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Research Article

EFFECT OF PLANT GROWTH REGULATORS AND pH OF MEDIUM ON *IN VITRO* REGENERATION OF *PINUS ROXBURGHII* SARG

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ABSTRACT
The genus *Pinus* has been an integral part of vibrant, but fragile ecosystem of Great Himalayan range in India. *Pinus roxburghii* is an economically important forest tree with lots of medicinal properties. The natural propagation of pine beset with many problems and thus, alternative tissue culture methods have been proposed for its large scale production and genetic improvement. The present research was undertaken to standardize the best phytohormone type and its concentrations for regeneration of *Pinus roxburghii* through Axillary bud induction and proliferation pathway. Five concentrations (5-25 µM) of three cytokinins *viz*. BAP, Kn and BPA and also the combination of BAP (5-25 µM) and NAA (0.5, 2.0 µM) were tested for induction and multiplication of axillary buds from nodal explants of *Pinus roxburghii*. The best results were obtained at 10 µM BAP, also when supplemented with 0.5 and 2.5 µM α-NAA. The best induction and multiplication was shown at pH 5.8. Optimum rooting was obtained at 2.5 µM NAA followed by IBA and IAA which could not root efficiently. Rooted plantlets were hardened by transferring them in hardening medium. The extract of this research was to develop an efficient *in vitro* regeneration protocol for sustainable regeneration of *Pinus roxburghii*. The outcome of the research will prove vital in improvement of *Pinus roxburghii* by regenerating the superior genotypes and also help in increasing the forest productivity.

Key Words: *Chirpine, Auxins, Axillary Bud, Pinus, In Vitro*

INTRODUCTION
Pine constitute one of the most divergent and economically important group of species, which provides valuable natural resources and contribute significantly to the local and industrial economy of the country. Application of tissue culture techniques in propagation and genetic improvement of Pines is especially desirable because of the commercial importance of this genus in forestry, its generally slow reproductive behaviour and wide variation between its individual trees and populations due to open-pollination (Chauhan *et al.*, 2005; Sharma *et al.*, 2002).

Axillary bud induction is one of the most successful procedures of micropropagation. Scientists have employed embryonic or very young seedling explants for induction of shoots. (Kaul and Kochhar 1987; Schwarz *et al.*, 1988; Thorpe and Biondi 1984; Webb *et al.*, 1988). Mainly cytokinin influences the induction of shoots (Arya *et al.*, 2012b). Lappm *et al.*, (1996) tested the effect of six cytokinins on induction of shoots in *Pinus monticola*. Baxter *et al.*, (1989) cultured shoots selected from low, medium and high producer clones of *Pinus oocarpa* for multiplication on BAP supplemented medium. Lower BAP concentrations stimulated shoot production but higher BAP concentrations were inhibitory. Elongation of the quiescent meristems induced by cytokinin treatment requires transfer to basal medium with or without growth regulators and added to promote shoot growth. Rooting is a very crucial step of micropropagation of many gymnosperms (Mohammed and Vidaver 1988a; Rancillac *et al.*, 2006). Efficiency of *in vitro* adventitious rooting is highly variable and is the key problem in conifer plantlet regeneration. Bergmann and Stomp (1994) reported that the *in vitro* raised shoots of 12 clones of *Pinus radiata* showed variation in percentage of shoots rooted, number of roots per shoot, shoot elongation rate and in requirement of *ex vitro* rooting conditions. Variation in the rooting response with the difference in

age groups in *Pinus pinaster* was reported by Dumas and Monteuuis (1955). Juvenile plant material exhibit greater potential for adventitious rooting, is also supported by earlier reports which also emphasized the inhibitory effect of explant maturation on the process of adventitious root formation. Several aspects of rooting phytohormones *viz*. type, concentration, mode of application and duration of response also need to be considered. For most of pine species α-NAA is reported as more potent auxin compared to IBA (Aitken *et al.,* 1981; Mehra-Palta *et al.,* 1978; Mott and Amerson 1981; Patel *et al.,* 1986; Rancillac *et al.,* 1982).

