

Indirect Organogenesis of *Platycladus Orientalis* L.

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Research Article

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Abstract

In conifer species vegetative propagation has many limitations, from low germination rates to the difficulty of rooting explants. Plant in vitro culture has long been used for multiplication, genetic improvement and conservation of elite traits. In this work it has been determined the influence of several parameters on the in vitro culture of *Platycladus orientalis* L.: the culture media, the type of explant, light/dark growth conditions and vernalisation period, specifically, on three different phases of development: germination, callus induction and indirect organogenesis. The best explant for callus induction was the cotyledon and the ideal medium for its culture was Murashige-Skoog under lighting conditions. In the case of indirect organogenesis, the better-differentiated explants grew up with light and LePoivre medium. In germination, the vernalisation of seeds during two months subjected to 4°C had a positive influence. The best medium, which produced the greatest number of roots and the greatest length, was Murashige-Skoog. Also, it has determined the important role of seed coats in germination.

Key Message

The indirect organogenesis of *Platycladus officinalis* L. has been achieved. Different segments from the seedling were used as explants, in several culture media and light/darkness growth conditions.

1. INTRODUCTION

CO₂ emissions due to the changes in the use of lands including deforestation and forest degradation account for between 12 and 29% of global greenhouse gas emissions (Fearnland, 2000; Myers, 2007; Van der Veerf et al., 2009). Changes in land use imply the reduction of gas emissions as one of the global objectives. Intensive forest plantations are currently considered one of the best defences against ecosystems threatened by deforestation. These plantations are included in one of the mechanisms of the Kyoto Protocol about control of global warming and climate change, as CO₂ sinks. The twenty-sixth United Nations Conference on Climate Change (COP26) held in Glasgow in November 2021 has forestry financing as one of its priority expectations. A global commitment to finance the protection and sustainable management of forests was agreed to protect natural habitats so that in the near future 90% of the world's forests will be covered by trees, a commitment by 130 countries to end deforestation by 2030.

Economically, plant conifers are very important timber forest plants, being the Pinaceae family the leading resource. The wood of pines, cedars and spruces is widely used in construction, pulp and wax production, furniture and wooden utensils, among other purposes. Also, several genera are used as ornamental plants in parks and gardens around the world.

Some conifer species are obtained by traditional vegetative propagation techniques through seed sowing, which is a slow process; grafting or cuttings from juvenile or adult individuals, both aimed at multiplying species with genotypes of high economic value, endangered or of scenic importance (Prieto, 1992;

Celestino et al., 2005). However, this system doesn't always guarantee the demand and doesn't always achieve a homogeneous and quality development of the planted trees, since these techniques have their own individual characteristics for each species and work under specific and exclusive conditions in each case (Gutiérrez, Ruiz and Bustamante, 1996). The germination of valuable genotypes, virus-free meristems or plants from transformed cells has no validity from an applied point of view if it is not possible to achieve mass multiplication of said selected genotypes, under economically viable conditions.

The first trees obtained in vitro were generated in the 1960s by the organogenic route of bud induction subsequent rooting. Conifers regeneration by organogenesis from explants such as apical buds, axillary buds, etc., have been not greatly studied alternatives, due to the problems of endogenous contamination. However, in some species of *Pinus* genus, regeneration from apical shoots has been achieved, for example in the case of *Pinus pinaster* (De Diego et al., 2008) or *Pinus caribaea* (De Fera et al., 2009 and 2010). Also, rhizogenesis or induction and development of a functional root system is one of the restraining barriers for micropropagation in certain woody species (Ríos et al. 2005), since multiplication coefficient and in vitro root formation rates for many species are really low (Oliveira et al., 2003; Stojicic and Budimir, 2004). Rooting is a slow process since the shoots of most conifers, root from callus or scar tissue or directly from vascular tissue, followed by their insertion in other media with auxins (Chavez, 2010). In some commercial micropropagation systems, losses during the acclimatisation stage of plants can be as high as 40% or more (Mathur and Nadgauda, 1999; Tang and Ou-Yang, 1999), being rooting the main obstacle to the success of conifer micropropagation (Mohammed and Vidaver, 1988).

Induction of adventitious buds after callus formation was a extended technique in conifer regeneration by organogenesis, mainly in genera like *Pinus*, *Picea* or *Pseudotsuga*. In the past, organogenesis, as a large-scale propagation method, was the preferred and most widely used method for many tree species. This technique is especially useful in species with high heterozygosity like *Pinus halepensis*, since the sexual reproduction of selected individuals has the disadvantage of producing a high proportion of undesired genotypes in the progeny (Benito, Manzaneda and Pardos, 1993).

In recent years, the forestry industry has opted for somatic embryogenesis, for the obtention of new plants for several reasons: 1) high multiplication potential, with the capacity to grow cultures in bioreactors, 2) Apply encapsulation techniques to manufacture synthetic seeds; 3) their regeneration after cryopreservation and 4) the possibility of using embryogenic cultures as suitable targets for genetic transformation, an essential step for any study of functional genomics. In genetic engineering, the two most frequently used methods for gene insertion in conifers are: 1) gene bombing with microprojectiles, i.e. accelerated particles covered with DNA, and 2) transformation with *Agrobacterium tumefaciens* carrying the desired genes in a transfer plasmid or Ti plasmid (Cyr and Klimaszewska, 2002). Potential applications of this methodology include the introduction of herbicide and pest resistant genes; genetic alterations that would provide desired wood characteristics and reproductive sterility, among others. Somatic embryogenesis has become important for tree breeding because of its potential to allow selection and propagation of elite lines. This is a way of increasing yield and reducing rotation age, i.e.

the age at which a forest mass is planned to be felled, which helps in the management of forest productivity.

