Multiple Shoot Formation From Mature Embryos of *Pinus wallichiana* A.B.Jackson

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Abstract*-Mature Zygotic embryos of Pinus wallichiana were cultured on MS(1/2) and LV(1/2) medium fortified with various Phytohormonal regimes. Embryo explants cultured on various auxin cytokinin combinations produced friable callus at radicle and hypocotyl regions. Multiple shoot proliferation was obtained on MS(1/2) supplemented with BAP(60µM). Axillary shoot regeneration was also observed on LV(1/2) medium enriched with BAP(10µM)+CH(500mg/l). Shoot elongation was observed after transferring the cultures on MS(1/2) basal medium. Rooting of microshoots was obtained on root induction medium containing MS(1/2)+ NAA(0.5µM)+BAP(2.5µM). Rooted Plantlets were transferred to pots and later accilimatized and transferred to green house.*

Keywords- Embryo, Pinus wallichiana, multiple shoot, MS medium (Murashige and Skoog,1962), LV medium (Litvay et al.1985), CH (casein hydrolysate)

1. INTRODUCTION

Pinus wallichiana A.B.Jackson (Pinaceae) also known as the Blue Pine or Kail is a beautiful evergreen tree. It is found in the Himalayas from Kashmir to Bhutan at an altitude of 2000-3500m. It is mostly exploited for timber, high quality resin and the fuel wood. Due to the ever increasing population, natural forests of Blue pine and associated trees of the region are being cleared for cultivation at an alarming rate. The conventional breeding of trees is not as straightforward as that of herbaceous plants (Merkle and Dean 2000). Trees have long life cycle, are self incompatible and highly heterozygous all of which make the fixation of an allele of commercial interest both very difficult and time consuming (Campbell el al. 2003). The economic value of a tree can only be assessed after it reaches maturity, maturation in itself induces changes in meristem behavior, thus reducing the propagation potential of the tree (von Aderkas and Bonga 2000, Greenwood 1995). Propagation of a superior mature individuals is an effective way of capturing genetic gain by exploiting all the components of the genetic variance (dominance, additive and epistatic) within a given generation without the need to proceed through long breeding cycles (Ahuja 1993). The regeneration of mature trees either alone, or in combination with conventional breeding programmes, could be a powerful tool to improve forestry management.

In vitro propagation is a suitable biotechnique for mass clonal production of trees in both coniferous and hardwood species. There have been several reports dealing with in vitro culture of different *Pinus species* (Abdullah et al. 1987, Konar and Singh 1980, Mathur and Nadgauda, 1999, Prehn e al. 2003, Arya et al. 2014, Kalia et al. 2007, Burns et al. 1991, Parasharami et al. 2003, Cortizo et al. 2009). The present study has been made to induce multiple shoot regeneration from mature Zygotic embryos under various phytohormonal regimes.

2. MATERIALS AND METHODS

Mature female cones of *Pinus wallichiana* were collected from the forest range of Gulmarg, Kashmir, India in the month of September-October. Seeds were separated from these cones and stored at 4^{o}C for one month prior to the excision of embryos. Seeds were thoroughly washed with detergent Cedpol

(1%) and few drops of tween-20 (surfactant). This was followed by their surface sterilization with $HgCl_2$ (0.1%) for 20 minutes and then rinsing three times with double distilled water. Embryos from sterilized seeds were excised aseptically under laminar air flow cabinet and then again sterilized with NaOCl (2%) solution for 5 minutes followed by their rinsing thoroughly three times with autoclaved double distilled water. Sterilized embryos were inoculated on MS and LV medium supplemented with various phytohormonal regimes. The pH of the medium was adjusted between 5.5-5.8 by using NaOH (0.1N) or HCl (0.1) before jelling the medium with 0.8% agar. The cultures were maintained at 25±3ºC with 16 to 18 hour photoperiod from cool while flourescent tube light at 50 to 65% relative humidity.