Efficient micropropagation protocol requires standardization of various phsico-chemical parameters and thus, is the aspect of present research. Phytohormone concentrations, their types and pH of medium were standardized for efficient *in vitro* regeneration of *Pinus roxburghii*.

MATERIALS AND METHODS

The explants used for axillary bud induction in the present study were seedling shoots (8-10 days old) and shoots from 20-25 day old seedlings. Seeds were surface sterilized using 0.1% $HgCl_2$ for 20 min and 3% (w/v) H_2O_2 for 10 min. This was followed by washing with autoclaved distilled water to remove traces of sterilizing agent. The surface sterilized seeds were aseptically inoculated on hormone free medium (0.8% agar and 3% sucrose) for germination. The cultures were incubated at temperature of 25 ± 2ºC and maintained at 16h light and 8h dark photoperiod timers. Light was provided by 40W white cool fluorescent tubes (Philips, India).

Induction of Axillary Buds

To study the effect of cytokinins on axillary bud induction in seedling shoots, three cytokinins *viz*. BAP, Kinetin and BPA were supplemented in MS medium at concentrations of 0, 5.0, 10.0, 15.0, 20.0 and 25.0 µM singly. The explants collected from hedges and mature trees were cultured on medium supplemented with 5-25 µM BAP. the effect of cytokinin-auxin combination was studied by adding α-NAA at 0.5 and 2.5 µM strength in MS medium containing 5-25 µM BAP.

MS medium was supplemented with 10 µM BAP and eight different pH of medium *viz*. 4.5, 5, 5.5, 5.8, 6, 6.5, 7, and 7.5 (prior to autoclaving) was adjusted using either 1M NaOH or 0.25 M HCl.

Multiplication of in Vitro Raised Shoots

The axillary shoot buds were multiplied for increase in number of shoots or rooted to produce complete plantlets for field transfer. The *in vitro* raised shoots (1.5-2.0 cm) were multiplied further and effects of various physico - chemical parameters on shoot multiplication were studied.

Effects of cytokinin and cytokinin-auxin combinations on multiplication of *in vitro* raised shoots were studied. The shoots were cultured on the medium supplemented with 5-25 µM BAP / Kn / BPA. α-NAA at 0.5 and 2.5 µM and BAP (5-25 µM) supplemented medium was used to assess the effect of cytokinin - auxin interaction on shoot multiplication.

The eight-pH range *viz*. 4.5, 5, 5.5, 5.8, 6, 6.5, 7, and 7.5 were investigate to find the best pH of MS medium for optimum bud multiplication.

Rooting, Hardening and Acclimatization of Plantlets

To complete plantlet regeneration elongated shoots (1.5-2.0 cm) were placed under various hormone regimes designed to induce adventitious root formation. Effects of many factors were studied on root inductions, which are mentioned as under.

Auxins *viz*. α-NAA, IBA and IAA were supplemented in ½x MS medium at 2.5, 5.0 and 7.5 concentrations singly or in combination to investigate their effect on rooting of *in vitro* raised shoots.

The *in vitro* raised plantlets were transferred to bottles containing liquid MS medium (¼x) supplemented with 1% sucrose and coir as support. Plantlets were placed in this medium for 5 weeks. For acclimatization, the *in vitro* raised plantlets were transferred to polybags containing sand, soil and farmyard manure (FYM) in 1:1:1 ratio and were supplied with ¼x MS medium without sucrose for 2 months.