Somatic embryogenesis from immature zygotic embryos is the most commonly used regeneration method in plants of *Pinus* sp. (Pullman et al, 2005; Salanova et al, 2007; Klimaszewska, 2007). However, other authors have reported that it's sometimes limited to callus formation, with few embryos produced per gram of fresh mass (Lelu et al., 2006; Maruyama et al., 2007). Plant biotechnology techniques have also been applied to other conifers in addition to the *Pinus* genus, for example, it has been described the micropropagation of *Cupressus sempervirens* var. *horizontalis* Mill. from secondary shoot explants (Khamushi et al, 2019); adventitious bud formation from needles of *Picea abies* L. Karst. (Von Arnold and Eriksson, 1979) or the case of the *Abies* genus, where somatic embryogenesis from vitro-explants and indirect organogenesis from mature zygotic embryos of *Abies guatemalensis* has been successfully achieved (Pérez and Demetrio, 2012). These somatic embryos have been initiated in conifers, mainly from embryonic tissues, as immature or mature zygotic embryos (Atree and Fowke, 1991). Also, Tautorus et al. (1991) described the production of embryogenic tissue from megagametophytes. What does appear to be common to most conifer species is that both explant and developmental stages are critical factors for embryogenic tissue induction, and explants from mature zygotic embryos are considered less successful. Newton et al. (1994) described that, in the case of *Pinus elliottii*, the best explant for induction was immature embryos in different phases of development. According to Celestino et al. (2005), in conifers, the presence of ABA in the culture medium is required for the maturation and multiplication of somatic embryos which before this treatment are globular clusters of a few cells. ABA is added to carry out their histogenesis, differentiation and maturation process until they reach the cotyledonary stage and germinate.

Platycladus orientalis L. Franco in Portugalae (Cupressaceae) also called the tree of life, is an arboreal species of the Cupressaceae family, the only accepted species of the *Platycladus* genus. It has highly branched leaves and a bushy or arboreal habit, reaching a height of no more than 12 meters. Used profusely as ornamental, especially for hedges.

In view of the limitations of traditional vegetative propagation and the background presented, the aim of this work has been to obtain from callogenic tissue the indirect organogenesis of *Platycladus orientalis* L. inducing directly the development of shoots from the callus. This technique is a variant of embryogenesis in which the first tissue developed has been the vegetative buds. In the second step, this explant has developed the root in the same culture medium. We have studied the development of the complete plant in several culture media, using several types of explants, light/dark conditions and the influence of vernalisation on in vitro establishment of this conifer plant.

2. MATERIAL AND METHODS

2.1. Plant Material

Seeds of *Platycladus orientalis* L. were used as initial explants. A total of 102 seeds were collected at “Las Lagunillas” Campus, Jaen (UTM coordinates: 37.785981, -3.774653) during the months of June to September 2020. To take the seeds of the cones, they were opened exposing them to direct sunlight for one week at room temperature (approximately 25°C), allowing the seeds to be obtained. Half of the seeds obtained were subjected to artificial vernalisation, inside the fridge, at a temperature of 5°C for two months, simulating the cold season they would experience in field conditions.

2.2. Culture media

Three culture media were used: Murashige-Skoog or MS medium (Murashige and Skoog, 1962), Le Poivre or LP medium modified by Aitken-Christie et al. (1988) and DCR medium (Gupta and Durzan, 1985). The pH of all culture media used was adjusted to 5.8. The MS and LP media were used at a half concentration of macronutrients, so they are referred to as MS/2 and LP/2.

2.3. Seeds germination

The seeds were disinfected by imbibition in a 20% solution of sodium hypochlorite (commercial bleach) for 10 min and rinsed with sterile distilled water. About half of the seeds were incised in the coat, under aseptic conditions in a laminar flow chamber, leaving another part of the seeds with the coat intact. The seeds, vernalised and non-vernalised, incised and non-incised, were sown in the three culture media described above (MS/2, LP/2 and DCR) without growth regulators. After sowing, the culture tubes were kept in darkness for 7 days, after which they were transferred to the phytotron type (ARALAB 600PHL-LED) for 60 days, with a photoperiod of 16 hours of light at 22°C and 8 hours of darkness at 22°C, relative humidity: 60%. After 60 days, were taken the following measures: germination percentage, root length, stem length, total length, and number of leaves of each plant and treatment.

2.4. Organogenesis induction

Asepsis of the seeds was carried out by imbibition in a 20% solution of sodium hypochlorite (commercial bleach) for 10 min. After this time they were rinsed with sterile distilled water. Then, to achieve germination, the seeds were put on sterile filter paper in sterile Petri dishes. A new sterile filter paper was put on top to completely cover them, the paper was humidified with sterile distilled water and, finally, the Petri dishes were put in darkness inside the heater at a temperature of 21°C for 21 days. After 21 days, the seedlings that emerged were disinfected following the same methodology as described above, in order to obtain explants for in vitro culture, and dissected obtaining three types of explants: cotyledon, hypocotyl and radicle. Each explant was cultured in Petri dishes containing the three culture media evaluated in the experiment.