3. RESULTS AND DISCUSSION

The response of mature zygotic embryos on MS (x1/2) medium supplemented with various phytohormonal concentrations and combinations is given in table 1. Embryo explants cultured on MS (x1/2) basal medium resulted in seedling formation after 6 weeks of culture period (Fig. 1). The cultured explants produced yellowish friable callus at radicle and hypocotyl regions on various BAP (2.5, 5, 10µM) concentrations. Emergence of primary needles and elongation of cotyledons was also observed in the same medium after 8 weeks of culture period (Fig. 2). With other BAP concentrations (20, 40µ M) low friable callus formation was recorded at the radicle end. However, high BAP (60 µM) fortified MS (x1/2) medium favoured direct axillary shoot regeneration (6± 0.5/explant) in 50% cultures (Fig. 3). Shoot development was noted after subculture of primary cultures on MS (x1/2) basal medium. Embryos cultured under the combined interaction of BAP(5,10,15,20,40 µM) and NAA (2.5,5,0.5 µM) showed friable callus formation towards hypocotyl and radicle regions, such calli did not show any differentiating potential even after repeated sub cultures onto basal medium.

Table 1. Effect of BAP, BAP&NAA on mature embryos of *Pinus Wallichina* cultured on MS(1/2) basal medium.

S.No.	BAP (µM)	NAA (µM)	Response*	Degree of callus formation	% age response
1.	2.5	-	Friable callus formation at basal end	+	20
2.	5	-	-do-	+	45
3.	10	-	-do-	++	70
4.	15	-	-do-	++	50
5.	20	-	-do-	+	40
6.	40	-	-do-	+	30
7.	60	-	Axillary shoot induction (6±0.5/explant)	+	50
8.	70	-	Friable callus formation at basal end	+	30
9.	5	2.5	-do-	++	60
10.	5	5	-do-	++	65
11.	10	5	-do-	++	80
12.	15	5	-do-	++	50
13.	20	0.5	-do-	+	40
14.	40	0.5	-do-	+	30

*** Ten replicates per treatment, data scored after 08 weeks of culture period.**

\+ Low callus growth, ++ Moderate callus growth

The response of cultured embryos on LV (1/2) medium enriched with BAP and 2,4-D /CH combinations is given in table 2. Cultured embryos produced low friable callus towards hypocotyl and radicle regions on BAP (5/5/10 µM) and 2, 4-D (2.5/5/2.5 µM) combinations. Moderate creamish white callus formation was recorded on BAP (10µM) + 2, 4-D (5µM) combination. Elongation of hypocotyl and cotyledons was also observed on the same medium. The usage of CH (500 mg/l) in BAP (5µM) enriched medium also favour low callus formation towards hypocotyl and radicle regions. However axillary shoot

regeneration (3±0.7/ explant) alongwith brown callus formation was recorded in 70% cultures on BAP (10µM) + CH (500 mg/l) enriched medium (Fig.4). With BAP (15/20 µM) + CH (500 mg/l) only friable callus growth was recorded.

The primary cultures with microshoots were subcultured on basal medium for shoot elongation. Microshoots were separated and subcultured on basal medium for further growth and elongation (Fig5). Isolated shoots showed only elongation on basal medium without any rooting response. Microshoots cultured on various concentrations of NAA/IBA (1.5,2.5,5.0,10,15 µM) produced callus of different degrees and textures, instead of roots towards basal ends. However, in presence of IBA (15µM) and NAA (0.5µM) 20% microshoots resulted in root regeneration. While as a combination of NAA (0.5µM) and BAP (2.5 µM) proved optimal for adventitious root regeneration (40%). Root initiation as well as elongation were observed on the same medium (Fig. 6). Plantlets were deflasked and transferred to green house for hardening and field trials (Fig7).

Table 2. Effect of LV (1/2)medium fortified with BAP & 2,4-D/CH on mature embryos of *Pinus wallichiana*.

S.No	BAP (µM)	2, 4-D (µM)	CH (mg/l)	Response *	Degree of callus formation	% age Response
1.	5	2.5	-	Friable callus formation towards hypocotyl and radicle regions	+	50
2.	5	5	-	-do-	+	65
3.	10	2.5	-	-do-	+	60
4.	10	5	-	-do-	++	70
5.	5	-	500	-do-	+	50
6.	10	-	500	Axillary shoot regeneration (3±0.7/explant), callus at basal end	+	70
7.	15	-	500	Friable callus formation at basal end	+	30
8.	20	-	500	-do-	+	40

*** Ten replicates per treatment, data scored after 08 weeks of culture period.**
\+ Low callus growth, ++ Moderate callus growth

Fig. 1 Seedling formation on MS(1/2) basal medium

Fig 2. Cotyledon elongation and callus formation at radicle and hypocotyl regions on MS (1/2) + BAP (10 µM)

Fig 3. Axillary shoot regeneration on MS (1/2) + BAP (µM)

