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Regeneration of Neem Plantlet by in Vitro Clonal Micropropagation

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Abstract: ***The present study was carried out during 2014-15 with Neem (Azadirachta indica) is an evergreen tropical forest tree that is a renewable source of various useful products, to investigate optimal concentrations and combinations of plant growth regulators in the medium for efficient micropropagation. The present observation revealed that the highest callusing response 22.3±0.92% was observed in MS medium supplemented with 0.3 mg/l 2, 4-D; 0.3 mg/l Kn and 0.3 mg/l NAA in leaf explant and in seed explant it is 42.5±0.97% was observed in MS medium supplemented with 0.5 mg/l of each 2, 4-D and BAP. The highest shoot regenerating capacity (94%) was recorded in MS medium supplemented with 1.0+0.5 mg/l (BAP+NAA). Best rooting (95%) was observed in ½ strength of MS medium supplemented with 3 mg/l NAA and the highest number of average roots per micro shoots and average length of root were 15 and 4.9 cm respectively. Acclimatization and hardening was successful with survival rate of 87.5%.***

Keyword: Azadirachta indica, MS- Murashige and Skoog, explant, harmones, micropropagation

I. INTRODUCTION

Neem (*Azadirachta indica*) is an evergreen tropical forest tree that is a renewable source of various useful products. The seed, flowers, leaves, bark and branches of the neem tree have multiple uses and several compounds of medical importance have been reported (Biswas *et al*., 2002). The neem holds the promise of providing highly effective, non-toxic and environmentally friendly means of controlling or eliminating insects and pests that cause losses in agricultural production (Govindachari *et al*., 1991). More importantly, neem has recently been recognized as a viable source of seed-oil for production of biofuel to meet the demand of the biofuel industry. However, the present production of neem seeds is grossly inadequate and planting of such oil bearing trees in a massive scale has the potential to provide a renewable energy resource, and in addition will alleviate the competitive situation that exists with food crops as biofuels and associated arable land and water use. Hence, there is an urgency to increase the production of such tree crops as neem which can be cultivated on marginal land. The prime aim of this objective study is to meet the renewable energy demand in the future; a large quantity of quality planting material will be needed. The micropropagation approach is considered to be the most appropriate viable alternative in tree improvement programs.

II. MATERIALS AND METHODS

A. Sterilization of Equipments and Glassware's

All operations for *in vitro* culture were carried out inside a laminar air flow cabinet under aseptic conditions using sterilized plant materials, equipments, glass materials and chemicals. A horizontal laminar flow cabinet with HEPA filter was used. The hood surface was wiped clean with paper towel soaked in 70 % ethanol and sterilized by ultraviolet light for 10 min prior to use. All surgical instruments, glassware and other accessories were sterilized in an autoclave at 121^0C with 15 psi for 30 min and then dried in oven. Surgical instruments like scalpels, forceps, and scissors were sterilized by dipping in 100 % ethyl alcohol and flaming prior to use.

B. Surface Sterilization of Explants

The young, healthy explants of *Azadirachta indica* were collected from Biodiesel Technology Park, Gulbarga University, Kalaburagi. The potential explants (the starting tissue originated from the donor plant) consist mostly of the shoot (internodes and nodal segments), leaf (lamina segments with ribs), apical meristem and seeds. Generally, younger, more rapidly growing tissue or tissue in early developmental stage is the most effective. Explants were brought to the lab and thoroughly washed under running tap water by adding a drop of detergent Tween 20 for half an hour to remove surface adhered particles. Explants were transferred to laminar air flow cabinet and surface sterilized with 0.1% sodium hypochlorite for 1 min followed by washing with distilled water 2-3 times (each wash is for 2 min) and wash with 70% ethanol for 1 min and washed with distilled water 2-3 times. After that explants were washed with 0.1% mercuric chloride for 5 min followed by washing with sterile water for five times.

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C. Culture media

Basic media based on the formulation of Murashige and Skoog (1962) were used throughout the investigation. The protocol provided for preparation of stock solutions, solubility of growth hormones and sequence of preparing the final solution was strictly observed.

