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AN EFFICIENT METHOD OF ROOTING FROM *IN VITRO* CULTURE OF ZYGOTIC EMBRYOS OF *PINUS ROXBURGHII* SARG.

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Abstract

This study was conducted to explore the possible rooting efficacy of the mature zygotic embryo of *Pinus roxburghii*. Megagametophytes with zygotic embryos were aseptically removed from surface-sterilized mature cones. After aseptic extraction, mature zygotic embryos (MZE) and megagametophytes (Mgt) were individually cultured on modified LP 505 media supplemented with 0.45 mg/L BAP, 2 mg/L NAA, and 0.43 mg/L Kinetin for initiation and extrusion of cultures in the dark for 20 days. The cultures were then changed to a 16-h photoperiod for an additional 20 days. In light (40-day-old cultures), embryos extruded 75% and Mgt 19.04%. The shoot length was 2 cm after 30 days of cultivation, while Mgt produced 3 cm shoots. Developing shoots (40 days old) were moved to DCR or LP semi-solid media enriched with 2, 4, 6, or 8 mg/L of indole-3-butyric acid (IBA) + α-naphthalene acetic acid (NAA) in a 16 h photoperiod. Rooting was unsuccessful in these media; however, shoots developed up to 4 cm in length on LP + 6 mg/L IBA + NAA after 20 days (using 2-month-old cultures). Both MS and DCR cultures exhibited 50% rooting with 2 cm long roots in half DCR, LP, or whole MS media. After a month, rooted shoots were transferred to 8 × 15 cm plastic pots filled with a 3:1 peat moss and sand potting media. Pots were wrapped in plastic bags to constrain moisture and kept under the culture room conditions. For one month, irrigation with ¼ DCR was done every 10 days. Plants from MS lasted 35 days, whereas DCR plants stayed green in the potting mix for 15 days and successfully grew under field conditions.

Key words: LP-505; Megagametophyte; Chir pine; *In vitro* rooting; Mature zygotic embryos; Root-shoot transition

Introduction

Forests are crucial for maintaining the balance of the environment and preserving the world's biodiversity (Simberloff, 1999). Through the process of absorbing carbon dioxide and storing it, they prevent temperature fluctuations (Alemu, 2014). In addition, forests are a source of food, wood, and medicinal plants. They also enhance the ground's productivity, regulate water cycles, and provide habitats for a diverse range of animals (Chamberlain *et al*., 2020). For the sake of the continuation of life on Earth and the sake of future generations being able to live in a healthy climate, these woods need to be preserved.

Distributed in Nepal, Bhutan, India and Pakistan, *Pinus roxburghii*, commonly referred to as chir pine, can be found growing at elevations ranging from 900 to 1,800 meters above sea level. It is a tree species of the Himalayas (Siddiqui *et al*., 2009). According to Ahmed *et al*., (2006), chir pines are found growing in the subtropical dry and wet temperate zones of Khyber Pakhtunkhwa, and Murri hill of Pakistan. Due to its durability and resistance to deterioration, it is a valuable material used in the production of paper, furniture, and construction. Additionally, chir pine contributes to the preservation of biodiversity, the management of watersheds, and the maintenance of soil stability (Sinha, 2002).

Chir pine is used in the landscaping industry for its attractive appearance and evergreen foliage. When it comes to cities, it is necessary to consider their space requirements and needle shedding (Bolund & Hunhammar, 1999). According to Khare (2007), it is also highly appreciated in traditional and Ayurvedic medicine for its capacity to reduce inflammation, eliminate pathogens, and protect cells from injury.

Pakistani chir pine stocks are at risk of being overharvested due to increased demand among consumers (Ansari *et al*., 2022). In situations where seeds are not viable, it is difficult to use the conventional methods of propagation, which are mostly dependent on the seed's vigor and germination (Sharma & Verma, 2011). Both micropropagation and axillary bud shoot proliferation have been proposed as potential alternatives; however, these methods are restricted due to the high terpenoid content of the plants, their resistance, and their inability to produce young explants.

It is possible that embryo rescue (ER) procedures could be utilized as a potential remedy. In the 18th century, Charles Bonnet was the first person who discovered this technique. Hanning *et al*., (1904) demonstrated that these techniques had been successful in preserving embryos in the ovum of other species. According to Lelu-Walter *et al*., (2016), entire megagametophytes are frequently utilized in the process of somatic embryogenesis (SE) in conifers. However, at this moment, there are no reports of *P. roxburghii* mature embryonic cultures taking root in a laboratory environment. The aim of the present research was to determine the rooting potential of *In vitro* grown mature zygotic embryos of chir pine.

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Material and Methods

Plant material and culture conditions: Female cones (green and immature) of two open-pollinated trees of *P. roxburghii* (Fig. 1) were harvested during August from sixty-year old trees at the Botanical Garden, University of the Punjab, Lahore. The cones were brought to the lab, kept in plastic bags, and stored at 4°C until use.