All three media were supplemented with growth regulators at concentrations of 2 mg/L 2,4-dichlorophenoxyacetic acid or 2,4-D and 1 mg/L benzylaminopurine or BAP. Once the dishes were seeded, they were divided into two groups; those that were to stay in darkness and those that were to be exposed to light. These latest were transferred to the phytotron under the same culture conditions described in the

previously during six weeks. The samples that stayed in darkness were six weeks at the same temperature and humidity conditions.

After 6 weeks of culture, it was measured the percentage of callus formation and the size of induced callus tissue.

2.4.1. Callus cells proliferation and organogenic structures elongation

The induced calluses were transferred to elongation medium under sterile conditions in the laminar flochamber. As elongation medium, the three cited culture media were supplemented with 0.4 mg/L 2,4-D and 0.5 mg/L BAP. The calluses were culture under the previously defined conditions for six weeks. After this time, they were determined the number of shoots or vegetative buds formed and the size of calluses.

2.5. Statistical análisis

STATSGRAPHIC Centurion XIX package (version 19.1.2) for Windows was used for statistical analyses. Specifically, Parametric Analysis of Variance (ANOVA) tests and Fisher's Least Significant Difference (LSD) procedure were applied.

3. RESULTS AND DISCUSSION

3.1. Seeds in vitro germination

Table 1 summarizes the effect of vernalization and culture media on the germination and growth parameters of *Platycladus orientalis*. Vernalisation had a significant effect on germination, because vernalised seeds increased the germination percentage (71.11%) compared to non-vernalised seeds (44.05%). Similarly, the culture media used also had an effect on germination. LP medium achieved the highest germination percentage (87.5%) compared to DCR (68.57%) and MS (16.67%).

Table 1

Effect of vernalization and culture media on germination and growth parameters in *Platycladus orientalis*. Different letters indicate statistically significant differences according to Fisher's minimum significant difference (LSD) procedure (* $p \leq 0.05$; ** $p \leq 0.01$).

Parameters	Treatment		Culture media		
	Vernalised	Non-vernalised	MS/2	LP/2	DCR
% <i>In vitro</i> germination	44,047 $\pm$ 2,259 a **	71,111 $\pm$ 2,259 b **	16,667 $\pm$ 2,767 a **	87,5 $\pm$ 2,767 b **	68,571 $\pm$ 2,767 c **
Root length (cm)	8,909 $\pm$ 1,582 a	5,870 $\pm$ 1,199 a	13,319 $\pm$ 3,152 a *	3,737 $\pm$ 1,014 b *	5,112 $\pm$ 1,082 b *
Shoot length (cm)	3,964 $\pm$ 0,309 a	3,702 $\pm$ 0,239 a	4,630 $\pm$ 0,615 a	3,595 $\pm$ 0,214 a	3,275 $\pm$ 0,211 a
Total length (cm)	12,759 $\pm$ 1,724 a	9,869 $\pm$ 1,335 a	17,745 $\pm$ 3,434 a *	7,811 $\pm$ 1,195 b *	8,387 $\pm$ 1,176 b *
Number of leaves	13,531 $\pm$ 2,915 a	13,369 $\pm$ 2,256 a	15,080 $\pm$ 5,805 a	11,645 $\pm$ 2,021 a	13,625 $\pm$ 1,989 a

With respect to pre-germinative treatments, as shown in Fig. 1, mechanical rupture of seed coats had a negative effect on germination (8.33%) compared to those with intact coats (64.72%). These results differ from those of published by Ardebol et al., (2006), where the presence of a coat in seeds of *Pinus cubensis* Griseb constituted a mechanical barrier between the embryo and media, limiting the germination process. However, the removal of the coat favoured it. Logically this is not very comparable since not all coniferous seeds have the same morphology and consistency and not the same amount of germination inhibitors in the coats. As shown in Table 1, the type of medium had an effect on root length and the total length of the plants. MS medium increased both root length (13.32 cm) and total length (17.75 cm) with respect to LP medium (3.74 and 7.81 cm, respectively) and DCR (5.11 and 8.39 cm, respectively) however the media had no significant effect on the number of leaves and shoot length. Vernalisation had neither significant effect on seed germination.

3.2. Organogenesis

3.2.1. *Callus induction*

After 6 weeks of explant growing in an induction medium, the mean percentage of callus tissue formation was 61.73% and the average size of the induced callus was 0.2605 cm. For the type of explant used (Fig. 2a), the use of cotyledon increased the percentage of callus formation (74.06%) compared to the hypocotyl (60.0507%) and radicle (41.69%). Roca and Mroginski (1991) obtained identical results with regard to the cotyledonary tissues, as the better explants to callus formation. Similarly, cotyledon (0.3669 cm) was the explant that got the largest callus size (Fig. 2d) compared to the hypocotyl (0.2120 cm) and radicle (0.14686 cm). Darkness (63.59%) increased callus formation respect to lighting condition

(53.61%) (Fig. 2b). These data are in consonance with the results obtained by Ramírez, who demonstrated in 2003 that darkness conditions produced a higher percentage of callus formation. Despite this, the largest callus size was obtained under lighting conditions (0.2955 cm), (Fig. 2e) compared to darkness (0.1883 cm). For the type of culture medium (Fig. 2c), LP medium (74.55%) increased the percentage of callus formation with respect to DCR (55.87%) and MS (45.38%). These latest results are in line with Ojeda-Carneros (2009), who described that LP medium supplemented with auxin and cytokinin got the best proliferation percentages in *Pinus pinea*. Regarding the size of the callus (Fig. 2f), DCR medium (0.2642 cm) induced the largest callus with respect to LP (0.2514 cm) and MS (0.2102 cm).

