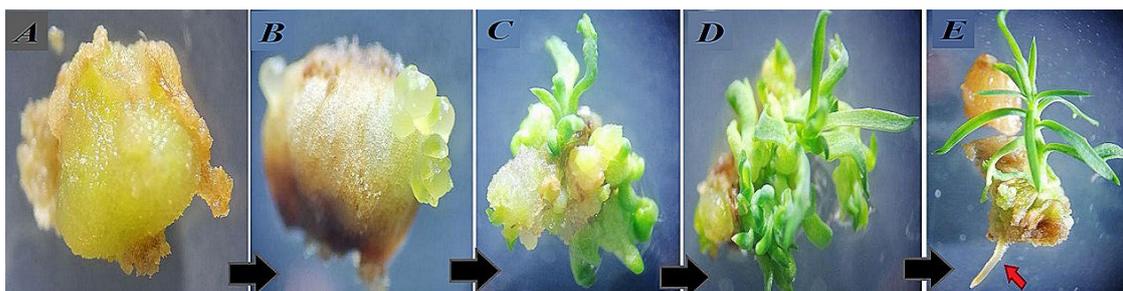


Fig. 5. Complete process of indirect organogenesis with each of the steps that comprise it. *A* - undifferentiated callus cells that have become competent to differentiate in the proliferation medium LP/2; *B* - appearance of the first pro-organogenic structures; *C* - development of these pro-organogenic structures and their differentiation towards the formation of the first shoots; *D* - leaves and stem perfectly differentiated and functional as photosynthetic organs; *E* - complete seedling in which the stem, several leaves and a root have differentiated (root indicated with the red arrow).

in vitro regeneration in conifers. Conversely, it has been observed the highest bud induction rates in *Pinus taeda* cultured in MS/2 medium compared to DCR or LP media (Barone et al., 2018). These findings suggest that each conifer species has specific nutritional requirements that must be considered when selecting an appropriate culture medium.

Neither light conditions nor explant type had a significant effect on the development of organogenic structures (Fig. 3E and Fig. 3D, respectively). Notably, radicle explants did not produce any bud induction.

Fig. 4 provides further detail on the organogenic structures formed (buds, shoots, and roots), all of which developed from calli derived from the two explant types mentioned above. Calli that gave rise to roots (Fig. 4A) originated from hypocotyl explants cultured in LP/2 elongation medium. Once the shoots differentiated, they began photosynthesizing, which is reflected in their green color (Fig. 4B,E,F). Fig. 4D shows a callus grown on MS/2 medium that failed to undergo organ differentiation, possibly due to a nutritional deficiency in the medium that either inhibits or delays morphogenesis. Calli in Fig. 4E,F, also derived from explants cultured on MS/2 medium, further illustrate this limited capacity for organ development, despite all three media sharing the same hormonal composition.

As a summary of the results obtained in this study, Fig. 5 illustrates the complete indirect organogenesis process of *Platycladus orientalis* – from the dedifferentiation of explant cells into an amorphous mass of callus tissue (Fig. 5A), to the development of the first competent cells or structures exhibiting some photosynthetic capacity, which appear green in color (Fig. 5B), and finally, to the differentiation of these previously determined cells into fully formed shoots or visible roots (Fig. 5C-E).

Discussion

In vitro regeneration of *Platycladus orientalis* remains an underexplored area despite its horticultural, ecological, and pharmacological importance. The findings from this study confirm that multiple variables (explant type, culture medium, light exposure, and pre-germination treatments)

play essential roles in the success of indirect organogenesis in this conifer.

The poor performance of mechanically scarified seeds, with a drastic reduction in germination percentage (from 64.72% to 8.33%), is consistent with other species where scarification proved detrimental. Similar outcomes have been observed in *Pinus lambertiana* (Shen and Cho, 2021), and it has been reported reduced germination in *Lepidocaryum tenue* following physical scarification (Huapaya, 2015). This suggests that, in some conifers, the seed coat may offer essential protection rather than represent a germination barrier (Ardebol et al., 2006). In contrast, other species like *Iliamna rivularis* benefit from scarification (Himanen et al., 2012), highlighting the species-specific response to pre-germinative treatments.

Vernalization was also counterproductive in *P. orientalis*, decreasing germination from 71.11% to 44.05%. While cold stratification typically enhances germination in Cupressaceae (Ma et al., 2022), the sensitivity of *P. orientalis* may relate to its adaptation to a broader climatic range, as vernalization-induced histone modifications can vary depending on species and ecotypes (Akter et al., 2018). In line with our results, it has been observed species-specific thermal sensitivity in seed viability (Rix et al., 2011).

Among the three tested culture media, LP/2 not only supported the highest germination rate (87.5%), but also the most effective callus induction (74.5%) and shoot regeneration (7.9 buds per explant). This reinforces the findings reported in *Pinus pinea*, in which LP medium was optimal for its culture (Carneros et al., 2009), similar results have been also obtained in *Abies koreana* (Guo and Jeong, 2021). Interestingly, although MS/2 supported limited germination (16.67%), it led to the tallest seedlings, as reported in *Moringa oleifera* (Ledea-Rodríguez et al., 2020). This suggests that while LP/2 favors early morphogenic events, MS/2 may better support post-germination elongation.