For *In vitro* culture establishment, four basic media (505) of Pullman & Johnson (2002), Gupta & Durzan, 1985 (DCR), Murashige & Skoog, 1962 (MS), von Arnold & Eriksson, 1979 (LP). Stock solutions for macronutrients, micronutrients, iron EDTA, and vitamins were prepared individually, stored at 4°C in dark glass bottles.

NAA (α-Naphthalene acetic acid), IBA (Indole-3-butyric acid (IBA), and Kin (Kinetin) were prepared as 1mM stock solutions by dissolving the required quantities in a small quantity of 0.1N NaOH or HCl, then adjusting the volume with distilled water. These were stored in brown bottles at 4°C.

For 1 liter of culture medium, the appropriate quantities of stock solutions, Myoinositol, and maltose (for 505) or sucrose (for DCR and LP) were mixed and stirred until the solution cleared. Growth regulators were added, and the final volume was adjusted with distilled water. The pH was adjusted to 5.7–5.8 using 0.1N NaOH or HCl. For solidification, 8g of agar (DCR and LP) or Gelrite (505) was added, and the medium was autoclaved at 121°C for 15 minutes. After autoclaving, 20 ml of medium was poured into each sterilized petri plate.

Sterilization of plant material and surgical tools: Cones were submerged in warm water for 30 min and surface was sterilized with ethanol (70%). The cones were cut longitudinally with the help of a sharp knife, and seeds were isolated by using forceps and scalpels (Fig. 2). The seeds were washed under running water for 10 minutes, sterilized in 10% hydrogen peroxide for 15 minutes, and then rinsed with sterilized water. Seeds were stored at 4°C.

All glassware was thoroughly washed with laboratory-grade detergent and sterilised at 70°C for one hour using a dry heat oven. Surgical tools were sterilized by autoclaving at 121°C for 15-20 minutes and further treated in a glass bead sterilizer at 250-350°C. The laminar flow cabinet was cleaned with 70% ethanol, and UV light was activated for 10 minutes before inoculation.

Embryo extraction, inoculation, and growth of cultures: The seed coat was removed with sterilized forceps, and the embryos were carefully extracted from the megagametophyte using a sterilized blade and forceps under a laminar flow cabinet (ESCO). MZE and Mgt were inoculated onto modified LP 505 medium in 90 mm² petri plates (Borosil, China) containing 20 ml medium. The plates were sealed with parafilm. Cultures were kept in the dark for 20 days, then transferred to light conditions (16-hour photoperiod, 35µmol m^{-2} s^{-1}) provided by cool-white tube lights (Philips) for further growth and development. After 20 days in light, cultures with green cotyledons were transferred to DCR and LP media supplemented with 2, 4, 6, and 8 mg/L combinations of NAA and IBA. Shoot elongation was noted in these media combinations. After 21 days, cultures with elongated shoots were transferred to ½ DCR, ½ LP, and MS media without growth regulators for root development. Rooting was observed in DCR and MS media, with 2-3 cm roots in 50% of cultures. No rooting was observed in LP medium.

Acclimatization: Rooted shoots were transferred into plastic pots filled with a potting mix composed of peat moss and sand in a 3:1 ratio. The pots were then covered with plastic bags to maintain a constant humidity level and stored in a culture room. Irrigation with ½ DCR was carried out at a ten-day interval for one month. Plants grown in MS media remained green for 35 days, whereas those produced in DCR persisted for only 15 days.

Fig. 1. Green, immature cone of chir pine.

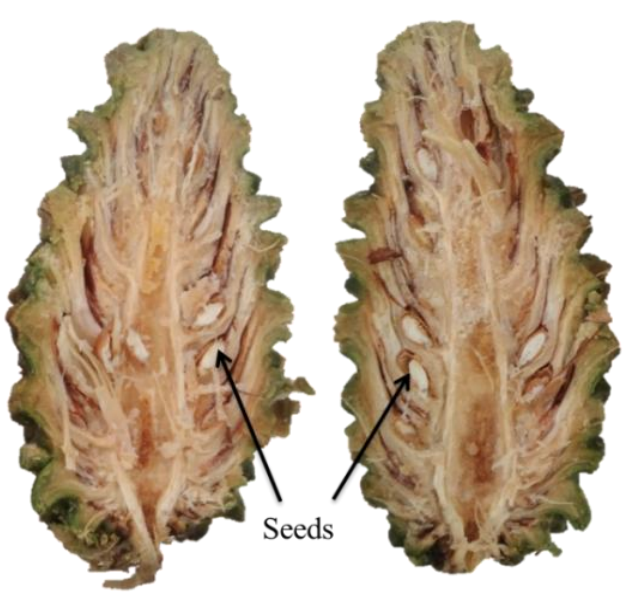

Fig. 2. Longitudinal cut of chir pine cone showing the location of seeds.