D. Growth regulators

Auxin and cytokinin were the two major phytohormones used in different concentrations and combinations in MS Media for callus and dir3ect regenerations.

1) *Auxin:* Powder of auxins (Himedia) were dissolved in 1N NaOH and made up the volume with sterilized distilled water and then used or stored in freezer as stock for further use. The auxins used in the present study were 2,4-Dichlorophenoxyacetic Acid (2,4-D), α- Naphthalene Acetic Acid (NAA), Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA).
2) *Cytokinin:* The cytokinin (Himedia) were dissolved in 1N NaOH and then used or stored as stock for further use. The two cytokinin used were 6-Benzyl amino purine (BAP) and Kinetin (Kn).
3) *Gibberellins*: Gibberelic acid (GA3).

E. Preparation of Media

The required quantity of stock solution of inorganic and organic salts, growth hormones and carbon source were added and the final volume was made up with distilled water. The medium was adjusted to pH 5.6±0.2 using 1N NaOH or 1N HCl prior to autoclaving at 0.7 Kg/cm^2 (15 psi) and 121^{0}C for 20 min. The composition of MS medium is shown in Table 1. The chemicals were dissolved in sterile distilled water to prepare stock solutions and further diluted as per the table during final preparation. All the stock solutions, growth hormones, vitamins and amino acids were stored preferably in the refrigerator. The sucrose concentration was maintained at 3% and that of agar to 0.8%.

F. Explants for Callus Induction

For callus induction, the sterilized leaf and seeds were cut and placed in the culture test tube touching the callus induction medium. All the culture tubes were wrapped with parafilm to prevent from contamination. The seed cultures were incubated in the dark at room temperature.

G. Callus Induction

For in vitro callus proliferation, explants were inoculated on MS media supplemented with various concentrations of plant growth regulators like 2, 4-D, Kn, NAA, BAP and 2, 4-D separately or in combinations as shown in Table 2-3. The cultures were maintained in the culture room [temperature 25-260C and photoperiod of 16 h light and 8 h dark]. Subcultures were done at 15 days interval.

Table 1: Composition of Murashige and Skoog (MS, 1962) medium

Sl.No	Nutrients	Ingredients	Concentration (mg/l)	ml taken for 1 litre
1	Macronutrients (10x/100ml)	NH_4NO_3 KNO_3 MgSO4.7H_2O KH_2PO_4 $CaCl_2$	1650.0 1900.0 370.0 170.0 440.0	100
2	Micronutrients (100x/100ml)	$MnSO_4.4H_2O$ H_3BO_3 $ZnSO_4$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$ $NaMoO_4.2H_2O$ KI	2230.0 620.0 860.0 2.5 2.5 25.0 83.0	10
3	FeEDTA (100x/100ml)	Na_2EDTA $FeSO_4.7H_2O$	3.73 2.78	5
4	Vitamins (100x/100ml)	Myo-inositol Thiamine HCl Nicotinic acid Pyridoxine HCl Glycine	100.0 50.0 50.0 50.0 100.0	10
5		Sucrose (g)	30.0	-
6		Agar (g)	0.8	-
7		pH	5.6 ± 0.2	-

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Table 2: Media formulation for callus induction from leaves

Sl. No.	Media	Growth regulators Conc.(mg/l)
1	MS1	MS + 2,4-D (0.1)
2	MS2	MS + 2,4-D (0.2)
3	MS3	MS + 2,4-D (0.3)
4	MS4	MS + 2,4,D (0.4)
5	MS5	MS + 2,4-D (0.5)
6	MS6	MS + 2,4-D (0.1) + Kn (0.1)
7	MS7	MS + 2,4-D (0.2) + Kn (0.2)
8	MS8	MS + 2,4-D (0.3) + Kn (0.3)
9	MS9	MS + 2,4-D (0.4) + Kn (0.4)
10	MS10	MS + 2,4-D (0.5) + Kn (0.5)
11	MS11	MS + 2,4-D (0.1) + Kn (0.1) + NAA (0.1)
12	MS12	MS + 2,4-D (0.2) + Kn (0.2) + NAA (0.2)
13	MS13	MS + 2,4-D (0.3) + Kn (0.3) + NAA (0.3)
14	MS14	MS + 2,4-D (0.4) + Kn (0.4) + NAA (0.4)
15	MS15	MS + 2,4-D (0.5) + Kn (0.5) + NAA (0.5)

