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REGENERATION OF PLANTLETS FROM IN VITRO CULTURED COTYLEDONARY NEEDLES OF PICEA SMITHIANA (WALL) BOISS.

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***Abstract**: Adventitious shoot induction from cotyledonary needles was investigated for Picea smithiana. The cotyledonary needles from in vitro raised seedlings were cultured on MS medium containing various cytokinin (BAP, Kn) concentrations and auxin (NAA) + Cytokinin (BAP, Kn) combinations. Explants produced green friable non regenerative callus on BAP (4.4, 10, 15 µM) enriched medium. Nodular callus proliferation and multiple shoot bud (5 ±0.4) induction was observed with 5µM BAP fortified medium. Combined influence of BAP and NAA also resulted in multiple shoot bud proliferation (3± 0.7) in the nodular callus cultures of explants. The primary cultures with multiple shoot buds were subcultured on MS basal medium for multiple shoot regeneration and elongation. Micro shoots were isolated and cultured on basal medium for further elongation and growth. In vitro raised shoots produced rooting response on IBA supplemented medium after 6-8 weeks of culture period. Rooted plantlets were transferred to green house for hardening and field trials.*

Key words: Cotyledonary needle, *Picea smithiana*, Multiple shoots, Callus

Abbreviations: BAP- 6-benzyl amino purine; Kn- kinetin; IBA- indole-3- butyric acid; NAA- napthaleneacetic acid; 2,4-D – 2, 4 dichlorophenoxyacetic acid; MS (x1/2)- Murashige and Skoog (half strength).

I. INTRODUCTION

Picea smithiana (Wall) Boiss. is a very large evergreen tree of pendulous habit and belongs to family Pinaceae. It is a tree of considerable economic importance on account of its multiple use in wood-based industries and manufacture of paper pulp for newsprint. The conventional method of propagation in *Picea smithiana* is through seeds. Since the conifers have along reproductive cycle, breeding and selection of phenotypically superior trees via seeds is usually very slow. Vegetative propagation by rooting of cuttings is difficult and has a low success rate especially when cuttings from mature trees are used (Arnold and Eriksson, 1986). Problems encountered with traditional reproduction and vegetative reproduction can be overcome by using the technique of plant tissue culture which has been successfully used for plant regeneration in many species of *Picea* (Arnold, 1982a; Bornman, 1983; Gupta and Durzan, 1986; Lu et al; 1991; Afele et al; and Yang et al; 1997).

The present work represents an attempt on in vitro culture of cotyledonary needles of *Picea smithiana* (West Himalayan Spruce). It describes a simple protocol for induction of nodular callus and multiple shoot buds on cotyledonary needle explants.

II. MATERIAL AND METHODS

Mature green cones were picked from the Gulmarg and Tangmarg forests of Kashmir valley in the months of September and October. Seeds separated from these cones were stored at 4°C for one month. The chilled seeds were thoroughly washed with running tap water after cleaning them with detergent (Labolene 10%) and a few drops of Tween 20 (surfactant). This was followed by their surface sterilization by using 0.1% $HgCl_2$ for 15 minutes and then rinsing three times with sterile double distilled water. These seeds were allowed to remain for soaking in sterile double distilled water in a refrigerator at 4°C for 2-4 days in small flasks with their mouth sealed. The sterilized seeds were finally again sterilized with a relatively mild concentration (0.01%) of $HgCl_2$ for 10 minutes under laminar air flow cabinet. The seeds were then inoculated on MS(x1/2) basal medium for germination and seedling formation. Cotyledonary needle explants were obtained from 4- 6 weeks old in vitro raised seedlings and cultured on MS(x1/2) medium supplemented with various phytohormonal regimes. The pH of the medium was adjusted between 5.5- 5.6 by using NaOH (0.1N) or HCl (0.1N) before jelling the medium with 0.8% Difcobacto agar. The medium was finally dispensed into culture vials which were plugged and autoclaved for 15-20 minutes at 15lb pressure and 121°C temperature. The cultures were maintained at a temperature of 25± 3°C, 16- 18-hour photoperiod provided by cool white fluorescent tube lights (3000 lux) and a relative humidity of 50- 65%

III. RESULTS AND DISCUSSION

Cotyledonary needle explants (1.5 - 2cm long) from 1- week old in vitro raised seedlings were cultured on BAP/Kn, BAP/Kn and NAA fortified MS(x1/2) medium (Table 1.1). The explants produced green friable callus with very low growth on BAP (4.4, 10, 15µM) supplemented medium. However, green nodular semicompact callus initiation was recorded in 50% cultures on BAP(5µM) augmented medium after 6-weeks of culture period (Fig. 1). Nodular callus proliferation was recorded upon subculturing the explants onto same medium. After 12 weeks of culture period shoot bud induction and shoot regeneration (5± 0.4) was observed in the nodular callus cultures (Fig. 2). Basal medium fortified with Kn (2.5,5, 10 µM) favoured low friable callus formation.

