Plant Research Journal, 01(01), 35-37 (2025)

Short Communication - Plant Tissue Culture

Selection of a suitable explant material for callus induction and in vitro morphogenesis in chir pine (*Pinus roxburghii*)

Sadia Kaleem, Aroosha Ashraf, Faheem Aftab*

Institute of Botany, University of the Punjab, Lahore-54590, Pakistan

*Corresponding author(s): E-mail: faheem.botany@pu.edu.pk

Received: 28-11-2025
Accepted: 10-12-2025
Published: 12-12-2025

Abstract

The present study aimed at standardizing in vitro conditions for suitable explants (shoot apical domes, secondary needles, mature seeds, immature seeds) for callus induction of *Pinus roxburghii* Sargent. Various factors, including the type of explant, callus induction medium, and explant collection time were investigated. Shoot apical domes were the most responsive for callus induction with the highest induction frequencies (82.5%) than secondary needles (75.80%) or other tissues on DCR medium supplemented with 22.62 µM 2, 4-Dichlorophenoxyacetic acid (2, 4-D), 26.85 µM α-Naphthalene acetic acid (NAA), and 2 µM Thidiazuron (TDZ) after 18 days. The maximum rate of callus induction was observed in January (for apical domes) and March (for secondary needles), indicating that collection times are suitable for callus induction from vegetative tissues. Off-white and friable calluses with excellent proliferation were achieved with TDZ better than 6-Benzylaminopurine (BAP) for callus induction and maintenance.

Keywords: Adventitious buds, bud-forming capacity, callus induction, chir pine, DCR medium

Key message: Callus induction in chir pine is vital to get subsequent in vitro morphogenesis

Abbreviations: DAS (Shoot Apical Dome), **DCR** (Gupta and Durzan), **KPK** (Khyber Pakhtunkhwa), **MS** (Mature Seeds), **PVP** (Polyvinylpolypyrollidone), , **SN** (Secondary Needles)

Citation: Kaleem, S., Ashraf, A. and Aftab, F. (2025). Selection of a suitable explant material for callus induction and in vitro morphogenesis in chir pine (Pinus roxburghii). Plant Research Journal, 01(01), 35-37.

Pinus roxburghii is also known as 'chir pine', a very important gymnosperm tree species of the Himalayas. It grows b/w 900 and 1800 meters above sea level of the subcontinent (Siddiqui et al., 2009). Chir pine is found in Khyber Pakhtunkhwa's (KPK) subtropical dry and moist temperate zones of Pakistan (Ahmed et al., 2006). It is a good source of softwood for making wood products and essential oils. Chir pine plays a role in soil stabilization, watershed management, and biodiversity conservation (Sinha, 2002). Overharvesting of chir pine is occurring due to its high demand in Pakistan (Ansari et al., 2022). Current propagation methods primarily rely on seed germination; however, this process is hindered by low seed viability (Sharma and Verma, 2011). Micropropagation has been suggested as an alternative to achieve fast growth. This study aimed to develop a protocol for efficient callus induction and in vitro morphogenesis by using different explants (Cardoso, 2018).

Different explants, including shoot apical domes (SAD), secondary needles (SN), mature seeds (MS) and immature seeds (ImS) were used on DCR (Gupta and Durzan, 1985) medium supplemented with various plant growth regulators 6-Benzylaminopurine (BAP 8.87 µM), 2, 4-Dichlorophenoxyacetic acid (2, 4-D 22.62 µM), α-Naphthalene acetic acid (NAA 26.85 µM), and Thidiazuron (TDZ 2 µM). SAD and SN were pretreated for 3 days at 4 ℃ on DCR + 0.2 g/L PVP and 0.3 % activated charcoal (AC), then transferred to callus induction medium. The DCR medium was prepared with PGRs, adjusted to pH 5.75, and contained 0.8% agar and 0.1% activated charcoal (AC). Ten milliliter (ml) of medium was poured into culture vessels (25 x 150 mm), capped, and autoclaved at 121 ℃ and 15 lb pressure for 15 minutes. Cultures were initiated under either complete darkness or light conditions (16 h photoperiod provided with white, cool fluorescent tube lights) at a temperature of 25 ± 2 ℃.

Results demonstrated that callus induction rate varied with different explants observed 18 days after the initiation of the experiment on different media. The callus induction was highest (82.55%) from SAD followed by SN (75.80%) on D2 (22.62 µM 2,4-D, 26.85 µM NAA and 2 µM TDZ) after 18 days (Table 1). Mature and immature seeds were less

responsive to both types of media. The callus was yellowish white (Fig. 1A) with a shiny surface but later became off-white and loose with a rough surface (Fig.1B). No considerable difference in callus morphology was observed with respect to the type of medium used and the explants. However, a notable observation was that the calli on D1 started turning brown after about 10 days of callus initiation (Fig. 1C). A change in the rate of callus proliferation was also observed at different collection times. Callus cultures initiated in December and January proliferated more rapidly compared to those undertaken in November, and the slowest callus proliferation was observed for cultures commenced in February.