Table 3: Media formulation for callus induction from seeds

Sl. No.	Media	Growth regulators Conc.(mg/l)
1	MS1	MS + BAP (0.1)
2	MS2	MS + BAP (0.2)
3	MS3	MS + BAP (0.3)
4	MS4	MS + BAP (0.4)
5	MS5	MS + BAP (0.5)
6	MS6	MS + 2,4-D (0.1) + BAP (0.1)
7	MS7	MS + 2,4-D (0.2) + BAP (0.2)
8	MS8	MS + 2,4-D (0.3) + BAP (0.3)
9	MS9	MS + 2,4-D (0.4) + BAP (0.4)
10	MS10	MS + 2,4-D (0.5) + BAP (0.5)
11	MS11	MS + 2,4-D (0.1)
12	MS12	MS + 2,4-D (0.2)
13	MS13	MS + 2,4-D (0.3)
14	MS14	MS + 2,4-D (0.4)
15	MS15	MS + 2,4-D (0.5)

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H. Shoot Regeneration

The callus mass subcultured on MS medium supplemented with different concentration of cytokinin (BAP and Kn) at 0.5, 1.0, 2.0 mg/l concentration and different combination of BAP+NAA [0.5+0.1-2.0+1.0 mg/l] and BAP+IBA [0.5+0.1-2.0+1.0 mg/l] for shoot regeneration. The Cultures were grown in 150×25 mm glass culture tubes, each containing 20 ml of medium. All the cultures were maintained at $25^0C \pm 2^0C$ and under diffuse light (1000-2000 lux) with 16h photoperiod.

I. Shoot Elongation, Multiplication and Rooting

For elongation, individual shoots of 0.5 cm length were separated from regeneration media and cultured on MS medium containing a lower concentration of BAP at 0.15 mg/l or 0.225 mg/l. BAP was tested alone or in combination with casein hydrolysates (CH) at 250 mg/l level or NAA (0.01 mg/l) or GA3 (0.175 mg/l). In another experiment, the effect of GA3 (1.0 mg/l) pretreatment was tested on shoot elongation. Once the shoots attained a sufficient length, they were cut into single node segments and transferred to fresh medium for further multiplication. The numbers of propagules obtained at the end of a multiplication cycle were regarded as the rate of shoot multiplication. For rooting, individual shoots, and measuring 3 cm and with 3-4 nodes, were excised and cultured on ½ strength MS media supplemented with different concentrations of auxin.

J. In vitro Rooting of Grown Microshoot Culture

The *in vitro* grown microshoot were inoculated into the ½ strength MS media supplemented with different concentration of auxins namely IBA, NAA, IAA [0.5, 1.0, 3.0, 5.0, 7.0 mg/l] and a combination of NAA+IBA [0.5+0.5, 0.5+1.0, 1.0+0.5, 1.0+1.0, 3.0+0.5, 3.0+1.0, 5.0+0.5, 5.0+0.5, 5.0+1.0, 7.0+0.5, 7.0+1.0 mg/l]. Simultaneously the effect of sucrose concentration along with the different pH range of rooting medium was studied by supplementing different concentration of sucrose [10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 g/l] and adjusting the medium at different pH levels [3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0].

K. Transplantation

The rooted plants were washed to remove the agar, transferred to soilrite in hycotrays and placed in a green house under high humidity and covered with cling film. After 10 days the cling film was removed and 3 weeks later the plants were transferred to soil in polythene bags and sprayed with a mixture of 0.1% Urea and Bavistin (1:1). After another 4 weeks, the plants were transferred to pots and shifted to a polyhouse. After 3 months of transplantation, the plants were shifted to a shaded area under natural conditions.