Combined influence of NAA(0.5µM) and BAP(5µM) resulted in green nodular callus initiation in 60% of cultures after 6- weeks of culture period. This callus continued growth and showed shoot bud induction after 12 weeks of culture period (Fig. 3). Multiple adventitious shoot regeneration and elongation was observed after transfer of nodular calli with shoot buds, on MS(x1/2) basal medium. Explants produced friable (non regenerative) callus on BAP (10,15µM) + NAA (0.5, 2.5, 5µM) combinations. Same response was recorded with Kn (5, 10µM) + NAA (0.5, 5µM) combinations.

The primary cultures with multiple adventitious microshoots were subcultured on MS(x1/2) basal medium for shoot elongation. Maximum shoot elongation (4±0.4 cm) was recorded after 10 weeks of culture period (Fig.4). Microshoots were separated and subcultrured on basal medium for further growth and elongation. For rooting trials elongated shoots were cultured under various phytohormonal regimes (Table 1.2). Friable callus formation at the basal ends of shoots was recorded on basal medium supplemented with various concentrations of IBA/NAA/2,4-D. However adventitious rooting of shoots (20%) was recorded on medium fortified with IBA (2.5µM) after 4- 6 weeks of culture period (Fig.5). Plantlets were deflasked and transferred to green house for hardening and field trials (Fig.6).

In vitro induction of adventitious buds in conifers was described on various explants including mature embryos, needle fascicles, cotyledons and hypocotyls. The best-known work demonstrating the importance of suitable explant selection for the regeneration of conifers is that by Aitken et al (1981). The authors proved experimentally that in *Pinus radiata* the cotyledon of aseptically growing seedlings are the most suitable material for organogenesis. Woody plant propagation by tissue culture has acquired a particular importance in recent years, and the factors regulating organogenesis have been the object of discussion in several research works. The effect of cytokinin in micropropagation protocols have been well documented in different conifer species (Bermudez and Sommer 1987, Kalia et al. 2001, Moncalean et al. 2005, Alonso et al. 2006, Kalia et al. 2007, Sabeena 2018). The results published by Mehra and Verma (1981) showed that callus (non differentiating) was formed from the cotyledons excised from sterile seedlings of *Picea smithiana* on MS medium enriched with NAA and Kn. In current studies quite similar response was observed by culturing cotyledonary needle explants on BAP/Kn and NAA combinations. However, with BAP (5µM) alone or in combination with low NAA(0.5µM) concentration cultured explants favoured nodular callus formation and shoot bud induction, which is almost in agreement with the results of Bornman

(1983) who observed bud induction in the same explant of *Picea glauca* by using same concentration of BAP. Toivonen and Kartha (1988) succeded in adventitious shoot production from seedling cotyledons of *Picea glauca* on low BAP(2µM) concentration. In the present studies, by using the same BAP concentration such response was not observed. With BAP (10, 15µM) only friable callus formation was achieved, which is contrary to the findings of Patel and Thorpe (1986) in *Picea engelmannii*. The present study reveals that the elongation of microshoots was observed on hormone free basal medium which is in conformity with the results of several other workers in various *Picea* species (John and Webb (1987; Ho, 1989; Budimir and Vujcic, 1995 and Kolevska and Buturovic, 1995).

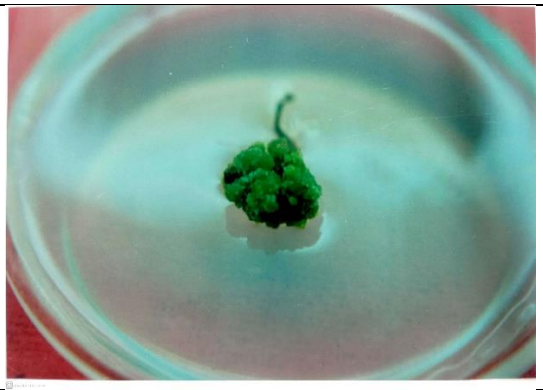

Fig.1 Nodular callus formation on MS(x1/2)+BAP(5µM)

Fig.2 Shoot regeneration on MS(x1/2) + BAP(5µM)

Fig.3 Nodular callus formation and shoot induction on MS(x1/2)+BAP(5µM)+ NAA (0.5µM)