3.2.2. Callus cells proliferation and organogenic structures elongation

Figure 3 shows the aspect of calluses and the first adventitious buds developed in in the induction medium. Figures 3b and 3d show the appearance of the calluses or amorphous masses of non-photosynthetic cells with rapid and disorderly growth, from which some denser masses or structures begin to be observed, indicating the beginning of differentiation into adventitious buds or into roots. On the other hand, in Figs. 3a and 3c, the numerous differentiated buds or adventitious shoots which have photosynthetic capacity can be observed almost from the beginning of their differentiation.

Culture in an elongation medium supplemented with 2,4-D and BAP fitohormones (Fig. 4) produced the increase of the calluses, as well as the development and elongation of buds, shoots and roots. After 6 weeks in elongation medium, the calluses got an average size of 0.4240 cm, so grew 62.7617% respect to the callus size obtained at final of induction stage.

In the study of indirect organogenesis (Fig. 5), all three factors studied in this work: type of explant, light/dark and culture media; affect callus increase (Figs. 5a, b and c), but only the culture media affected to the number of shoots or buds developed from the callus (Figs. 5d, e and f). In the case of the explants, cotyledon (0.447493 cm) and hypocotyl (0.398674 cm) increased callus size compared to radicle (0.243976 cm) (Fig. 5a). Callus size was higher under lighting conditions (0.417154 cm) than in darkness (0.309608 cm) (Fig. 5b). LP medium (0.437841 cm) gave better results than MS (0.318333 cm) and DCR (0.333969 cm) (Fig. 5c). Also LP medium (7.88885) increased the number of shoots or buds compared to MS (3.29879) and DCR (1.45423) (Fig. 5d). Neither radiation nor explant type had any effect on organogenesis. Radicle didn't produce bud induction (Figs. 5e and f).

Figure 6 shows as a summary of the results of this work the complete indirect organogenesis process, from the dedifferentiation of the explants (cotyledon) to form amorphous masses of callus cells (Fig. 6a). The development of the first competent cells, with photosynthetic capacity, in green (Fig. 6b) and finally, the differentiation of the cells can be seen until they form perfect stems or roots in Figs. 6c, d and e.

4. CONCLUSIONS

The results of this work defined different in vitro culture conditions for *Platycladus orientalis*. On the one hand, the induction of callus from juvenile explants and their subsequent determination and differentiation in the different plant organs is a viable form of regeneration, with the possibility of applying genetic engineering techniques, such as the insertion of genes of interest in the callus phase. LP medium achieved 7.89 shoots per explant in the organogenesis process, being the best of the evaluated media for this purpose. Cotyledon was the most efficient explant for callus induction, and culture under light conditions produced the best induction. MS medium increased the size of calluses to a greater extent.

In the elongation stage, light and LP medium were the factors that achieved the greatest callus cell proliferation and organogenic structures formation (stems and roots), with cotyledon being the explant that provided the better results.

With respect to the germination process, vernalisation had a positive effect on the seed germination capacity of this species. It has been demonstrated the important role of seed protective coats during germination. Vernalisation did not have a significant effect on any of the measured parameters. Plants grown on MS medium had longer root lengths and greater total lengths compared to plants grown in DCR and LP medium.

Abbreviations

BAP, benzylaminopurine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **MS**, Murashige-Skoog; **LP**, LePoivre

Declarations

Data availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Competing interests

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AUTHOR CONTRIBUTIONS: Both authors contributed to the study conception and design.

Conceptualisation: Ana Maria Fernández Ocaña (AMFO) and Luis Lucena Soriano (LLS); Material preparation and data collection were performed by LLS. Data analysis was performed by LLS and AMFO. The first draft of the manuscript was written by LLS; Final draft AMFO. commented on previous and following versions of the manuscript AMFO. Both authors read and approved the final manuscript.

