Original Article
Journal of Phytology 2014, 6: 7-13
http: //scienceflora.org/journals/index.php/jp/

# Root initiation in cuttings and *in vitro* raised shoots of *Pinus roxburghii*

**Arvind Arya[1]*, Sandeep Kumar[2], M. S. Kasana[3]**

[1]Department of Biotechnology, Meerut Institute of Engineering and Technology, Meerut, Uttar Pradesh, India, [2]National Institute of Engineering and Technology, NIMS University, Jaipur, Rajasthan, India, [3]Department of Botany, IP College, Bulandshar, Uttar Pradesh, India

*Address for correspondence:
Arvind Arya, Department of Biotechnology, Meerut Institute of Engineering and Technology, Meerut, Uttar Pradesh, India.
E-mail: arvindarya@hotmail.com

## ABSTRACT

Rooting of *in vitro* produced buds and shoots is often the limiting step during micropropagation. Therefore, a better understanding of the various stages before and during root formation is needed. Reviewing the work done so far on pines the present investigation was carried out to study the factors that affect *in vitro* rooting. Different parameters that influence rooting viz. donor age, phytohormones, and substrate were investigated. Shoots took from the field; it was found that the juvenility of the explant and position on the mother plant greatly affects the *in vitro* responses. *In vitro* raised shoots and hypocotyl cuttings of *Pinus roxburghii* showed the best response over the other explants on ×½ DCR medium. Among the various auxins used in the present investigation, N-acetyl-aspartate (NAA) at lower concentrations found best for root initiation. Agar at 0.6% concentrations resulted in more healthy roots. Further elongation was achieved on ×½ DCR medium supplemented with lower concentrations of NAA. The present investigation was an attempt to establish an operative micropropagation protocol by improving the rooting of "hard to root" *P. roxburghii*. *In vitro* rooting studies on *P. roxburghii* will be vital for enhanced multiplication and genetic improvement of this economically important forest tree species.

KEY WORDS: Auxins, hypocotyl, *Pinus roxburghii*, rooting

## INTRODUCTION

Two difficult stages of conifer tissue culture are rooting and acclimatization. The efficiency of *in vitro* adventitious rooting is highly variable [1] and is the key problem in conifer plantlet regeneration. Juvenile plant material exhibit greater potential for adventitious rooting [2]. The difference in the rooting response by the difference in age groups in *Pinus pinaster* was reported by Dumas and Monteuuis (1955) [3]. The size of shoot (more than 5 mm) [4] and the removal of callus from the base of shoots also found to show the improved effect on rooting [5].

Several aspects of rooting phytohormones viz. type, concentration, mode of application, and duration of response needs to be considered. For most of pine species, N-acetyl-aspartate (NAA) is reported as most potent auxin compared to indole-3-butyric acid (IBA) [4,6-9]. Low concentration of NAA generally found most effective for root induction in pines [10,11]. Combinations of NAA, indole-3-acetic acid (IAA), or IBA are more effective [6,9]. Sometime results in the formation of callus [11]. Root induction is also affected by the shoot quality [12], donor age, and genetic origin [13,14].

The shoot induction medium, type of cytokinins, and their concentrations also reported having profound effect on rooting [4,11] and sometime attributed as "carry-over-effect" of cytokinins at higher concentration [15]. Similarly, elongation medium also influences the rooting as reported for *Pinus radiate* by Horgan and Aitken-Christie (1981) [16].

*In vitro* rooting is usually done using the agar-solidified medium as the substrate. Advantages include uniform distribution of auxins and nutrients and good contact between shoots and substrate that results in more synchronous rooting. However, the quality of root produced on agar is not always acceptable [2]. Agar hinders gas exchange as well as the production of root hairs and development of the vascular system. Permeable substrates like peat:perlite or peat:vermiculite found more recommended [17]. Peat:vermiculite (1:1) moistened with ×¼ medium was reported better by Pulido *et al*. (1994) [11] in *Pinus canariensis*. Similar findings were

reported by Horgan and Holland (1989) [5] in *P. radiate*, he suggested that peat:perilite:vermiculite (1:1:1) is better over agar. 50% rooting in soil under mist chamber in *Pinus taeda* was reported by Mehra-Palta *et al.* (1978) [4].