Results

Three basal media were investigated for the *In vitro* development of chir pine embryonic structures: DCR, LP, and LP 505. The most promising treatment for both improved embryo culture formation and a high rate was LP 505 supplemented with BA (0.45 mg/L) + Kin (0.43 mg/L) + NAA (2 mg/L). In the subsequent initiation and culture improvement studies, LP 505 medium was utilized due to its promising potential.

Growth and development of developing cultures: Two different kinds of plant materials were utilized as chir pine explants for *In vitro* culture and cotyledonary structure development to assess the rooting capacity subsequently. The whole Mgt (Fig. 3A) showed no physical change after 5 days in the dark, but MZE (Fig. 3B) displayed the appearance of cotyledons in LP 505 media during the same culture period.

Initiation and extrusion: There were 10 Petri plates, each of which contained three explants, and each of these plates was used to culture either MZE or Mgt in a distinct manner on LP 505 media supplemented with 0.45 mg/L BA, 0.43 mg/L Kin, and 2 mg/L NAA. After five days of being kept in dark settings, the rate of culture initiation with visible embryonic masses was found to be at its most significant level (84.21%). The remainder of the cultures were contaminated, and some of them did not exhibit visible sign of initiation (Table 1). After 5 days, MZE (Fig. 3B) displayed the visible appearance of cotyledons, which continued to develop and eventually became green after ten to thirteen days of initial culture (Fig. 4A). On the other hand, cultures of Mgt did not exhibit any discernible change after five days having been subjected to the same culture conditions of medium and environment. An evident appearance of a white elongated structure of radicle (Fig. 4B) began to form in the dark on LP 505 media on day 10 of continuous culture after the culture had been going on constantly. According to Table 1, the rate of initiation and extrusion of Mgt was 21.14%.

It was not possible to find any cotyledons on Mgt. Still, the hypocotyl had significantly grown to a length of 12.41 mm after 20 days of continuous cultivation on the same medium composition (Table 1). When these cultures were exposed to light, they exhibited tremendous growth in their unique way (Fig. 5). The cotyledons of Mgt, which were green in color, emerged and grew longer in light very quickly (Fig. 5A). It was also noted that the cultures of MZE exhibited a rapid development of cotyledons and the creation of shoot primordia (Figs. 3B, C).

Effect of NAA & IBA: Forty-day-old MZE cultures were transferred to either LP or DCR media supplemented with auxins for a pulse treatment aimed at promoting rooting. This treatment enhanced the growth of both cotyledons and hypocotyls in MZE and Mgt (Table 2). Overall, cotyledon growth was more pronounced in MZE compared to Mgt, while hypocotyl growth was better in Mgt than in MZE when cultured on LP medium (Table 2). Maximum cotyledon length in MZE was observed on LP medium supplemented with IBA (6 mg/L) and NAA (5.50 mg/L), reaching 34.32 mm, followed by Mgt with 19.41 mm (Fig. 6A, B, C). This same auxin combination also promoted the longest hypocotyl growth, with Mgt reaching 39.22 mm and MZE 9.33 mm. The DCR medium also facilitated substantial growth in both cotyledons and hypocotyls, with further improvement was noted with the increase auxin concentrations (Table 2). However, some MZE cultures displayed abnormal and stunted growth, with both cotyledons and hypocotyls showing reduced lengths when cultured under the same medium conditions (Fig. 6D, E).

In the current study, browning and necrosis were observed in various MZE cultures across different treatment conditions. The formation of necrotic tissue was notably more prominent in the hypocotyl region of MZE, particularly under all tested conditions (Fig. 7A). Although the cotyledons had started to expand in 5-day-old MZE cultures, the hypocotyl exhibited browning when cultured on LP 505 medium supplemented with 0.43 mg/L kinetin, 2 mg/L NAA, and 0.45 mg/L BA, (Fig. 7B). Additionally, in 40 days, callus formation was evident in the hypocotyl region when cultures were maintained on LP medium containing 2 mg/L of either IBA or NAA (Fig. 7C).

The extent of browning and necrosis became more pronounced with the passage of time. By 55 days, cultures on DCR + 4 mg/L IBA and NAA showed increasing necrosis, with the hypocotyl region whitening and the cotyledons continued to elongate (Fig. 7D). Upon transferring the cultures to PGR-free medium, the initial growth was satisfactory, but necrosis developed later during the early rooting phase on MS medium at 76 days old (Fig. 7E). At day 80, severe browning and pathological changes were observed in the hypocotyls and cotyledons, which included curling, rough epidermal texture, and granulation, particularly on ½ DCR medium without PGRs (Fig. 7F, G). Furthermore, abnormal growth was noted in some cultures, with the hypocotyl ends becoming blunt and cotyledons showing browning, particularly when treated with 8 mg/L NAA + 8 mg/L mg/L IBA in LP basal medium (Fig. 7H).