Fig.4 Isolated microshoots on basal medium

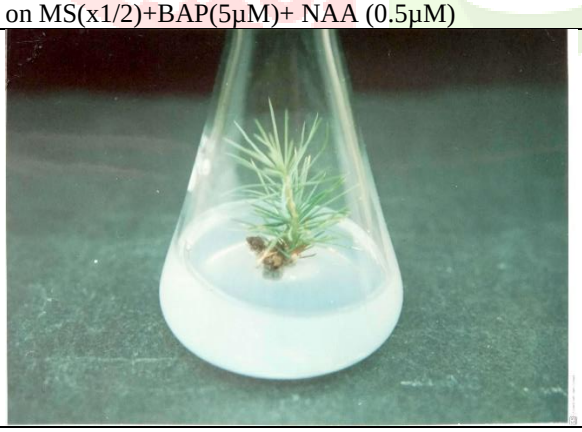

Fig.5 Root initiation on MS(x1/2) + IBA(2.5µM)

Fig. 6 Transferred Plantlet

Table1.1. Effect of BAP/Kn ; BAP/Kn and NAA combinations on cotyledonary needle explants of *Picea smithiana* cultured on MS(x1/2) basal medium

S.No	BAP (µM)	Kn (µM)	NAA (µM)	Response*	Degree of callus formation	%age response	Average No. of shoots /explant
1.	1	-	-	-	-	0	-
2.	2	-	-	-	-	0	-
3.	4.4	-	-	Callus(F)	+	30	-
4.	5	-	-	Callus(N) Shoot bud induction	++	50	5± 0.4
5	10	-	-	Callus(F)	++	50	-
6.	15	-	-	Callus(F)	+	30	-
7.	-	1	-	-	-	0	-
8.	-	2	-	-	-	0	-
9.	-	4.4	-	-	-	0	-
10.	-	5	-	Callus(F)	+	20	-
11.	-	10	-	Callus(F)	+	20	-
12.	5	-	0.5	Callus(N) Shoot bud induction	++	60	3± 0.7
13.	10	-	0.5	Callus(F)	++	50	-
14.	10	-	2.5	Callus(F)	++	60	-
15.	10	-	5	Callus(F)	++	80	-
16.	15	-	0.5	Callus(F)	+	50	-
17.	-	5	0.5	Callus(F)	+	20	-
18.	-	10	0.5	Callus(F)	+	20	-
19.	-	10	5	Callus(F)	+	30	-

*Ten replicates per treatment, Data scored after 8- weeks of culture period
F; friable N; nodular +; Low ++; moderate

In conifers the potential and efficiency of adventitious rooting is highly variable and remains one of the key problem in plantlet regeneration in vitro (Kalia et al .2007). Rooting has been achieved in number of species but the rooting percentage has been generally low 4% in *Pinus resinosa* (Noh et al. 1988), 3% in *P. monticola* (Stiff et al. 1989) 25 % in *Picea abies* (Arnold and Eriksson,1986) and 3% in *Abies* hybrids (Gajdosova and Vookova, 1994). In present studies in vitro raised microshoots favoured 20% rooting on IBA (2.5µM) fortified medium. In contrast to this study Kolevska and Buturovic (1995) recorded only 12% rooting in microshoots of *Picea omorika* on IBA (10µM) enriched MS medium. However, Arnold (1982b) reported better rooting in *P. abies* with IBA enriched medium. Similar to present findings Patel and Thorpe (1986) also observed excessive amount of callus formation instead of roots in microshoots of *P. engelmannii* on IBA and NAA combination. Different rooting potential in various species of *Picea* may be attributed to different endogenous hormonal levels and stimulus which triggers the root induction.

Table1.2. Morphogenetic response of microshoots of *Picea smithiana* on various auxins

S. No.	Auxin (µM)	Nature of response*	%age response
1.	IBA (1.5)	No response	-
2.	IBA (2.5)	Adventitious root initiation and elongation	20
3.	IBA (5)	Callus + (F)	60
4.	IBA (10)	Callus + (F)	60
5.	IBA (15)	Callus + (F)	40
6.	NAA (1.5)	Callus + (F)	50
7.	NAA (2.5)	Elongation of shoots and callus at basal end	100
8.	NAA (5)	Do	100
9.	NAA (10)	Do	90
10.	NAA (15)	Callus +(F)	60
11.	2,4-D(2.5)	No response	-
12.	2,4-D (5)	Do	-
13.	2,4-D(10)	Callus +(F)	40

* Ten replicates per treatment, Data scored after 8- weeks of culture period

+; Low F; Friable

IV. CONCLUSION

The present study demonstrates a protocol for multiple shoot regeneration through callus cultures of cotyledonary needle explants of *Picea smithiana*. Such an effort of micropropagation can be employed for multiplication of the genotype and significantly increases the forest productivity.

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