III. RESULTS

The present study was conducted to investigate optimal concentrations and combinations of plant growth regulators in the medium for efficient micropropagation in neem. Three different parts of neem including leaves, nodular stem sections and mature embryos (seeds) were used as explants to raise these cultures. The explants were collected from neem plants growing in Biodiesel Park, Gulbarga University, Kalaburagi.

A. Callus Proliferation Response Of Leaf Explants On Different Media

The sterilized leaf explants were inoculated on MS media with 3.0% sucrose and fifteen different concentrations of plant growth regulators of like 2, 4-D, Kn and NAA (Table 2). The data was collected and summarized after four weeks of inoculation. Our results shows that, MS medium supplemented with various hormonal concentrations depicted overall good callusing response between 1.0 to 22%. The highest callusing response 22.3±0.92% was observed in MS medium 13 (supplemented with 0.3 mg/l 2, 4-D; 0.3 mg/l Kn and 0.3 mg/l NAA) followed by 19.21±0.55 and 17.23±1.08 in MS media 14 and 10 respectively as data presented in Fig. 1. It was observed that 2, 4-D and Kn in combination with NAA showed a good callusing response when compared to other combination in MS Media. Similarly, among different aged leaves, young green leaves showed better callus proliferation as compared to older darker green leaves. Similarly, early subculturing also enhanced callusing response, when the leaves were subcultured after 12 to 15 days of inoculation showed better response as compared to 18 to 21 days subculturing after inoculation.

B. Callus Proliferation Response Of Seeds (Mature Embryos) On Different Media

The sterilized mature seeds were inoculated on MS media containing different concentrations of 2, 4-D and BAP separately and in combinations (Table 3). All the hormonal concentrations showed a variable response of callus proliferation from 7.1 to 42.2%. The highest callus proliferation response (42.5±0.97%) was observed in MS medium 10 supplemented with 0.5 mg/l of each 2, 4-D and BAP (Fig. 2), while the lowest callus proliferation response (7.8±0.4%) was observed in MS1 medium supplemented with 0.1 mg/l BAP.

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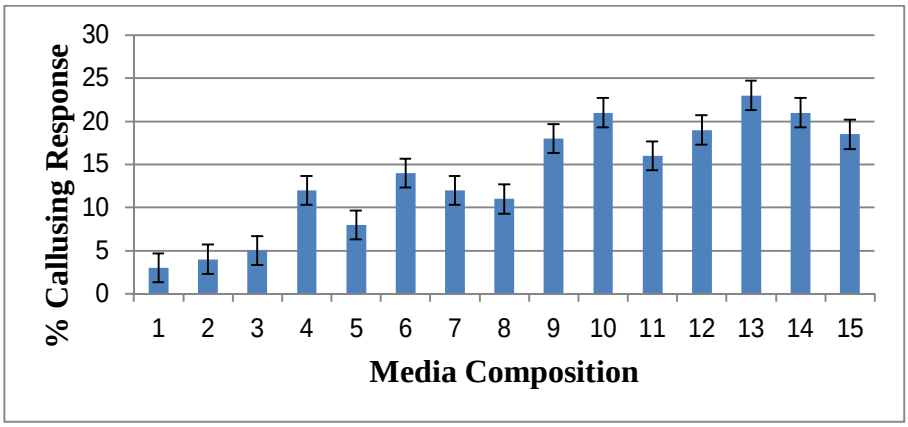


Fig 1: Percentage callusing response of neem leaves inoculated on MS media supplemented with 3.0% sucrose and different concentrations of 2, 4-D, Kn and NAA separately or in combinations (Mean±S.E) ($P \leq 0.05$).