***In vitro* rooting and hardening:** Roots were developed in both types of cultures using the three different media types - DCR, MS, and LP, without the addition of growth regulators. Mgt developed a primary root while MZEs did not reach the rooting stage (Fig. 8A, B, C). Half LP and MS media produced robust primary roots and secondary roots, whereas other media components developed primary roots (Fig. 8A, C, D). After 10 days in the same medium, the inner whorl of the buds also began to form in the Mgt cultures. The root-shoot transitional region began to darken in color after 20 to 25 days in the media (Fig. 8D).

Table 1. Twenty-day-old cultures of mature zygotic embryos of chir pine on different basal media fortified with plant growth regulators.

Media (mg/L)	Growth (%)	Length of structures (mm)		Morphology of cultures
	MZE	Cotyledons	Hypocotyl	
LP 505 + BA (0.45) + Kin (0.43) + NAA (2)	84.21 ± 2.41	8.2 ± 1.21	4.11 ± 1.01	Cotyledons opened and started to increase in length
	Mgt 21.14 ± 3.31	Nil	12.41 ± 3.32	The micropylar end is responsible for pushing the embryo's radicle out, while the cotyledons continue to be contained within the Mgt

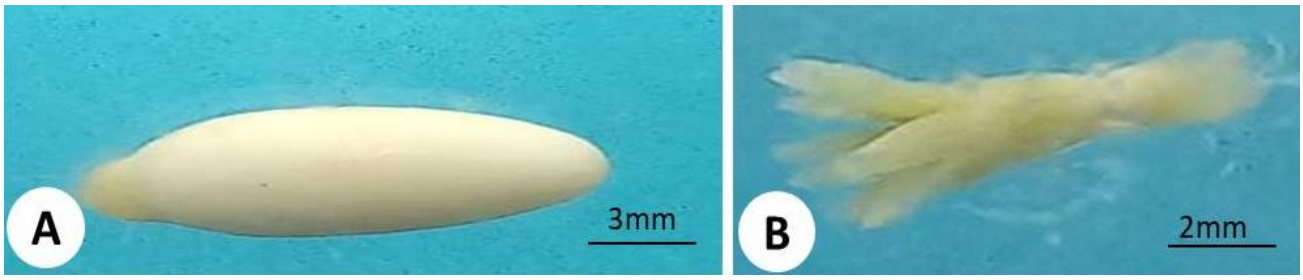

Fig. 3. **(A)** Tender and soft tissue of mature seed of chir pine placed on LP 505 medium; **(B)** After five days, the Mgt culture developed to cotyledonary stage.

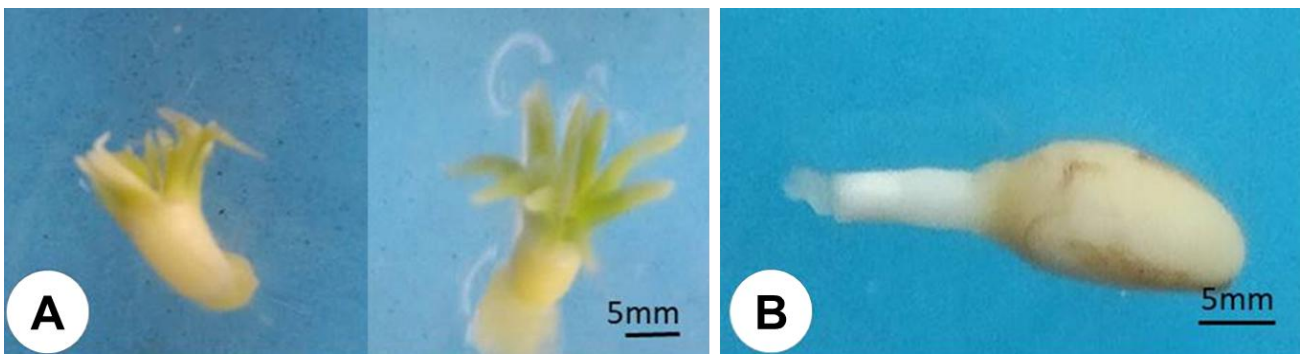

Fig. 4. Extrusion and further development of embryonic structures from zygotic embryos of chir pine under the dark conditions. **(A)** A white elongated radicle is emerging from Mgt; **(B)** Further development of the cotyledonary structures MZE resulted in the production of a green color.