Fig 4. Axillary shoot regeneration on LV (1/2) + BAP (10µM) + CH (500mg/l)

Fig 5. Elongated shoots on MS (1/2) basal medium

Fig 6. In vitro rooting of micro shoots on MS(1/2) + NA(0.5µM) + BAP 2.5µM)

Fig 7. Plantlet in a pot

Woody plant propagation by tissue culture has acquired a particular importance in recent years, and factors regulating organogenesis have been the object of discussion in several works. The effect of cytokinin in micropropagation protocols have been well documented in different *Pinus* species (Bermudez and Sommer 1987, Kalia et al. 2001, Moncalean et al. 2005, Alonso et al. 2006, Kalia et al. 2007, Sabeena 2018). Moreover the culture medium, culture physical conditions and exogenously applied cytokinins influence the process of organogenic induction (Thorp et al.1991). Other factors such as the concentration, the application method and the incubation period affect the ability of explants to develop healthy shoots with root forming ability. In the present investigation mature embryos have shown multiple shoot regeneration on high BAP (60µM) fortified medium. While working on the same lines almost similar results were achieved by various workers on high BAP enriched media Reilly and Aitken (1981) in *Pinus radiata*, Jain et al. (1988b) in *P. sylvestris*, Bermudez and Sommer (1987) in *P. elliottii*, Lapp et al (1995) in *P. monticola*, Que et al. (1997) in *P. taeda*, *P. elliottii* and *P. serotina*.

In cultured mature embryos of *Pinus massoniana* L.(Zhang et al.2006) adventitious buds were recorded on BA+ IBA/NAA supplemented medium. However in present study only friable callus formation was observed on various auxin cytokinin combinations. Green friable callus proliferation was also recorded on 10µM BAP concentration which is quite similar with the findings of Ishi et al. (2008) in cultured mature embryos of *Pinus armandii*.

The investigations carried out elsewhere on mature embryos of *Pinus strobes* (Schwarz et al.1988) and *P. merkussi* (Okamura and Kondo,1998) revealed adventitious shoot regeneration on BAP(10µM) enriched medium. Such reports are more or less in agreement with the current studies in which axillary shoot regeneration from the mature embryos was recorded on LV (1/2) medium fortified with BAP (10 µM) and CH (500 mg/l). Further the capacity of embryos to produce adventitious buds differs depending on the seed

lot used. About 90% of embryos derived from controlled crossing formed adventitious bud primordia while only 60% of embryos from open pollinated trees formed adventitious bud primordial(von Arnold,1982b)

The present study revealed that the elongation of microshoots was observed on MS(1/2) basal medium which is in conformity with the results of Gonzalez et al. (1998) in *Pinus pinea*, Bermudez and Sommer (1987) in *P. elliottii*, Kalia et al. (2007) in *P. roxburghii*. In conifers adventitious rooting is highly variable and remains one of the key problem in plantlet regeneration in vitro (Kalia et al. 2007). In present observation rooting (20%) of microshoots was observed on MS (1/2) + IBA (15µM) + NAA (0.5 µM). This observation is in accordance with the findings of Ellis and Bilderback (1991) in *Pinus ponderosa* who also achieved low rooting (14%) on IBA and IAA fortified medium. In *Pinus caribea* (Halos and Go, 1993) rooting of microshoots was also reported on IBA +NAA combination. The formation of roots in shoots is often dependent on the cytokinin/auxin ratio in the medium(Skoog and Miller,1957;Nemeth,1986).The rate of rooting in the shoots of *Pinus brutia* was enhanced by the inclusion of low level of BAP with a mixture of NAA+IBA at a concentration of 1+2mg/l respectively (Abdullah et al.1984). This study confirms our investigation where 40% rooting response was recorded on NAA(0.5µM)+BAP(2.5µM) enriched medium. The findings of Chang et al.(1991) also display root induction on auxin(NAA1.3µM,IBA 1.2µM) and cytokinin (BAP 0.4µM) combination in *P.virginia*. In *P.taeda* root formation was observed on IBA(0.1mg/l)+BAP(1mg/l)+GA_3(0.5mg/l) combination (Tang and Ouyang,2000).

4. CONCLUSION

The present investigation describes a protocol for micropropagation of *Pinus wallichiana* from mature embryo explants. Such an effort of regeneration can significantly boost the forest productivity with the production of selected genotype

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