## MATERIAL AND METHODS

### Rooting of *In Vitro* Raised Shoots

*In vitro* raised shoots, hypocotyl cuttings from juvenile seedlings, shoots from 1 + year old plants, and hedge shoots (7 + year old) collected from different positions on mother plant were used as explants. All the explants were subjected to *in vitro* rooting. Detailed studies of rooting behavior *in vitro* because of different parameters: Shoot quality, donor age, growth regulators, and substrate were studied.

#### *Effect of basal medium*

Three basal media viz. Murashige and Skoog's (MS), DCR, and Woody plant medium (WPM) were used in the present investigation and supplemented with 2.5 μM IAA.

#### *Effect of medium strength*

Four strengths of basal medium (DCR) viz. ×¼, ×½, ×1, and ×2 were used for rooting of different types of shoots of *Pinus roxburghii*.

#### *Effect of auxins*

Auxins viz. NAA, IBA, and IAA were supplemented in ×½ DCR medium at 2.5, 5.0, and 7.5 μM concentrations to investigate their effect on rooting.

#### *Effect of agar concentrations*

The gelling agent reported to have an important role in adventitious rooting of shoots. To study the effect of agar concentrations on root induction, half strength DCR medium supplemented with 2.5 μM NAA was gelled with 0.6, 0.7, or 0.8% agar.

#### *Effect of pulse treatment*

Mode of application of auxin is also known to effect rooting of the shoots. Auxins viz. NAA and IBA were provided at a concentration of 50 μM for 24 h in semisolid medium followed by culturing in semisolid ×½ DCR basal medium gelled with 0.6% agar.

#### *Effect of liquid medium on elongation of root primordia*

The shoots with root primordia produced on semisolid auxin supplemented media or in response to pulse treatment were transferred to either liquid (with Filter Paper Bridge) to study their effect on the elongation of root primordia. Liquid and semisolid DCR medium was supplemented with 2.5 μM NAA or IBA singly and in combination (1.25 μM NAA + 1.25 μM IBA).

## RESULTS

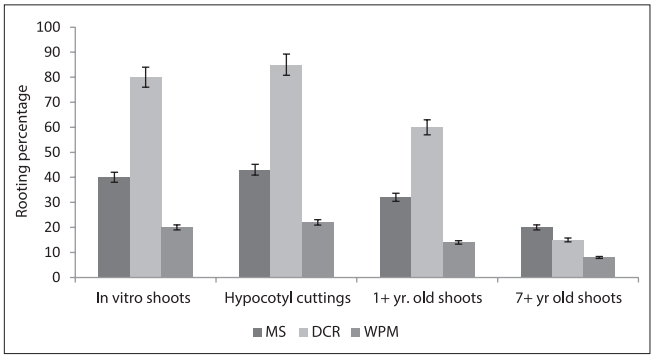
### Rooting of *In Vitro* Raised Shoots

#### *Effect of basal medium*

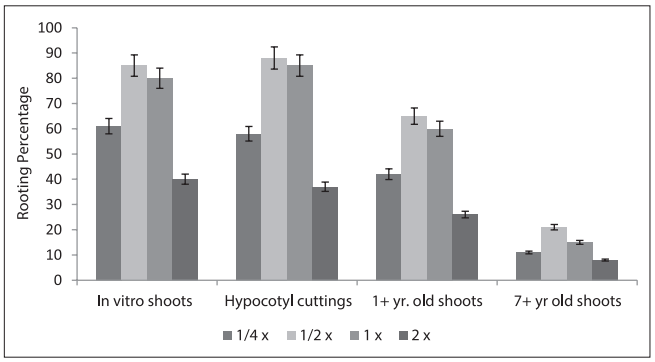
Basal medium showed differences in rooting from different types of shoots. DCR medium showed a best rooting response in all types of shoot material, especially the *in vitro* raised shoots, and juvenile seedling shoots showed a best rooting response (Graph 1). MS and WPM medium failed to produce optimum results. Mature shoots of *Pinus* from 1 + year and 7 + year old plants could not produce appropriate roots *in vitro* in any of three basal medium.