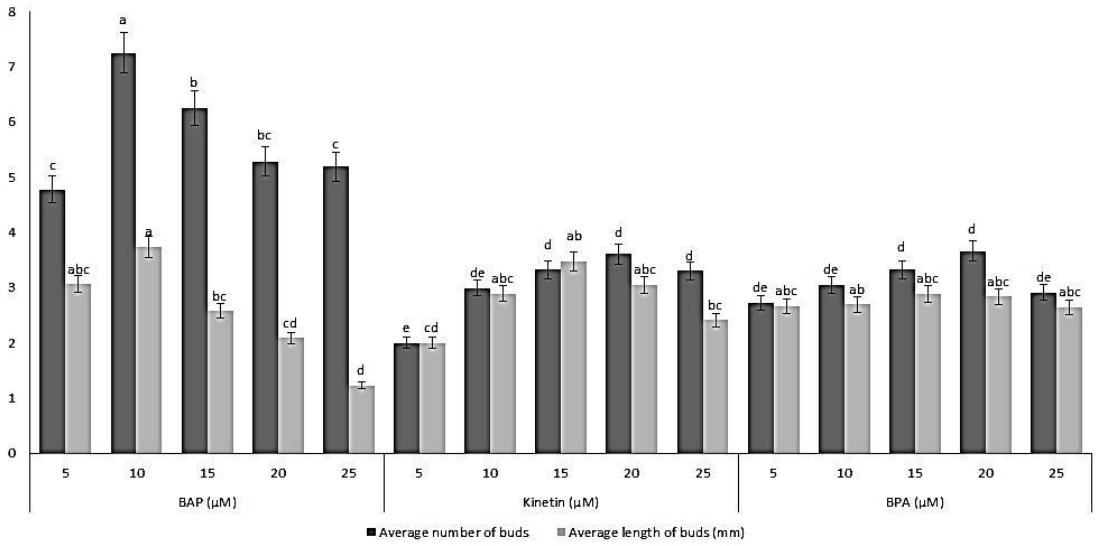
Statistical Analysis

All the experiments were repeated three times with a minimum of hundred explants per treatment. Experiments were analyzed in a factorial based, completely randomized design. Analysis of variance (ANOVA) was performed and comparisons of means were conducted by Duncan's Multiple Range Test. All analyses were performed using IBM SPSS v 20, DSAASTAT add-ins in MS EXCEL 2010 and data analysis tool of MS EXCEL 2010. Differences were regarded as significant at P = 0.05.

RESULTS

Induction of Axillary Buds

The induction of axillary buds was found to be regulated by the level of phytohormones in the medium. All the three cytokinins i.e. BAP, Kn and BPA effectively induced axillary buds on the cultured shoots. The shoots cultured on basal medium devoid of cytokinins produced no axillary buds. The maximum average numbers of buds were induced in the explants cultured on 10 µM BAP. Other cytokinins i.e. Kn and BPA helped to produce lesser response compared to BAP inducing a maximum of 3.61 ± 1.85 and 3.67 ± 1.88 buds in 60% and 73.33% explants respectively on medium supplemented with 20 µM of Kn and BPA (Graph 1). The induced shoot buds were longer on medium containing BAP compared to the shoot buds induced on medium containing Kn and BPA. The average length of induced shoot buds was maximum (3.74 mm) in medium supplemented with 10 µM BAP and decreased with increase in BAP concentration.