Apical dome explants collected in January resulted in the highest callus induction frequencies. This response may be related to the release of bud dormancy during this month, as observed in six-month-old loblolly pine (Pinus taeda L.) seedlings, where bud dormancy was at its maximum in December and rapidly released in early January (Boyer and South, 1989). Similarly, in Scots pine (Pinus sylvestris L.), explant viability and callus growth from the shoot apex of vegetative and male buds were good in January, which was associated with the breakdown of winter metabolism at the cellular level during this month (Hohtola, 1988). The use of apical domes for callus induction has been previously reported in several other pine species (Malabadi and Van Staden, 2003; Malabadi et al., 2004; Malabadi and Nataraja, 2006). The use of BAP (8.87µM) in combination with NAA (26.85 µM) and 2,4-D (22.62µM) for callus induction in *P. roxburghii* has earlier been reported (Malabadi and Nataraja, 2006). But during the present investigation, these auxins in combination with BAP did not prove suitable for callus induction from shoot apical dome and secondary needle explants of *P. roxburghii*. However, the replacement of BAP (8.87µM) with TDZ (2 µM) was proved to be more effective with high rate of callus induction. Moreover, the callus proliferation was also good on TDZ, whereas the calli on BAP showed browning. TDZ is considered very efficient for in vitro propagation of many recalcitrant woody species (Lu, 1993). TDZ is more active than amino purine cytokinins (Akram and Aftab, 2008) and can stimulate callus formation at concentrations higher than 1µM (Huetteman and Preece, 1993). However, there are no previous reports about the use of TDZ for the callus induction in *P. roxburghii Sarg*.

Table 1. Effect of media and explant collection time on callus induction (%) from shoot apical domes of *Pinus roxburghii* under dark conditions at 25 ± 2 ºC.

Explants	Callus induction %		Days to Callus Initiation	Callus Morphology	Callus proliferation
	D1	D2			
Shoot apical domes	64.22 ± 1.40^{bc}	82.55 ± 3.18^{a}	18	Off-white, loose and friable with a rough surface	+++
Secondary needles	37.51 ± 2.55^{d}	75.80 ± 2.49^{b}	18		++++
Mature seeds	75.28 ± 1.44^{a}	70.14 ± 2.55^{c}	18		++++
Immature seed	58.33 ± 7.16^{cd}	76.66 ± 5.22^{b}	18		++

D1 = DCR + 22.62µM 2,4-D, 26.85µM NAA and 8.87µM BAP. D2 = DCR + 22.62µM 2,4-D, 26.85µM NAA and 2µM TDZ. ±SE, ++: Moderate, +++: Good, ++++: Excellent. Small letters indicate significantly different results at $p \leq 0.05$. Data was recorded for 40 replicates per treatment, and the experiment was repeated three times.

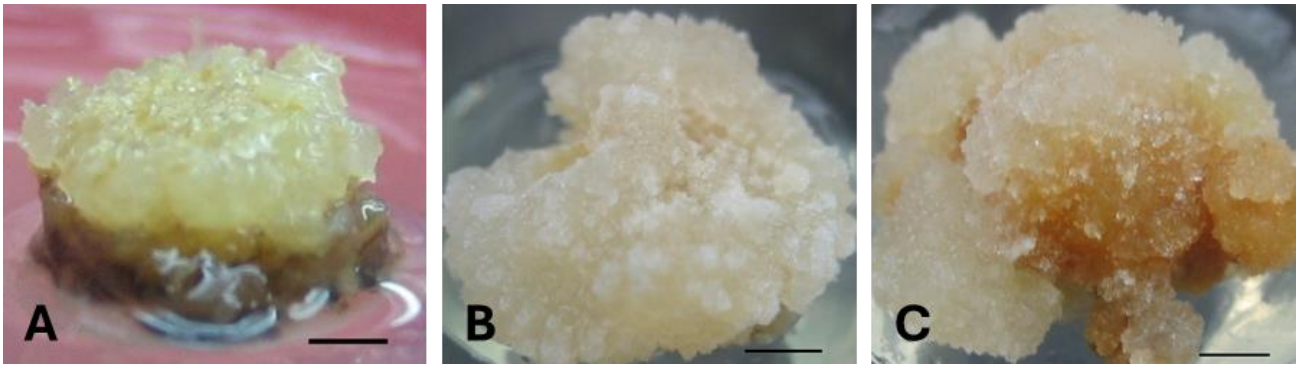

Fig.1. Callus induction from shoot apical dome on D2 medium (DCR + 22.62µM 2,4-D, 26.85µM NAA and 2µM TDZ) after 18 days of culture. Bar = 2.5mm

Acknowledgment

This paper is part of MS thesis of first author is grateful to institute of Botany University of the Punjab for the provision of lab facilities.

Author(s) contribution

Conceptualization, methodology, software, **SK**; validation, **FA**; formal analysis and investigation, data curation, **SK**; writing—original draft preparation, **AA**; writing—review and editing, **FA**; supervision, **FA**. All authors have reviewed and approved the manuscript.

Declarations

Consent for publication

Not applicable

Conflict of interest

The authors declare no conflict of interest.

Competing interests

The authors declare no competing interests.

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