#### *Effect of medium strength*

Among the four strengths of basal medium (DCR) used for rooting of different types of shoots of *P. roxburghii,* the best rooting was obtained on ×½ strength of DCR medium. At this strength, all types of shoots showed good rooting response except the 7 + year mature shoots of *Pinus*. Hypocotyl shoots of *Pinus* seedlings showed the best rooting percentage (88%) followed by *in vitro* raised shoots (85%) and 1 + year old shoots (65%) (Graph 2).




**Graph 1:** Effect of basal media on rooting of different shoots of *Pinus roxburghii.* Basal media were supplemented with 2.5 μM indole-3-acetic acid




**Graph 2:** Effect of strengths of DCR medium on rooting of different shoot types. Basal medium was supplemented with 2.5 μM indole-3-acetic acid

### *Effect of auxins*

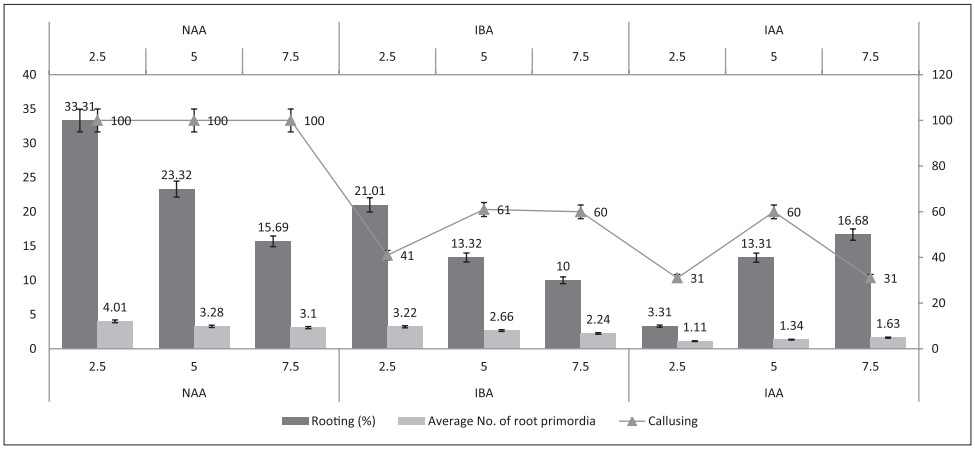
Shoots from the different origin of 2.0-3.0 cm length were used for *in vitro* rooting. Auxins namely NAA, IBA, and IAA were used at varied concentrations in half strength DCR medium for induction of rooting. Success was obtained only in formation of root primordia at the base of the hypocotyl shoots and *in vitro* raised shoots when cultured under different experimental combinations of the auxins. It was found that NAA although initiates root primordia but later callus develops which overgrowth the developing root primordia. Root primordia were initiated in 33.31% shoots cultured on 2.5 µM NAA supplemented medium (Graph 3). Increase in NAA concentration led to a decrease in the number of root primordia induced with a simultaneous increase in callus formation. The root primordia produced in all NAA concentrations could not elongate on subculturing to fresh medium, as well as on transfer to the basal medium.

IBA and IAA when used at 2.5-7.5 µM levels produced root primordia in a lesser percentage of shoots. Similar to NAA the root initials formed could not elongate into roots (Graph 3).