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Figures

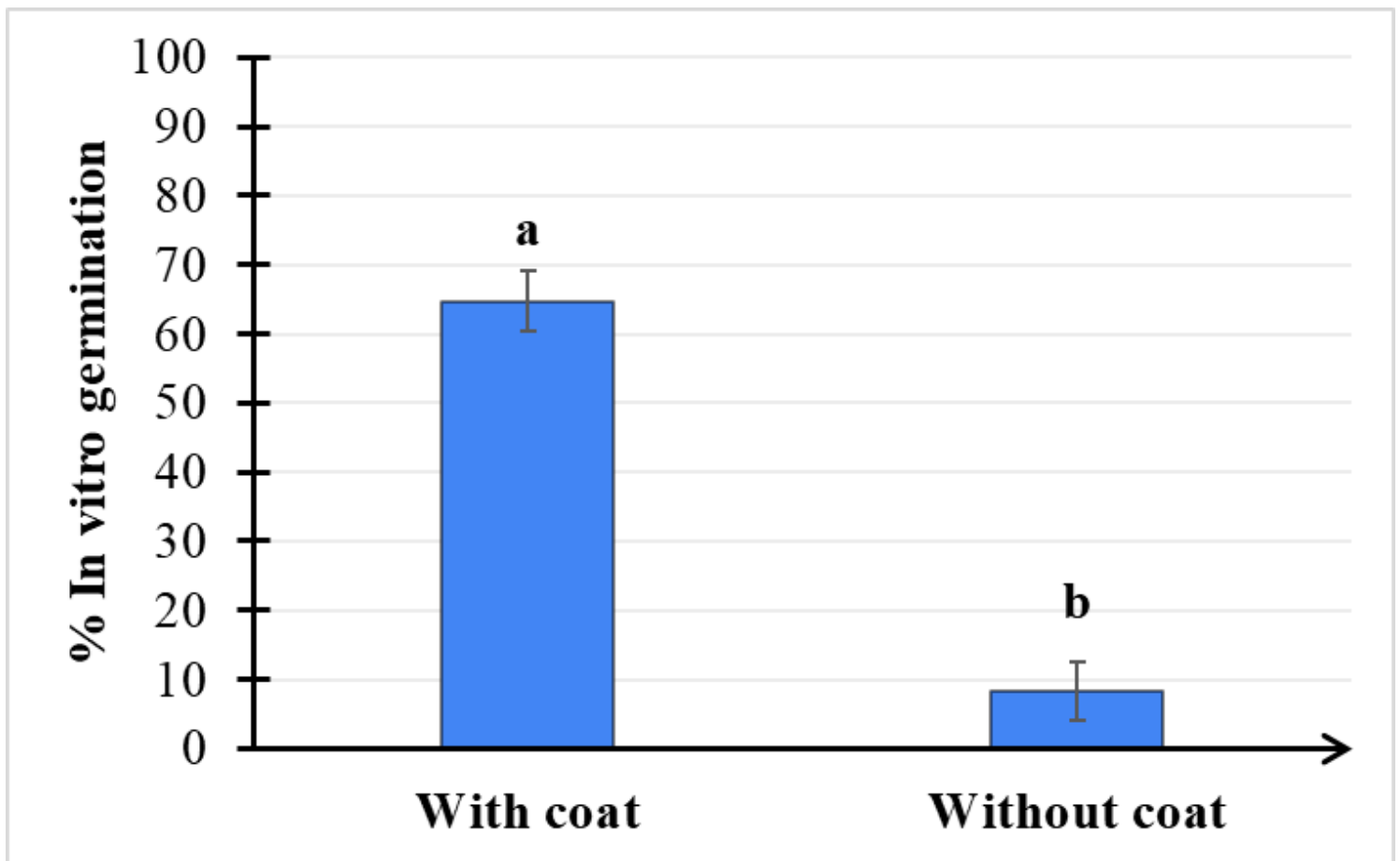


Figure 1

Effect of pre-germinative treatment on in vitro germination in seeds of *Platycladus orientalis*. Different letters indicate statistically significant differences according to Fisher's minimum significant difference (LSD) procedure with $p \leq 0.01^{**}$.