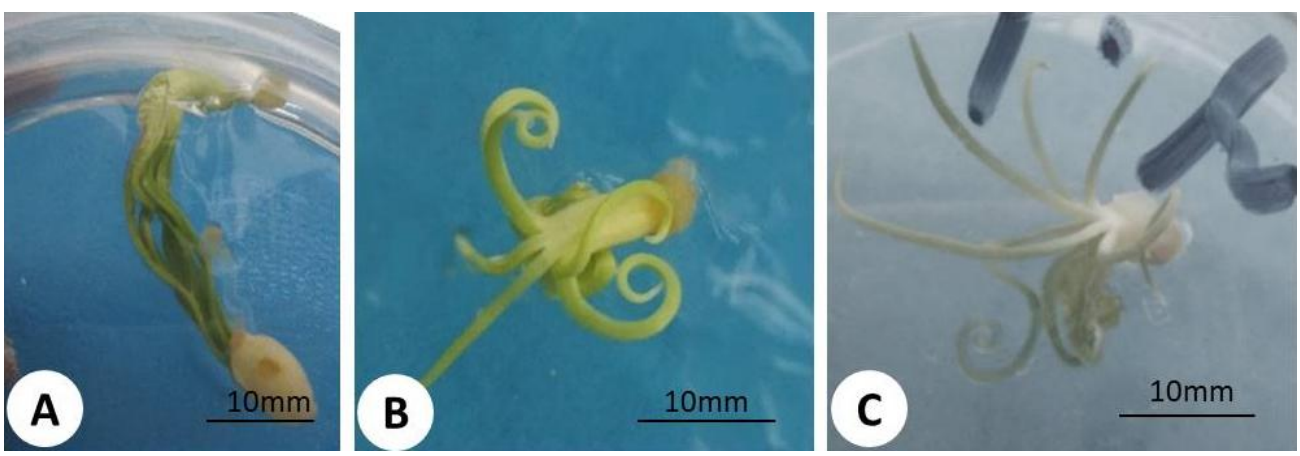

Fig. 5. Forty-day-old cultures (20 days light + 20 days pre-incubated in dark) of chir pine Mgt. Dark green elongating cotyledons (A). Curled cotyledons become light green during the subsequent period.

Table 2. Effect of basal media fortified with NAA + IBA on growth and development of mature zygotic embryos of chir pine after sixty 60 days of culture.

Media	Plant growth regulators (mg/L)		Mature zygotic embryos		Megagametophyte	
	IBA	NAA	Cotyledon length (mm)	Hypocotyl length (mm)	Cotyledon length (mm)	Hypocotyl length (mm)
LP	2	1.80	06.44 ± 2.11^{e}	05.55 ± 2.01^{bc}	11.44 ± 1.22^{d}	13.22 ± 1.233^{de}
	4	3.70	11.22 ± 3.33^{de}	04.44 ± 0.44^{cd}	15.11 ± 2.41^{c}	16.44 ± 2.45^{d}
	6	5.50	34.32 ± 4.22^{a}	09.33 ± 2.42^{a}	19.41 ± 4.11^{a}	39.22 ± 4.22^{a}
	8	7.40	08.22 ± 2.22^{e}	03.22 ± 0.11^{d}	11.44 ± 1.32^{d}	21.55 ± 1.45^{cd}
DCR	2	1.80	11.11 ± 1.11^{de}	04.44 ± 2.31^{cd}	06.66 ± 2.41^{e}	11.22 ± 3.33^{e}
	4	3.70	14.44 ± 2.22^{cd}	05.51 ± 2.44^{bc}	08.43 ± 3.33^{de}	09.44 ± 2.41^{ef}
	6	5.50	22.11 ± 3.14^{bc}	04.21 ± 2.65^{cd}	04.22 ± 1.44^{ef}	04.44 ± 3.33^{f}
	8	7.40	11.22 ± 1.41^{de}	03.33 ± 1.11^{d}	18.41 ± 2.52^{b}	26.44 ± 3.24^{bc}

Small letters along with means indicate significantly different values tested by Duncan's multiple range test (DMRT) followed by ANOVA. Standard error (±SE). Each means consists of ten replicates, and the experiment was repeated thrice