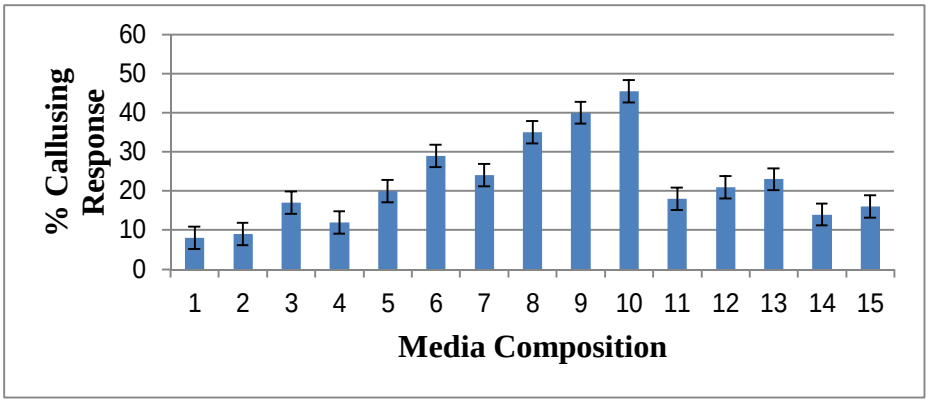


Fig 2: Percentage callusing response of neem seeds (mature embryos) inoculated on MS media supplemented with different concentrations of 2, 4-D and BAP (Mean ±S.E) ($P \leq 0.05$).

C. Shoot Regeneration From Callus

Among the different concentration and combination of BAP, Kn, BAP+NAA and BAP+IBA for the shoot, the highest shoot regenerating capacity (94%) was recorded in 1.0+0.5 mg/l (BAP+NAA) combination. The minimum (32%) shoot regeneration was observed in 1.0 +1.0 mg/l (BAP + NAA) combination. In case of BAP, 1.0 mg/l showed maximum (74%) of shoot production. Whereas the minimum (18%) was noticed in 0.5 mg/l concentration and with Kn the media showed maximum (40%) of shoot production and the minimum (12%) was noticed in 0.5 mg/l concentration. Among the all experimental combination 1.0+0.5 (BAP+NAA) and 1.0+0.5 mg/l (BAP+IBA) showed better performance in neem shoot regeneration. The average number of shoots in BAP+NAA (1.0+0.5 mg/l) was 15.5 and BAP+IBA (1.0+0.5 mg/l) was 6.4 respectively (Fig. 3).

D. Elongation and Multiplication of Shoots

The above shoot regeneration media did not support satisfactory shoot elongation. Therefore, the individual shoots were excised and transferred to MS basal medium or basal medium supplemented with a lower concentration of BAP (0.15 mg/l, 0.225 mg/l) either alone or in combination with an auxin NAA (0.01 mg/l) and/or CH (250 mg/l) or GA3 (0.175 mg/l, 1.0 mg/l). In one of the treatments, MS was tested with GA3 (1.0 mg/l) alone. On all the treatments, yellowing or abscission of leaves was observed with insufficient elongation of shoots. However, pre-culture of shoots for 12 days on MS+GA3 (1.0 mg/l), followed by their transfer to MS+BAP (0.15 mg/l) alone or supplemented with CH (250 mg/l) remarkably improved the shoot elongation. With this procedure, the best shoot growth was observed on MS+BAP (0.15 mg/l). On an average, the original shoots attained a length of 4.5 cm, with 4 nodes per shoot, after 6 weeks. Once the shoots acquired sufficient length, further multiplication was achieved on MS medium supplemented with BAP (0.225 mg/l) and CH (250 mg/l). At the end of the passage, each elongated shoot was cut into single nodal segments and planted on MS+BAP (0.225 mg/l) + CH (250 mg/l). Each node again produced a single, 6 cm long, multimodal shoot, which provided 5-6 cuttings after 6 weeks. Thus, 6-fold shoot multiplication could be achieved in 6 weeks on MS+BAP+CH by cutting the solitary shoot into single node segments and culturing them on fresh medium.