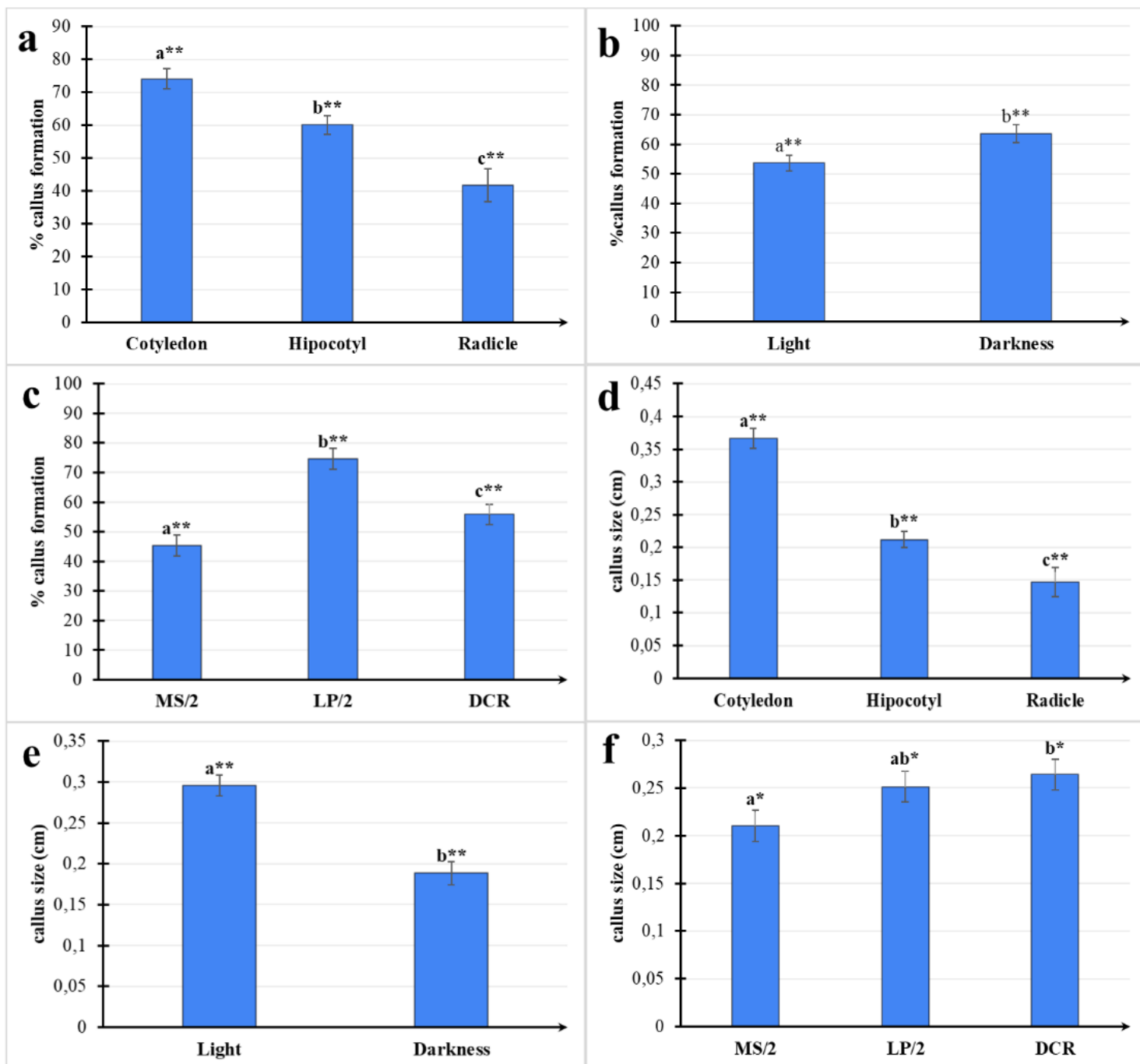


Figure 2

Percentage of callus formation and their size for the three types of explants used (cotyledon, hypocotyl and radicle), the radiation which cultures were exposed and the type of culture media used. **Graphs a and d:** effect of explant on the % of callus formation and callus size, respectively.; **graphs b and e:** effect of radiation on % callus formation and callus size, respectively; **graphs c and f:** effect of culture media on the % of callus formation and callus size, respectively. Different letters indicate statistically significant differences according to Fisher's minimum significant difference (LSD) procedure (* $p \leq 0.05$; ** $p \leq 0.01$).

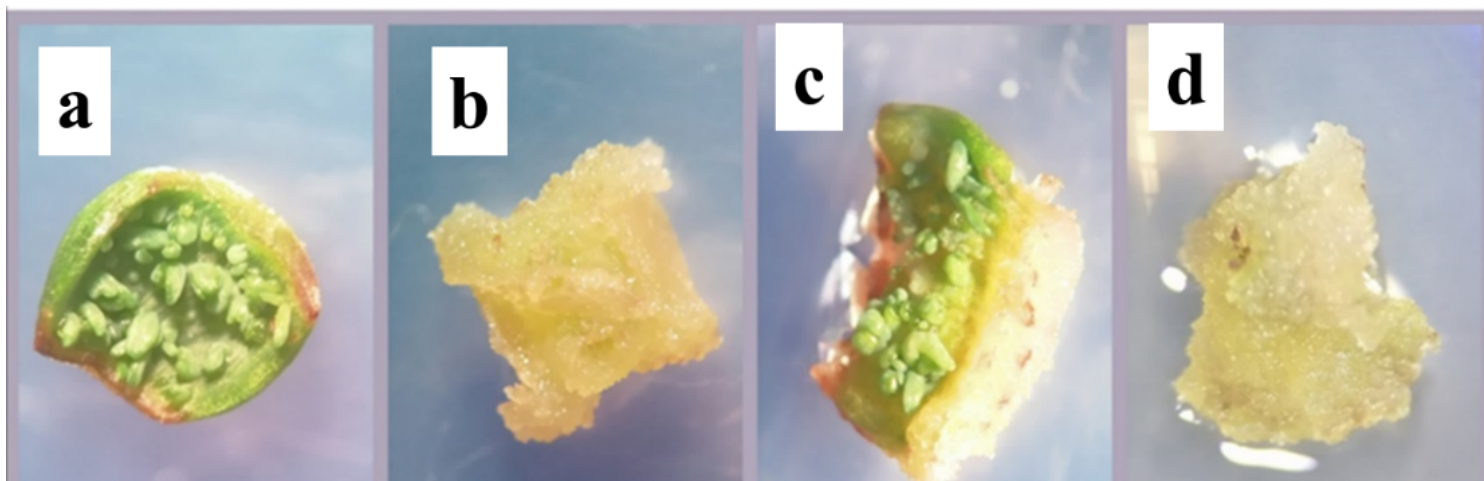


Figure 3

Callus and adventitious buds from cotyledon of *Platycladus orientalis* after 6 weeks of culture in induction medium under lighting conditions. **Images a and c:** adventitious buds induction on callus cultured in LP medium; **images b and d:** callus induction on explant culture in DCR medium.