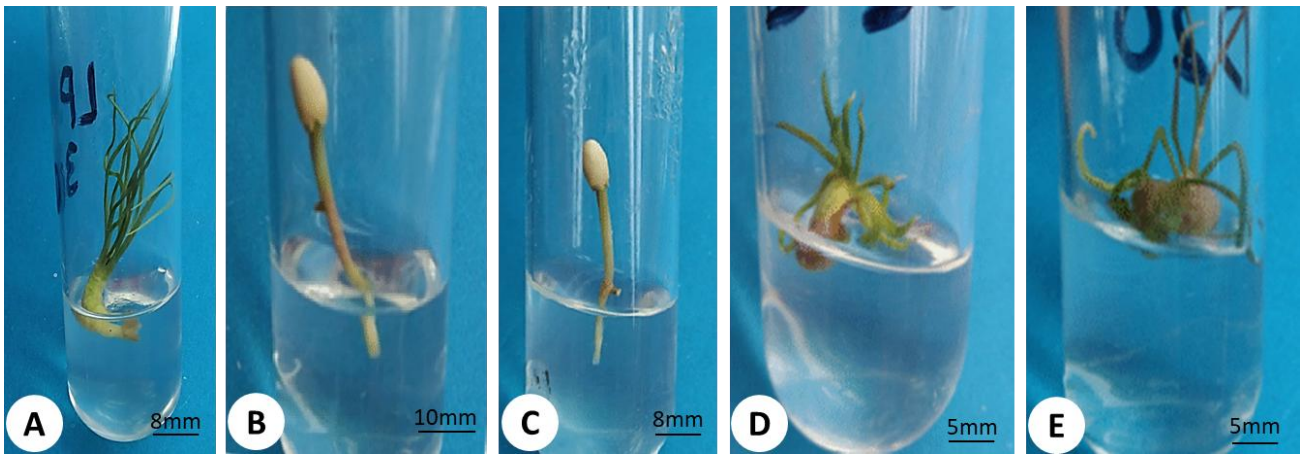

Fig.6. **(A)** Sixty-day-old cultures of *P. roxburghii* Mgt on LP and DCR basal media supplemented with NAA and IBA. Culture growth on LP + 6 mg/L IBA + 5.50 mg/L NAA; **(B, C)** Cultures on DCR + 6 mg/L IBA + 5.50 mg/L NAA; **(D.E)** Abnormal and stunted growth of MZE cultures on DCR + 6 mg/L IBA + 5.50 mg/L NAA.

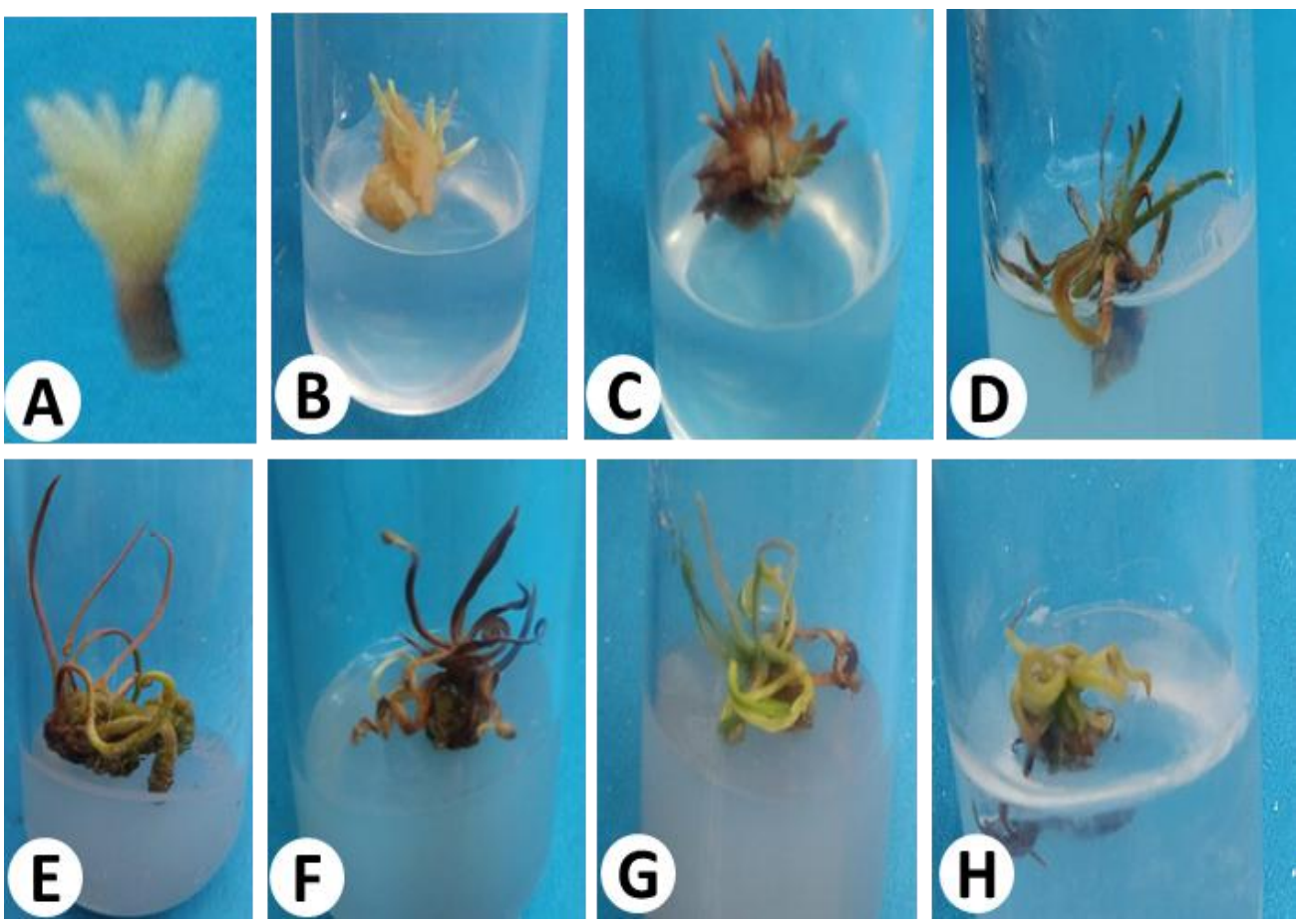

Fig. 7. **(A)** LP-505 enriched with kin (0.43 mg/L) + NAA (2 mg/L) + BA (0.45 mg/L), the hypocotyl of a 5-day-old MZE turns brown; **(B)** Callus initiation from the hypocotyl region of 40-day-old MZE on LP + 2 mg/L of NAA + IBA; **(C)** Browning occurred in cotyledons and on white patches on the hypocotyl of 55-day-old cultures treated with DCR + 4 mg/L of NAA and IBA; **(D)** A 76-day-old culture on MS showing necrosis in the cotyledon; **(E)** Granular epidermis observed on cotyledons and hypocotyl on ½ DCR; **(F,G)** 76-day-old culture showed abnormal growth of cotyledons on ½ DCR; **(H)** Abnormal growth of cotyledons with blunt hypocotyl ends in 56-day-old culture on LP with 8 mg/L of either NAA + IBA.