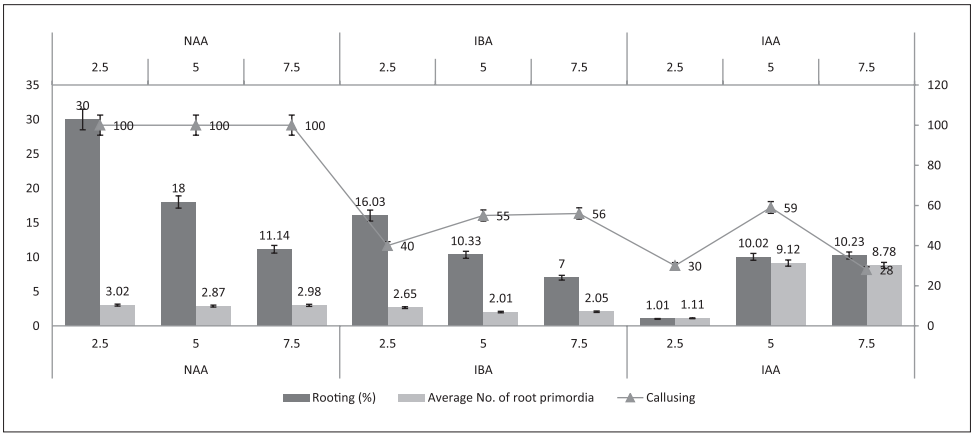
Hypocotyl cuttings also responded toward rooting when cultured on DCR basal medium with various concentrations of auxins. NAA (2.5 µM) produced a best rooting response (30%) over other concentrations and auxin types (Graph 4).

### *Effect of agar concentration*

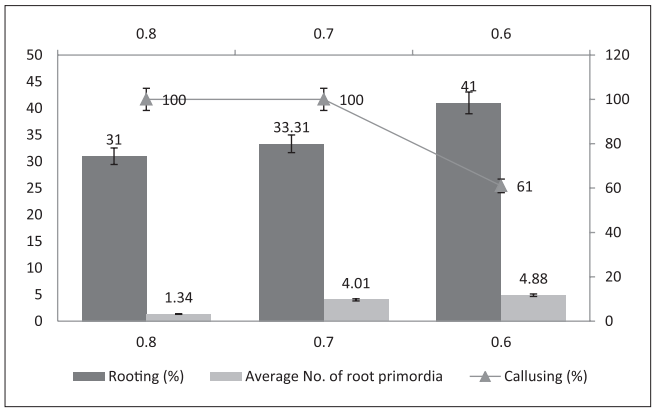
The shoots when cultured on medium gelled with 0.8% agar (Bacteriological grade agar, BDH) produced stunted root primordia with profuse callusing while the shoots cultured in liquid medium were vitrified with no root formation. Thus, attempts were made to check the excessive callusing and induce roots in *in vitro* raised shoots by culturing them on a gelled medium of softer consistency. To study the effect of agar concentration on rooting, the *in vitro* raised shoots and hypocotyl cuttings were cultured on medium gelled with 0.8, 0.7, and 0.6% agar (Graph 5 and Graph 6). A limited success was achieved



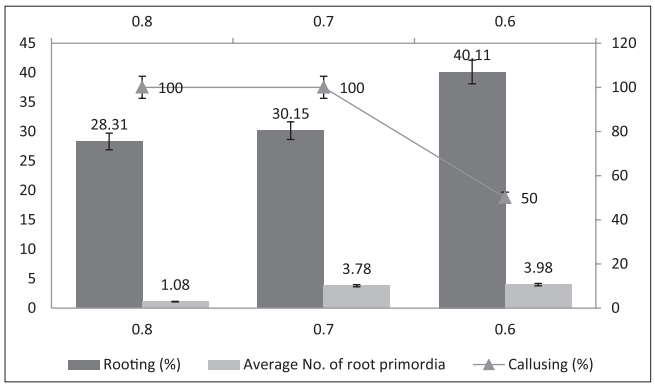

**Graph 3:** Effect of different concentrations of auxins on rooting percentage, number of root primordia and callusing percentage in *in vitro* raised shoots