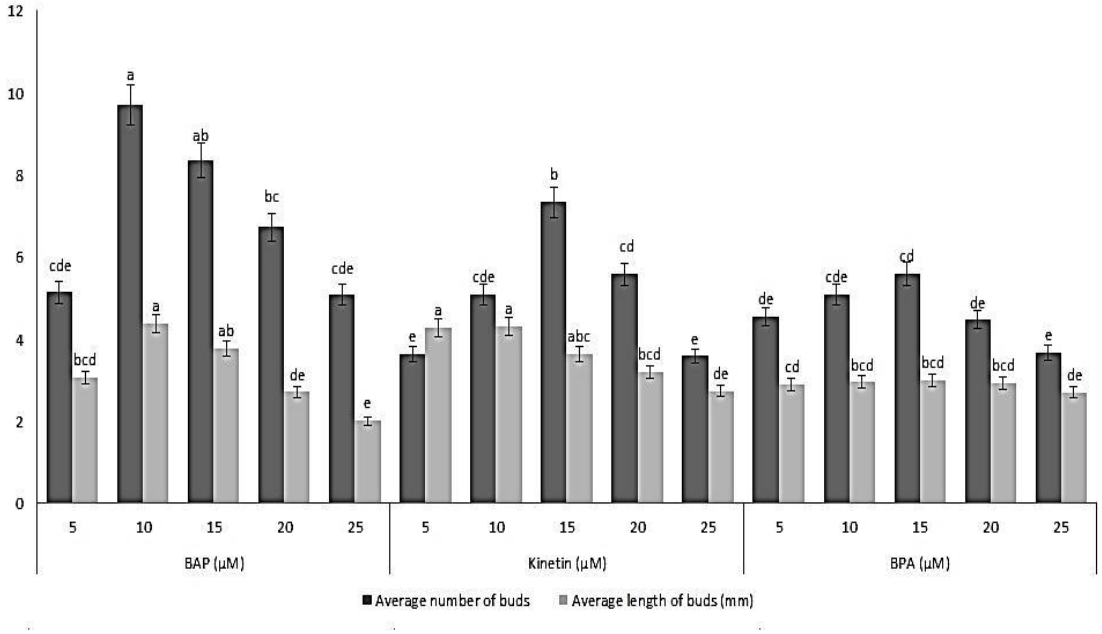


Note: Bars having the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Duncan's Multiple Range Test.

Graph 1: Effect of cytokinins supplemented in MS medium on induction of axillary buds on shoot apices *Pinus roxburghii*. Data recorded after 6 weeks of culture.

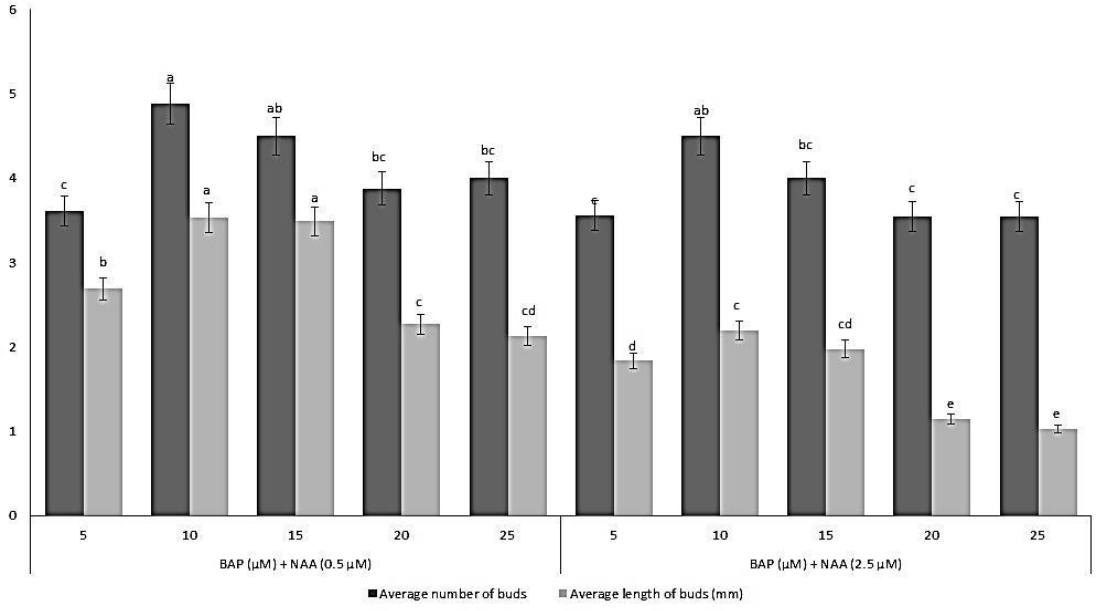
It was found that the interaction of BAP and α-NAA was not synergistic and had an adverse effect on percent response, average bud number as well as average bud length. Addition of 0.5 µM α-NAA reduced the average percent response to 80% (compare to 90-100% in BAP supplemented media) and average bud number was reduced from 7.25 to 4.88 per explants (Graph 3). The amount of callus produced increased when α-NAA concentration was increased from 0.5 to 2.5 µM with a corresponding decrease in percent response (53.33% compared to 80%), average bud number (4.50 compared to 4.88) and a drastic decrease in average bud length (from 3.53 to 2.20). Shoots were green and healthy in medium supplemented with BAP alone but showed deterioration in health and vigour in α-NAA supplemented media. The shoots

turned light green to pale green with swollen apices, many needles turned brown and even some explants died.