Different media had varying rooting rates after 30 days. Half LP (B) produced the highest rooting (79.44%) with a root length of 44.14 mm, followed by ½ DCR (A) and MS media (C) (Table 3). The rooted shoots were moved to the plastic pots with the sand (3:1) and autoclaved peat moss (Fig. 8E, F). Perlite and vermiculite mixture (1:1) was also utilized (Fig. 8G). All plantlets were healthy, green, and formed their first leaves throughout the first 20 days. These cultures became brown after 30 days and were unable to survive in identical settings.

Table 3. Rooting of Mgt on basal media after another 30 days of culture.

Media type	Root induction (%)	Length of root (mm)	Cotyledon length (mm)	Hypocotyl length (mm)
A	66.44 ± 5.55^{c}	36.44 ± 4.44^{b}	41.22 ± 4.32^{a}	50.20 ± 5.41^{a}
B	79.44 ± 7.14^{a}	44.14 ± 5.55^{a}	32.11 ± 3.33^{c}	35.50 ± 6.56^{c}
C	75.55 ± 4.33^{b}	31.21 ± 4.21^{c}	39.41 ± 4.32^{b}	40.52 ± 5.45^{b}

Small letters along with mean values indicate significantly different values tested by DMRT followed by ANOVA. Standard error (±SE). Each means consists of ten replicates, and the experiment was repeated thrice. A = ½ DCR, B = ½ LP, C = MS