**Graph 4:** Effect of different concentrations of auxins on rooting percentage, number of root primordia and callusing percentage in hypocotyl cuttings




**Graph 5:** Effect of agar concentrations on rooting percentage, average number of root primordia and callusing percentage in *in vitro* raised shoots

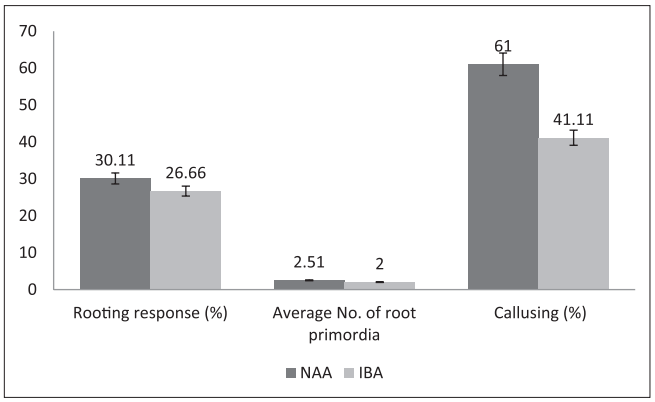



**Graph 6:** Effect of agar concentrations on rooting percentage, average number of root primordia and callusing percentage in hypocotyl shoots

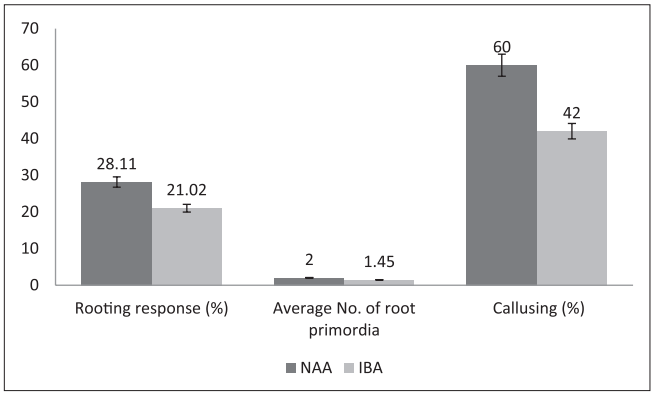
in reducing callus formation at basal ends of shoots. In addition, the percentage of root primordia formation was increased slightly. The root primordia induced in 5-7% shoots elongated into roots on further subculturing on basal medium. It was observed that the shoots producing no or moderate callus at shoot base developed root primordia but the shoots. Producing excessive callus at the base produced no root primordia or primordia, which could not elongate to form roots.

### *Effect of pulse treatment*

Attempts were also made to induce rooting by providing a pulse treatment to individual shoots before transferring them for rooting on half strength basal medium. The *in vitro* raised shoots were cultured on semisolid medium containing high concentration (50 µM) of NAA or IBA individually for 24 h and then transferred to basal medium without auxins. *In vitro* shoots and hypocotyl cuttings showed the formation of root primordia at the base of 30.11% and 28.11% explants, respectively, (Graph 7 and Graph 8), some of these primordia elongated to a length of 1.0-2.0 cm.