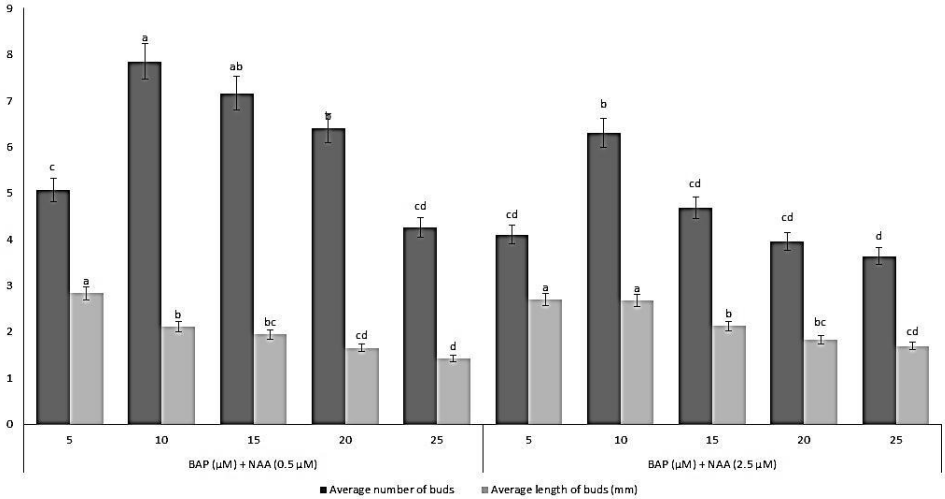
Note: Bars having the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Duncan's Multiple Range Test.

Graph 2: Effect of cytokinins supplemented in MS medium multiplication of *in vitro* raised shoots of *Pinus roxburghii*. Data recorded after 6 weeks of culture.



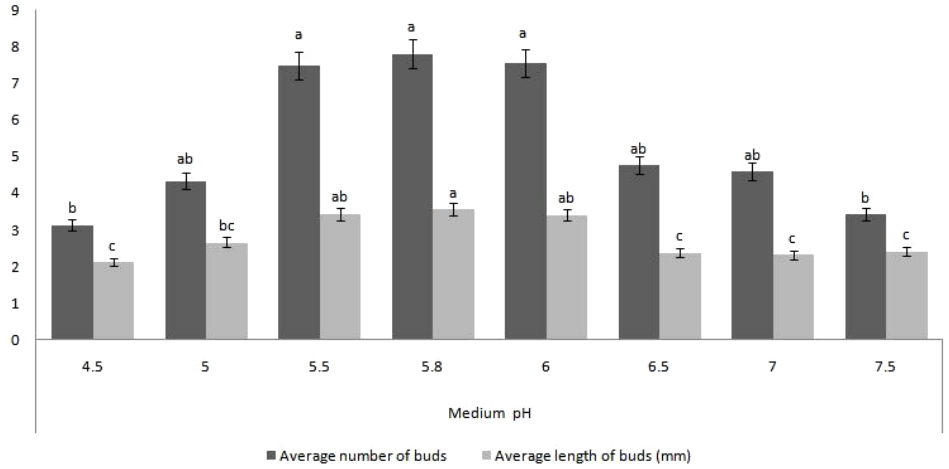
Note: Bars having the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Duncan's Multiple Range Test.

Graph 3: Effect of BAP- α-NAA combination supplemented in MS medium on induction of axillary buds on shoot apices of *Pinus roxburghii*. Data recorded after 6 weeks of culture.



Note: Bars having the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Duncan's Multiple Range Test.

Graph 4: Effect of BAP- α-NAA combination supplemented in MS medium on multiplication of *in vitro* raised shoots of *Pinus roxburghii*. Data recorded after 6 weeks of culture.



Note: Bars having the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Duncan's Multiple Range Test.