In terms of explant responsiveness, cotyledons consistently outperformed hypocotyls and radicles in callus induction and organogenic potential, as shown in this and previous studies (Roca and Mroginski, 1991; Furmanek and Banas, 2011; Konate et al., 2013). Interestingly, superior results have been found in hypocotyl from *Arctium lappa*

(He et al., 2006), again confirming the species dependency of explant behavior.

Dark incubation significantly improved callus induction, in agreement with findings in *Abies guatemalensis* (Ramírez, 2003), *Pinus koraiensis* (Gao et al., 2021; 2023), and *Abies pindrow* (Bhat et al., 2014). However, maximum callus size was obtained under light conditions, indicating that light exposure supports cell proliferation and differentiation in a dual-phase model (Sarmast, 2018; Tavares, 2019).

Regarding the regeneration phase, LP/2 medium not only supported callus proliferation but also maximized shoot differentiation, corroborating the results of Rojas-Vargas et al. (2023) in *Pinus ponderosa*. However, MS/2 was superior in *Pinus taeda* (Barone et al., 2018), contrasting with our results and underscoring the need to fine-tune protocols for each conifer species, as nutrient demands are genotype-dependent (Celestino et al., 2005).

Notably, no regeneration occurred from root-derived calli, a limitation observed in other gymnosperms and possibly linked to reduced competence or hormonal responsiveness in radicle tissues (Mehbub et al., 2022). The ability of cotyledon and hypocotyl-derived calli to produce photosynthetically active shoots confirms their higher morphogenic potential, with early pro-organogenic structures observed under light (Fig. 5), as also described previously in *Pinus pinea* (Sarmast, 2018).

In this study, the effects of various factors on germination, callus induction, and the differentiation of new organs from callus tissue were evaluated. To analyze the germination process, the influence of mechanical scarification and vernalization, as well as the impact of different culture media, were assessed. In *Platyclusus orientalis*, mechanical scarification was found to be highly detrimental, reducing germination capacity from 64% to just 8%. This result underscores the critical role of the protective seed coat during the germination process.

Regarding vernalization, this treatment also negatively affected seed germination in this species, decreasing the germination percentage by 62% compared to non-vernalized seeds. Among the culture media tested, LP/2 medium (with macronutrients at half-strength) produced the highest germination rate, exceeding 84%, which was significantly higher than that obtained in DCR medium (68.5%). In turn, DCR significantly outperformed MS/2 medium, which resulted in a germination rate of only 16.6%. However, seedlings grown in MS/2 medium were significantly taller and more developed than those grown in the other two media. Given the well-documented challenges in germinating seeds from the Cupressaceae family, this finding represents a significant advancement in achieving complete plant development from *P. orientalis* seeds.

In the context of callus induction, several factors influencing callus development in *P. orientalis* were studied. Cotyledons proved to be the most effective explants, producing significantly higher callus induction rates and larger callus size, followed by hypocotyl-derived explants. Darkness substantially enhanced explant dedifferentiation, increasing callus formation by 10%

compared to light conditions (from 53.6% to 63.6%). The most suitable culture medium for callus induction was LP/2, which produced 74.5% callus formation, with significantly larger callus size than MS/2 and comparable size to that obtained with DCR medium.

Finally, during the differentiation phase of callus cells into organogenic structures, calli derived from cotyledons showed superior proliferation and significantly higher differentiation rates compared to those from hypocotyls and roots. No organogenesis was observed in calli originating from root explants. Light played a key role in the formation of early pro-organogenic structures with photosynthetic capacity, although its effect was limited to the initial stages of differentiation. LP/2 medium again proved to be the most effective, promoting the largest callus size during the acclimatization phase and the highest number of differentiated shoots.

Overall, our findings establish a reproducible and efficient regeneration system for *P. orientalis*, which may facilitate its conservation and clonal propagation. This protocol also lays the groundwork for future studies on somatic embryogenesis, cryopreservation, or metabolite enhancement (Fan et al., 2012; Ahn and Choi, 2017; Ho et al., 2022), particularly in ancient or high-value genotypes (Chang et al., 2023; Dong et al., 2023).

In conclusion, this study provides valuable insights into the factors affecting germination, callus induction, and organ differentiation in *Platyclusus orientalis*. The findings highlight the critical roles of scarification, vernalization, and culture medium selection in optimizing germination, with LP/2 medium emerging as the most effective for both germination and callus development. The results also emphasize the importance of darkness for efficient callus induction and the role of light in initiating organ differentiation. Overall, these advancements contribute significantly to overcoming propagation challenges in the Cupressaceae family, paving the way for more efficient and reliable cultivation methods.

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