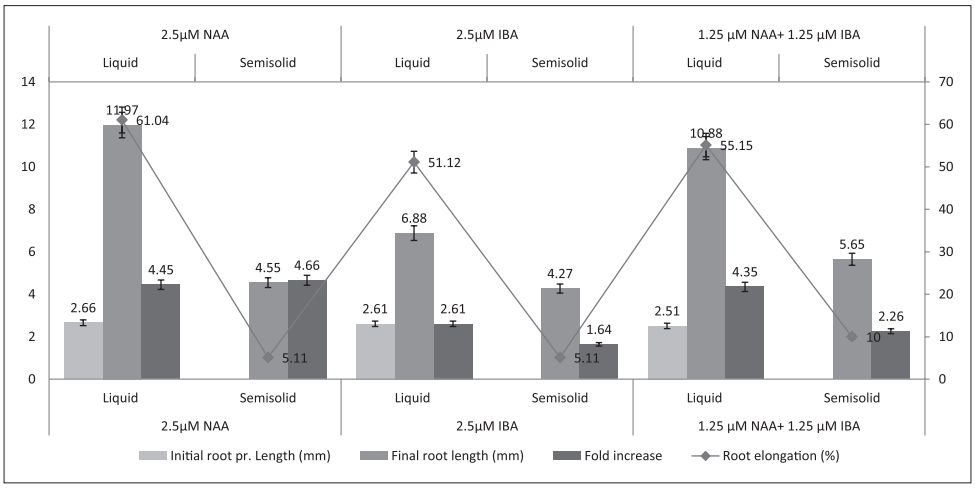

**Graph 7:** Effect of pulse treatment with 5 0 µM N-acetyl-aspartate/ indole-3-butyric acid supplemented in ×½ DCR medium for 24 h followed by culturing on basal ×½ DCR medium (gelled with 0.6% agar) on root primordia induction in *in vitro* raised shoots of *Pinus roxburghii.* Data recorded after 5 weeks



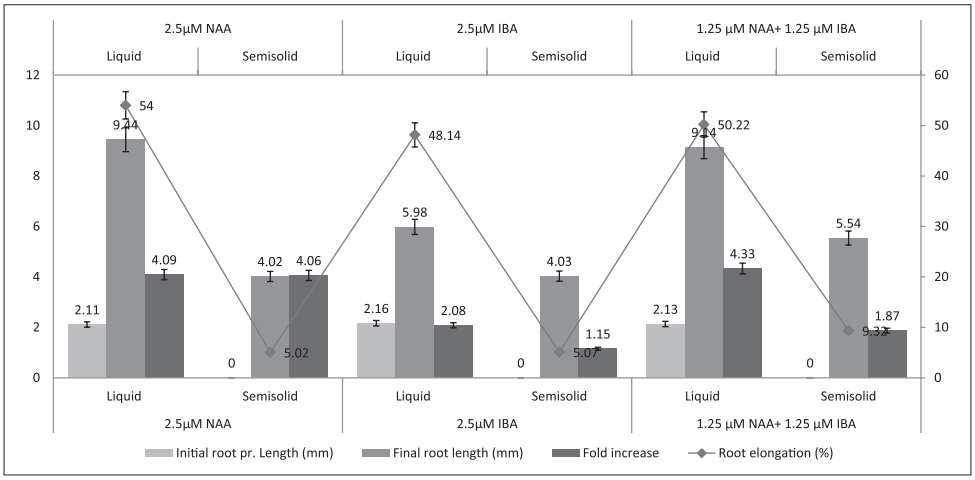

**Graph 8:** Effect of pulse treatment with 50 µM N-acetyl-aspartate/ indole-3-butyric acid supplemented in ×½ DCR medium for 24 h followed by culturing on basal ×½ DCR medium (gelled with 0.6% agar) on root primordia induction in hypocotyl cuttings of *Pinus roxburghii.* Data recorded after 5 weeks

### *Effect of liquid medium and auxins on elongation of root primordia*

The shoots with developing root primordia required transfer to basal medium for elongation of primordia into roots. Better results obtained when the shoots with induced root primordia were transferred to liquid medium with filter paper bridges. The stunted root primordia of 50-60% shoot elongated into roots compared to only 7-10% in medium gelled with 0.6% agar (Graph 9). All the roots were white to off-white with no branching. Hypocotyl shoots also showed root elongation on liquid DCR medium. The rooting in hypocotyl cuttings was observed at all concentrations and combinations of NAA and IBA. The best rooting was observed on 2.5 µM NAA when supplemented in liquid medium followed by a combination of 1.25 µM NAA + 1.25 µM IBA again in liquid medium (Graph 10).