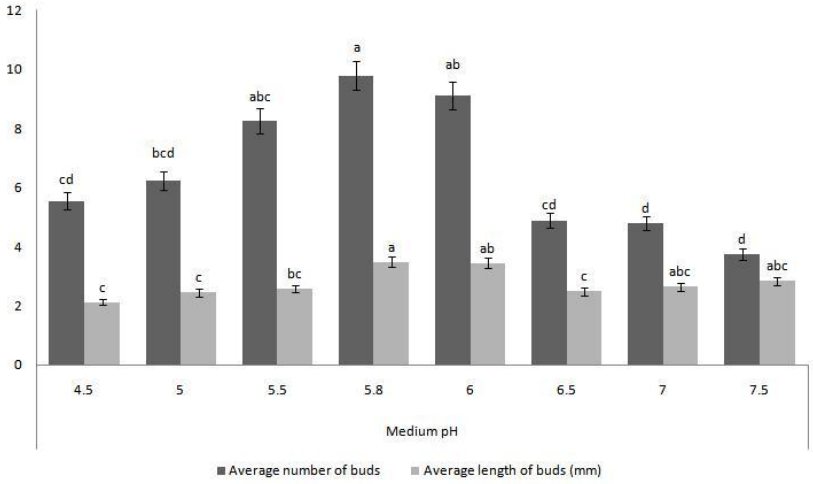
Graph 5: Effect of pH of MS medium on Induction (A) and Multiplication (B) of Axillary buds and *in vitro* regenerated shoots of *Pinus roxburghii*.

Different pH of MS medium had significant differences in the induction of buds form seedling shoots of *Pinus roxburghii*. Acidic pH of medium (4.5-5.8) showed much better results as compared to alkaline pH (6.0 -7.0). However, the best pH was 5.8 at which maximum buds 7.8 ± 0.89 were induced (Graph 5)

Multiplication of in Vitro Raised Shoots.

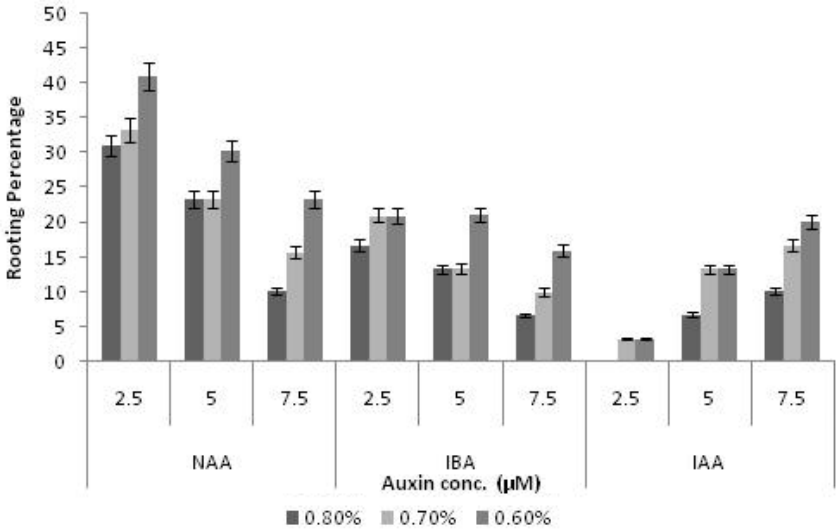
The effect of cytokinins on multiplication of *in vitro* raised shoots of *Pinus roxburghii* was studied. The results revealed that shoot multiplication is regulated by the presence of cytokinins in the medium as the shoots cultured on cytokinin free medium showed an increase in length without increase in number of shoots. Maximum shoot multiplication response was obtained in BAP supplemented medium. The optimal BAP concentration for multiplication of shoots was 10 µM, which supported the development of

9.70 buds per cultured shoot in 4 weeks. At increased BAP concentrations, (15-25 µM) reduction in multiplication rate was observed along with a decrease in average bud length. The multiplication response decreased sharply on further increase in BAP concentration (Graph 2).