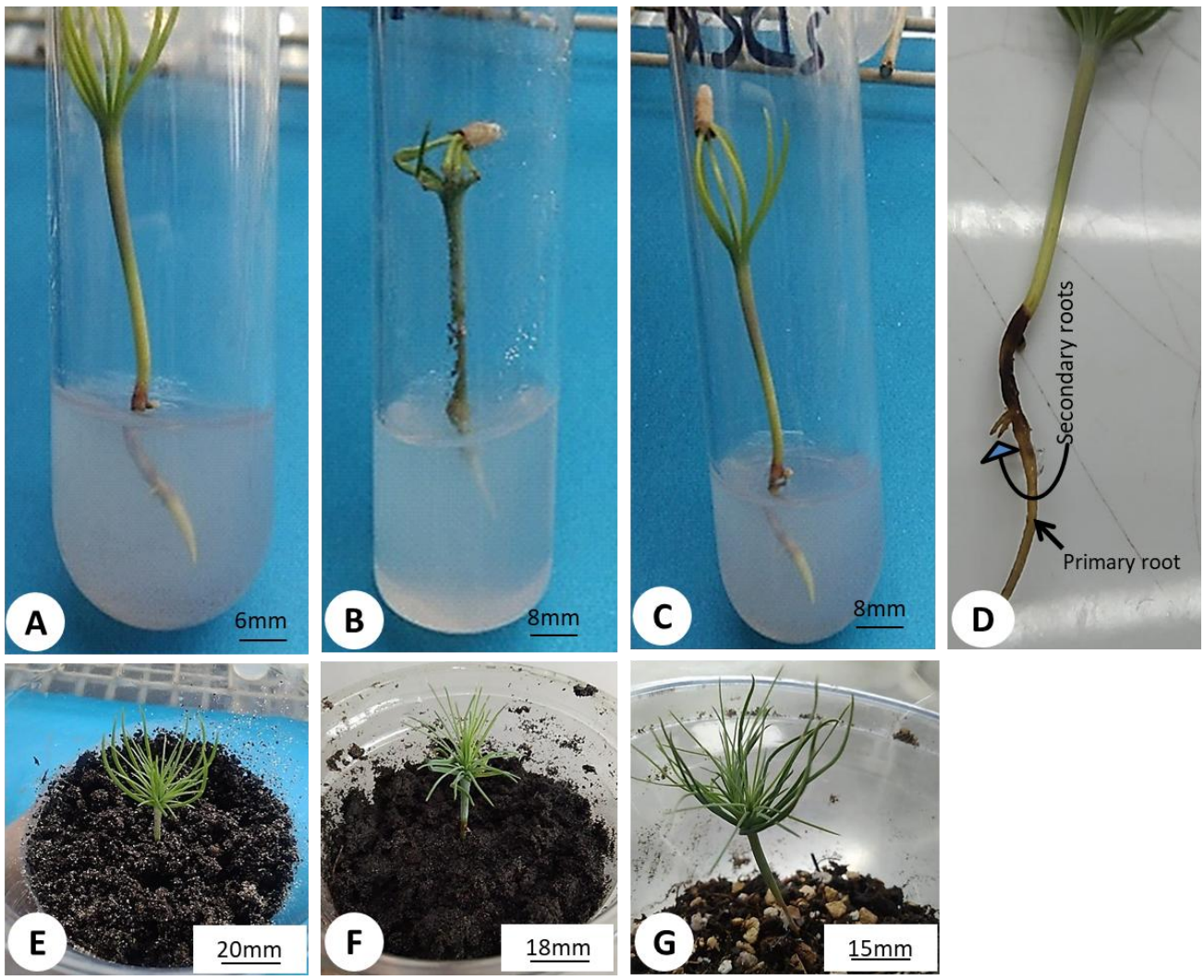

Fig. 8. Rooting and hardening of the chir pine of 74-day-old cultures from Mgt. **(A)** ½ LP, **(B)** ½ DCR, **(C)** full MS devoid of PGRs, **(D)** Secondary root formation, (**E, F)** Hardening of plantlets in peat moss and sand, **(G)** Hardening of plantlets in perlite and vermiculite. The current study demonstrated the success of *In vitro* rooting of *P. roxburghii* rescued embryos, with significant implications for forest regeneration, genetic conservation, and sustainable management. The choice of basal medium and growth regulator supplements notably influenced the initiation and proliferation of cultures.

Discussion

The medium 505 supplemented with 0.45 mg/L BA, 0.43 mg/L Kin, 2 mg/L NAA, and 50 mg/L AC (activated charcoal) proved the most effective treatment for shoot bud induction. Our results are supported by the work of Gupta & Pullman (1991), who reported that AC is the most effective agent in the medium necessitating the adjustments in cytokinin concentrations. The optimal initiation of shoots from the mature embryos in this study required both hormones, as they play essential roles in triggering growth responses (Hutchison & Kieber, 2002; Kepinski & Leyser, 2005). On the other hand, Aitken-Christie & Parkes, 1996, demonstrated that initiation of shoots from the embryo can also occur without the addition of auxins or cytokinins in the medium. The morphological differences between MZEs and Mgt highlight the importance of explant source and embryo maturity in tissue culture. MZEs displayed cotyledon emergence and elongation, while Mgt cultures showed significant hypocotyl elongation. These distinct developmental patterns suggest that different hormonal treatments may be necessary for shoot and root development in chir pine embryos. DCR and LP media supplemented with various concentrations of NAA and IBA (2+2, 4+4, 6+6, and 8+8 mg/L) significantly impacted Mgt, while MZEs exhibited necrosis in the presence of auxin combinations.

The longest growth (2 cm long) of cotyledons as well as hypocotyls (3.5 cm long) was observed in Mgt cultures at 6 + 6 mg/L NAA + IBA. At lower concentrations (2 + 2 mg/L), however, neither type of explant showed any clear signs of growth. It was found that ½ LP, ½ DCR, or MS media worked well to help Mgt plants grow roots. From this information, we can infer that these media provide nutrients that plants need to grow roots. Along with sprouting, secondary root growth happened, especially in MS and ½ DCR. This means that plants have gotten more nutrients and become more stable. This shows that these media can support both root and shoot growth even more. For example, after 10 days, inner whorl buds appeared in Mgt cultures, indicating that shoot production is possible.

The culture browning in pine tissue culture is not well understood. However, the literature demonstrates that polyphenol oxidase and peroxidase enzymatic oxidation were physiological defensive responses that mitigate stress and wounding during explant preparation of *Pinus* spp. (Amente & Chimdessa, 2021; Liu *et al*., 2024; Permadi *et al*., 2024).

The root-shoot transition developed colour changes within 20-25 days of culture. This might be due to the tissue maturation and differentiation. The appearance of this colour change corresponds to changes in the physiological parameter across different developmental stages. These changes must be made to optimise gardening practices and help plants grow properly. Controlled field tests demonstrated that the known regeneration and cryopreservation methods for *P. roxburghii* were successful. Plants grown from tissue culture did better than seedlings and rooted cuttings. Tissue necrosis and browning of seedlings were also observed during the present investigation. We did not fully understand the cause of browning and tissue necrosis in developing shoots. We are conducting further research on this aspect as part of our ongoing project to understand tissue apoptosis.

The present study demonstrated an efficient method for *In vitro* establishment of mature zygotic embryos of chir pine on DCR medium. Shoot development was also possible from megagametophyte. The shoots showed good root initiation, which developed at the highest rate on ½ LP medium compared to other basal media. Acclimatization of plantlets was quite possible under the culture room conditions, where the new root primordia development in the preexisting roots remained obscure.

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Author Contribution: AA conducted experiment; **MA** writing and communicating the article; **FA** supervised the research.

Conflict of Interest: The authors declared no conflict of interest.

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