**Graph 9:** Effect of liquid DCR medium (×½) on the elongation of root primordia induced at the base of *in vitro* raised shoots of *Pinus roxburghii* cultured on ×½ DCR medium supplemented with auxins. Data recorded after 8 weeks




**Graph 10:** Effect of liquid DCR medium (×½) on elongation of root primordia induced at the base of hypocotyl shoots of *Pinus roxburghii* cultured on ×½ DCR medium supplemented with auxins. Data recorded after 8 weeks

## DISCUSSION

Rooting is one of the major steps of micropropagation and affects by a number of factors. Phytohormones, nutrient medium, and physiology of shoots have a profound effect on *in vitro* rooting. Rhizogenesis requires a lower concentration of nutrient in the medium [2] and hence half strength DCR medium used in the present research was found successful for *in vitro* rooting response in chirpine. However, different medium with or without modification has been reported to give best rooting response from a variety of explants in *Pinus* [5,8,18]. Reduction in the medium strength resulted in enhanced rooting response as also reported earlier [11,19].

Three auxins viz. NAA, IBA, and IAA were added alone and in combination to study their effect on root induction. For chirpine, NAA (2.5 µM) was observed as best rooting hormone which coincide with the previous findings [9,18,20]. A lower concentration of NAA showed a best rooting response in present investigations which is also in line with previous findings by Murithii *et al*. (1993) [10]. However, callus formation was observed as a usual problem in rooting of pines. To overcome callusing, explants were exposed to pulse treatment of hormones. Exogenous application of auxin is generally required for rooting [21]. However, without the pre-treatment of auxin no significant difference in rooting was observed [22,23]. Continuous exposure of NAA (2.5 µM) in medium showed more number of root primordia formation over the pulse treatment of shoots with 50 µM NAA or IBA. Kaul (1987) [24] and Schwarz *et al*. (1988) [13] also found the same effect of pulse treatment on *in vitro* rooting.

Agar concentration (0.8%) was reduced to 0.6% as it was not allowing roots to penetrate into the medium and result in their radial growth and callusing at the base of shoots. This change resulted in more number of root formation per micro-shoots. Root primordia produced under the influence of auxins were small and needed elongation, and liquid media was found best for this compared to semisolid medium. The superiority of liquid medium for elongation of root primordia is possibly due to better aeration of the growing roots which is poor in the semisolid medium [2].

*In vitro* developmental stages of plants are having low light level, aseptic conditions and high amount of sugar and nutrient and growth factors and controlled light and humidity in the environment. Such a heterotrophic environment makes these plants more delicate and hard to sustain *in vivo*. Gradual acclimatization of *in vitro* plantlets makes them harder to sustain harsh field condition and provide minimal stress for plant multiplication. *In vitro* rooted plantlets of pine were exposed to further hardening and acclimatization.

## CONCLUSION

In the present investigation, we attempted *in vitro* rooting of *P. roxburghii* shoots from different aged material as well as from *in vitro* raised shoots. Such an effort of tissue culture of coniferous species can significantly increase forest productivity with the production of selected genotypes. This *in vitro* regeneration protocol will provide an alternative to rooted cuttings for the propagation of conifers.