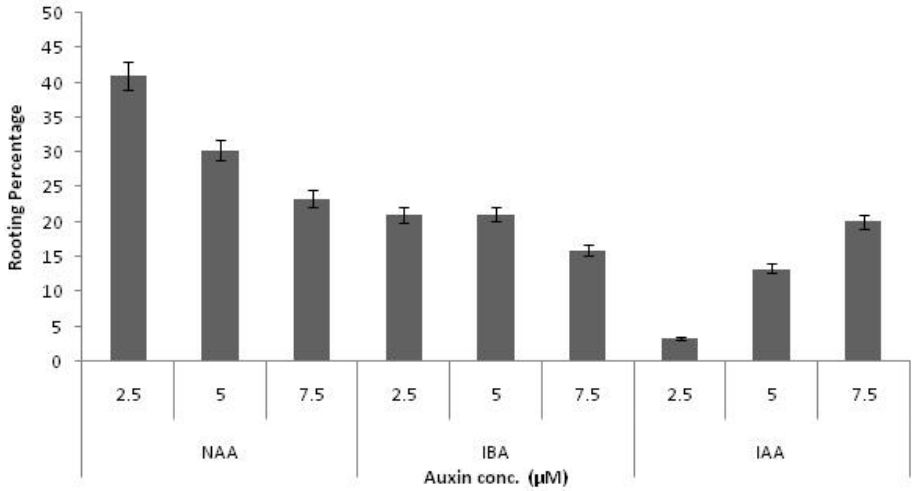
Note: Bars having the same letter do not differ significantly (P = 0.05) as indicated by one-way ANOVA followed by Duncan's Multiple Range Test.

Graph 6: Effect of pH of MS medium on Induction (A) and Multiplication (B) of Axillary buds and *in vitro* regenerated shoots of *Pinus roxburghii*.



Graph 7: Effect of different concentrations of auxins and agar concentrations on rooting of *in vitro* raised shoots.

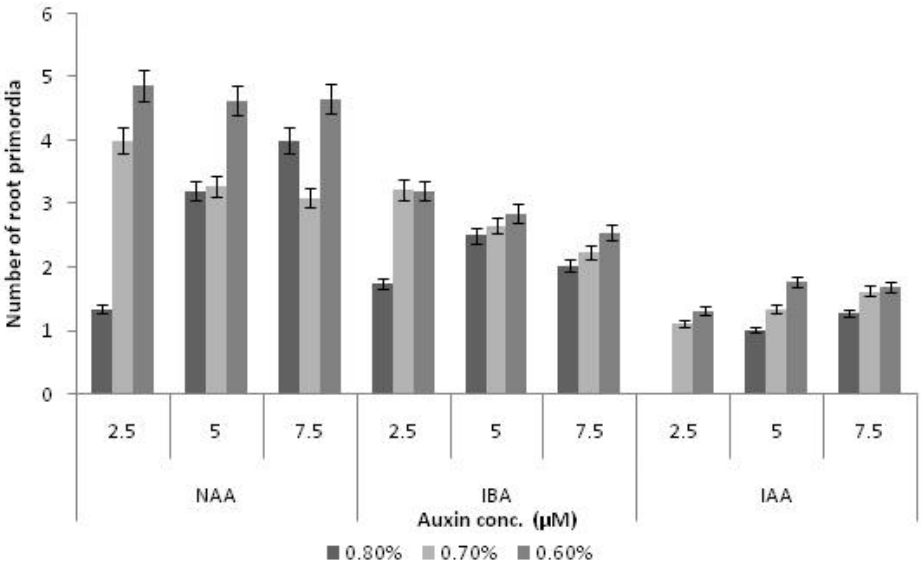
Incorporation of small amounts of auxins along with cytokinins in shoot multiplication medium may have a promotory effect as has been reported in some other plant species. Addition of α-NAA at 0.5 µM and 2.5 µM concentrations produced maximum average bud number of 7.85 and 6.30 respectively on 10 µM BAP supplemented MS medium (Graph 4). The best multiplication of *in vitro* induced axillary buds was obtained at 5.8 pH. However, at higher and lower pH the multiplication response was stunted (Graph 5). Before transferring to rooting medium the multiplied shoots were subjected to elongation on low strength MS medium (data not given).



Graph 8: Effect of different concentrations of auxins on rooting of *in vitro* shoots when cultured on 0.6% agar.

Rooting, Hardening and Acclimatization of Plantlets

The *in vitro* multiplied shoots of 1.5-2.0 cm length were tried for *in vitro* rooting. Success was obtained only in formation of root primordia at the base of the shoots cultured under different experimental combination treatments of the auxins. It was found that α-NAA although initiates root primordia first, but afterward callus develops which overtakes the growth of the developing root primordia. Root primordia were initiated in 31% shoots cultured on 2.5 µM α-NAA supplemented medium (Graph 6).