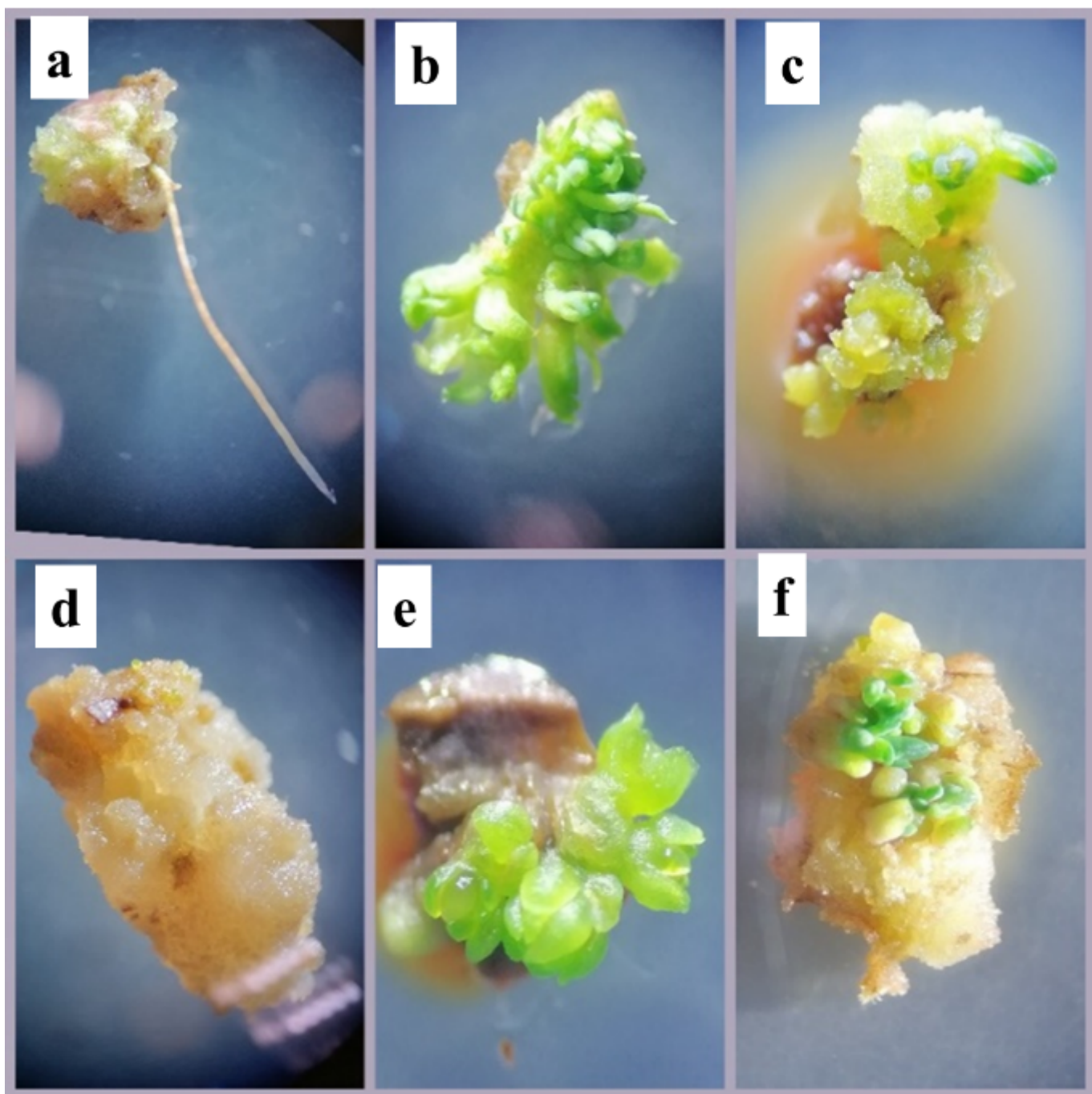


Figure 4

Callus and organogenesis in different explants of *Platycladus orientalis* after 6 weeks in elongation medium under lighting conditions. **Image a**: apparent root induced from hypocotyl in LP medium; **images b, c, e and f**: buds and shoots elongation from cotyledon cultured in LP medium; **image d**: callus cells proliferation from cotyledon cultured in MS medium.