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E. Rooting Of In Vitro Microshoot Culture

For rooting, 3 cm long terminal portions of 6-week-old elongated shoots were cut and transferred to MS medium of different concentration and combination of Auxin, NAA and IBA were found to be a comparatively better response than IAA for producing roots. NAA+IBA combination showed positive results. Best rooting (95%) was observed in ½ strength of MS medium supplemented with 3 mg/l NAA and the highest number of average roots per micro shoots and average length of root were 15 and 4.9 cm. The plantlets with well developed roots were successfully transplanted in soil and the percentage survivability was 70. In case of IBA, the best rooting (88%) was observed in concentration 3 mg/l and highest number of average roots/microshoots were 12.6 and the average length of roots were 3.7 cm. ln combination of NAA+IBA best rooting (85%) was observed at concentration 3.0+1.0 mg/l and the highest number of average roots/microshoots were 14.5 and the average length of roots was 4.1. The poor response of minimum or no development was noticed in media supplement with 0.5 mg/l, 7.0 mg/l of IAA and NAA+IBA with concentration of 0.5+0.5 mg/l and 7.0+1.0 mg/l (Fig. 4).

F. Different Concentration of Sucrose

Neem microshoots originated from callus inducted tissue could be rooted on media containing a wide range [0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 g/l] of sucrose. The maximum percentage (96%) of microshoots rooted was seen in the media supplement with a sucrose concentration of 30 g/l. However the rooting percentage on media containing no sucrose or very high level concentration (70 g/l) of sucrose were lower and showing 20% of microshoots rooted and 15% microshoots rooted in them respectively (Fig. 5).

G. Different pH Level

The pH of the medium may be a limiting factor for growth media, pH was adjusted between 3.0 and 7.0. Between these pH levels, the highest percentage (96%) rooting was recorded on the medium adjusted to pH 5.7 and plants showed poor quality of rooting at pH 3.5, 4.0, 6.5 and 7.0 respectively, no roots were formed at pH 3.0 (Fig. 6).

H. Transplantation

Following the protocol described under Materials and Methods, 40 plants from ½ MS+NAA were transferred out of culture. Of these, 35 plants survived. Thus, transplantation survival of micropropagated plants was 87.5%.

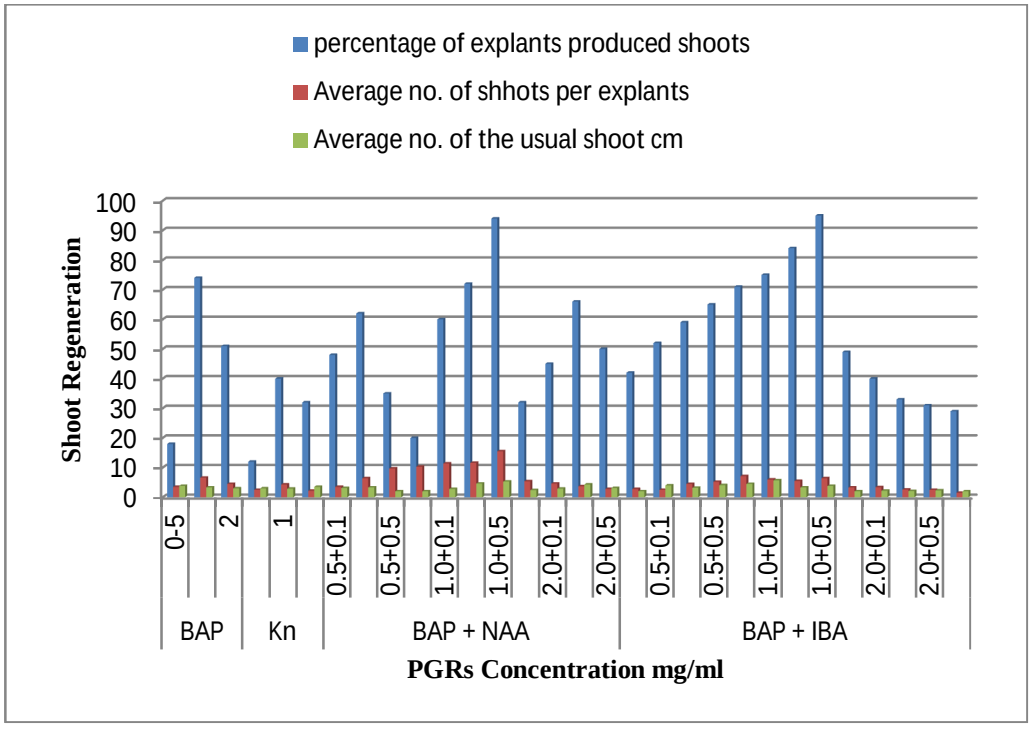


Fig 3: Effect of the cytokinin and auxin at different concentrations and combination in Ms medium on shoot regeneration from the callus tissue of neem.