Graph 9: Effect of different auxins concentrations on number of root primordia.

Increase in α-NAA concentration led to a decrease in the number of root primordia induced and with a simultaneous increase in callus formation. At higher α-NAA levels (7.5 µM) the root primordia produced did not elongate on subculturing to fresh medium as well as on transfer to basal medium. IBA and IAA when used at 2.5-10 µM levels produced root primordia in lesser percentage of shoots. Similar to α-NAA the root initials formed could not elongate into roots (Graph 7 and Graph 8).

DISCUSSION

Enhanced axillary branching was utilized for micropropagation *Pinus roxburghii*. *In vitro* culture response in conifers is dependent on the age of explants. In present investigation the axillary bud induction was achieved from pre-existing quiescent meristems from seedling shoots of *Pinus roxburghii*. Juvenile plants of *Pinus roxburghii* showed very good response though; experiments were also conducted to achieve bud induction from different aged plants as well as mature plants (data not given). The importance of juvenile explants was reported in pine by many researchers (Bonga 1987; Webb *et al.,* 1988; Zel *et al.,* 1988).

Growth regulators have profound effect on *in vitro* bud induction process. Cytokinins are the major class of growth regulators responsible for *in vitro* shoot induction (Skoog 1971). Among all the cytokinins used, BAP showed best response in the proliferation of axillary buds. Many of the regeneration studies have also acknowledged the importance of BAP (Abdullah *et al.,* 1986; Arya *et al.,* 2012a; Arya *et al.,* 2012b; Baxter *et al.,* 1989; Gupta and Durzan 1985; Lappm *et al.,* 1996; Lin *et al.,* 1991; Schwarz *et al.,* 1988).

In Chirpine, cytokinin concentration (10 µM BAP) was found best for bud induction and multiplication. In *Pinus strobus*, 5 µM BAP (Baxter *et al.,* 1989) and 2 or 4 mg/l BA (Kaul 1990) was reported best while for *Pinus ponderosa* 4.4 µM BAP (Lin *et al.,* 1991) reported as best concentration. Addition of little amount of auxins was found to have stimulatory effect on bud initiation. The optimum concentration of auxin and cytokinin in the medium needs optimization as it varies from species to species. α-NAA along with BAP was investigated for bud induction in *Pinus roxburghii* and resulted in decreased bud induction. Findings of present study are in agreement with (Lin *et al.,* 1991).

The pH of medium is very important as it influences the uptake of nutrients and plant growth regulators (Owen *et al.,* 1991). A pH higher than 6 give a fairly hard medium and a pH below 5 do not allow satisfactory gelling. In present investigation the optimum induction and multiplication of shoots was obtained at 5.8 pH. The results also verified that the plant were not able to sustain a wide range of pH.

Rooting of *in vitro* developed shoots is one of the major steps of micropropagation and need the support of auxins in the medium. Auxins are well studied and an established rooting hormone (Scott 1972). Rhizogenesis requires a lower concentration of nutrients in the medium. Hence, half strength MS medium was used in present investigation and found successful for *in vitro* rooting response in Chirpine. Three auxins *viz.* α-NAA, IBA and IAA were added alone to study their effect on root induction. For Chirpine the α-NAA (2.5 µM) was observed as best rooting hormone which concur with the findings of (Cheng 1979).

Gradual acclimatization of *in vitro* plantlets makes them hardened to sustain harsh field conditions and provide minimal stress for plant multiplication. In Chirpine, for hardening, ¼x MS medium containing 1% sucrose and coir as support was used. Reduction of medium's nutrients for hardening as proposed first by Murashige (1974) followed by low carbohydrates and higher level of light makes *in vitro* plants use their photosynthetic apparatus for nutrition (Kozai *et al.,* 1988). In present investigation the success was achieved for *in vitro* propagation of plantlets from juvenile explants. However, more exhaustive study is required especially at rooting part to get sustained supply of Chirpine.

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