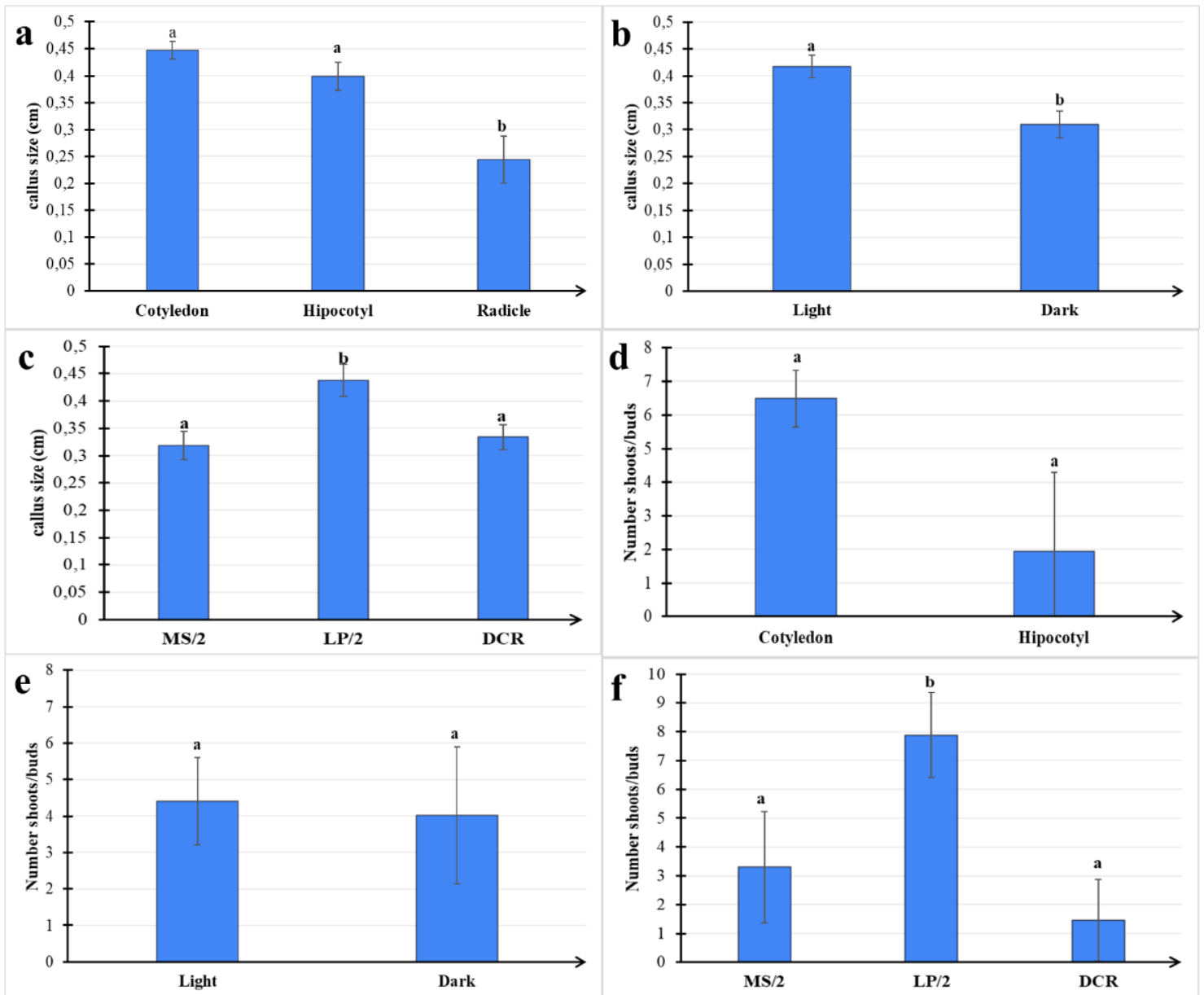


Figure 5

Effect of the type of explant, radiation and culture media on callus increase and the number of buds/shoots after 6 weeks of culture in an elongation medium. **Graphs a and d:** effect of explant on the size of callus and the number of shoots/buds, respectively; **graphs b and e:** effect of radiation on callus size and number of shoots/buds, respectively; **graphs c and f:** effect of culture media on callus size and number of shoots/buds, respectively. Different letters indicate statistically significant differences according to Fisher's Minimum Significant Difference (LSD) procedure with $p \leq 0.01$.

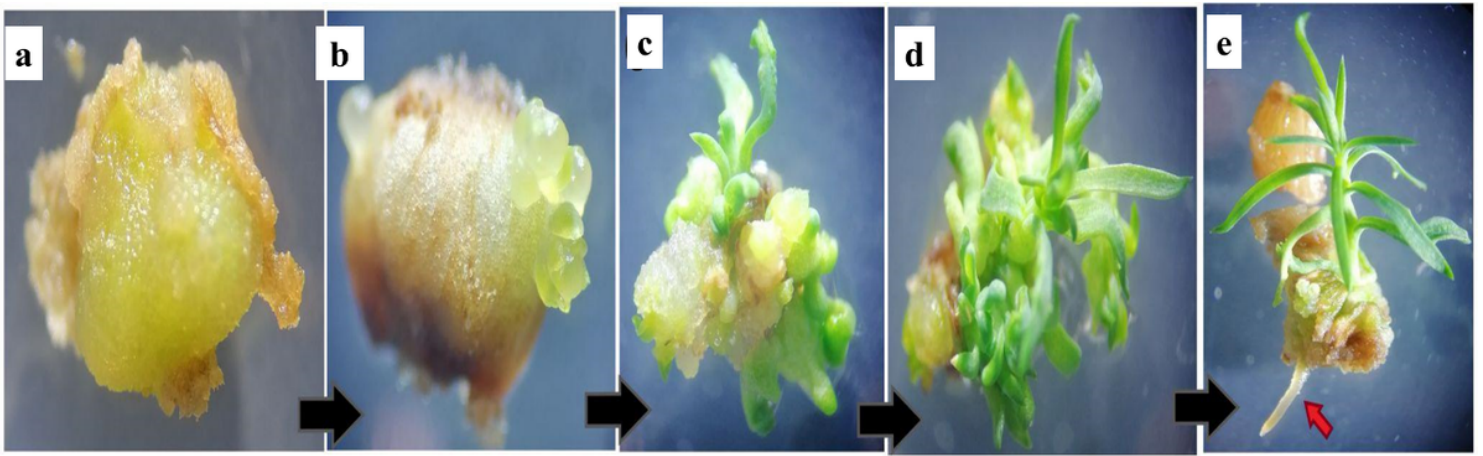


Figure 6

From left to right, **image a** shows callus cells determination in chlorophyllic cells; **image b** shows the appearance of the first buds; **image c** shows the development of these buds and the emergence of shoots; **image d** already shows the leaves and the stem in elongation and **image e** shows a stem with leaves and the red arrow points to an apparent root.