## REFERENCES


1. Bergmann BA, Stomp AM. Effect of genotype on rooting of hypocotyls and *in vitro* produced shoots of *Pinus radiata*. Plant Cell Tissue Org Cult 1994;39:195-202.
2. Mohammed GH, Vidaver WE. Root production and plantlet regeneration in tissue cultured conifers. Plant Cell Tissue Org Cult 1988;14:137-60.
3. Dumas E, Monteuuis O. *In vitro* rooting of micropropagated shoots from juvenile and mature *Pinus pinaster* explants: Influence of activated charcoal. Plant Cell Tissue Org Cult 1955;40:231-5.
4. Mehra-Palta A, Semltzer RH, Mott RH. Hormonal control of induced organogenesis from excised plants parts of loblolly pine (*Pinus taeda* L.). TAPPI 1978;61:37-40.
5. Horgan K, Holland L. Rooting micropropagated shoots from mature radiata pine. Can J Forest Res 1989;19:1309-15.
6. Aitken CJ, Horgan KJ, Thorpe TA. Influence of explant selection on the shoot forming capacity of juvenile tissue of *Pinus radiata*. Can J Forest Res 1981;11:112-7.
7. Mott RL, Amerson HV. Tissue culture process for the clonal production of loblolly pine plantlets. N C Agric Res Serv Tech Bull 1981;271:1-14.
8. Rancillac M, Fay M, David A. *In vitro* rooting of cloned shoots of *Pinus pinaster*. Physiol Plant 1982;56:97-101.
9. Patel KR, Kim HR, Thorpe TA. Plantlet formation in pitch pine (*Pinus rigida* Mill.) by tissue culture method. Forest Ecol Manag 1986;15:147-60.
10. Murithii WT, Harry IS, Yeung CC, Thorpe TA. Plantlet regeneration in Chir pine (*Pinus roxburghii* Sarg.): Morphogenesis and histology. Forest Ecol Manag 1993;57:141-60.
11. Pulido CM, Harry IS, Thorpe TA. Effect of various bud induction treatments on elongation and rooting of adventitious shoots of Canary Island pine (*Pinus canariensis*). Plant Cell Tissue Org Cult 1994;39:225-30.
12. Abdullah AR, Grace J, Yeoman MM. Rapid micropropagation of calabrian pine from primary and secondary buds on shoot explants. Can J Forest Res 1986;16:637-41.
13. Schwarz OJ, Schlerbaum SE, Beaty RM. Plantlet regeneration from mature zygotic embryos of eastern white pine (*Pinus strobus* L.). Plant Cell Rep 1988;7:174-7.
14. Webb DT, Flinn BS, Georgis W. Micropropagation of Eastern white pine (*Pinus strobus* L.). Can J Forest Res 1988;18:1570-80.
15. Chesick CA, Hackett WP. Plantlet production from white pine (*Pinus strobes* L.) embryos *in vitro*: Bud induction and rooting. Plant Cell Tissue Org Cult 1991;26:107-14.
16. Horgan K, Aitken-Christie J. Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. Physiol Plant 1981;53:170-5.
17. Cheng TY. Clonal propagation of woody species through tissue culture techniques. Proc Int Plant Propag Soc 1978;28:139-55.
18. Amerson HV, Mott RL. Improved rooting of western white pine shoots from tissue cultures. Forest Sci 1982;28:822-5.
19. Patel KR, Thorpe TA. *In vitro* differentiation of plantlets from embryogenic explants of lodgepole pine (*Pinus contorta* Doug. Ex. Loud). Plant Cell Tissue Org Cult 1982;3:131-42.
20. Cheng TY. Recent advances in development of *in vitro* techniques for Douglas fir. In: Sharp WR, Larsen P, Paddock E, Raghavan V, editors. Columbus: Ohio State University Press; 1979.
21. Haissig BE. Influence of aryl esters of Indole 3 acetic and Indole- 3 butyric acids on adventitious root primordium initiation and development. Physiol Plant 1979;47:29-33.

22. Baxter R, Brown SN, England JF, Ludlow CH, Taylor SL, Womarck RW, Dunstan DI. Production of clonal plantlets of tropical pine in tissue culture via axillary shoot activation. Can J Forest Res 1989;19:1338-42.
23. Halos SC, Go NE. Micropropagation of *Pinus caribaea* Morelet. Plant Cell Tissue Org Cult 1993;32:47-53.
24. Kaul K. Plant regeneration from cotyledon: Hypocotyl explants of *Pinus strobes* L. Plant Cell Rep 1987;6:5-7.