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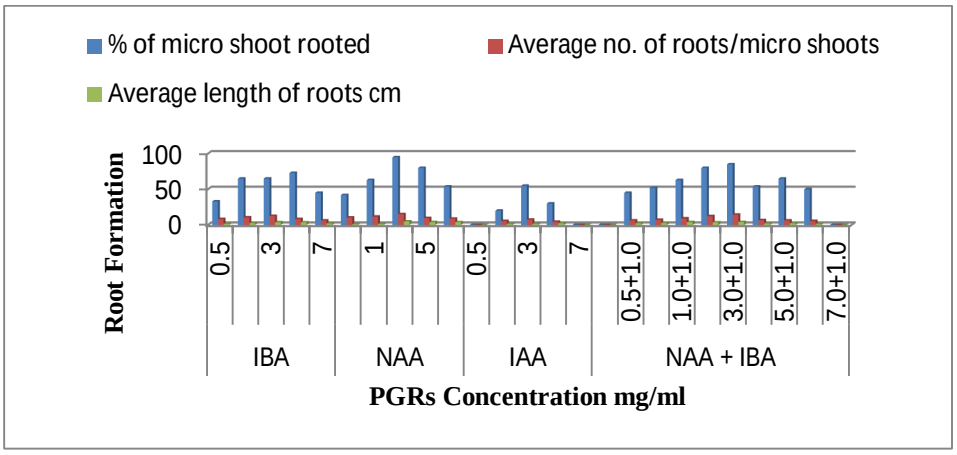


Fig 4: Effect of different concentration of auxin on formation of root on *in vitro* grown micro shoots culture.

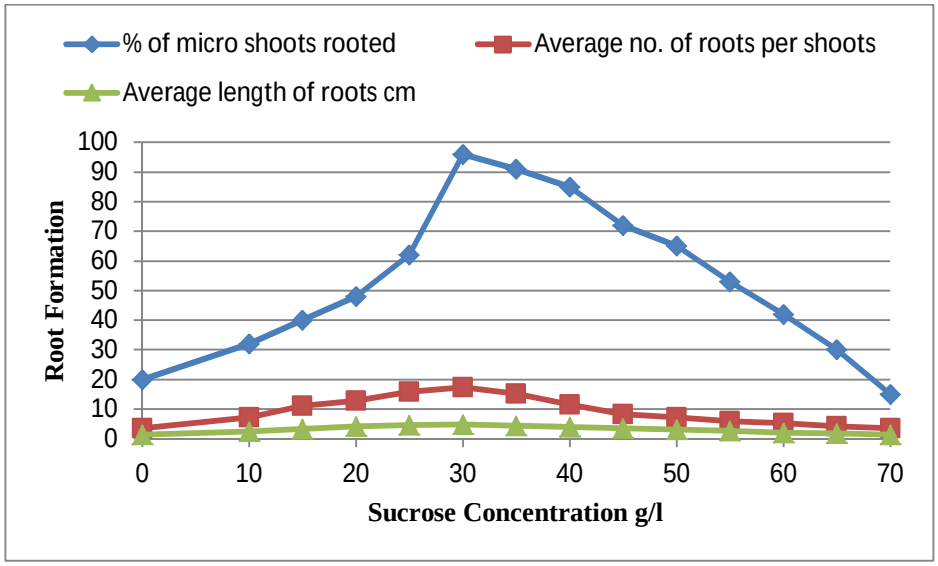


Fig 5: Effect of sucrose concentration on rooting of *in vitro* differentiated shoots.

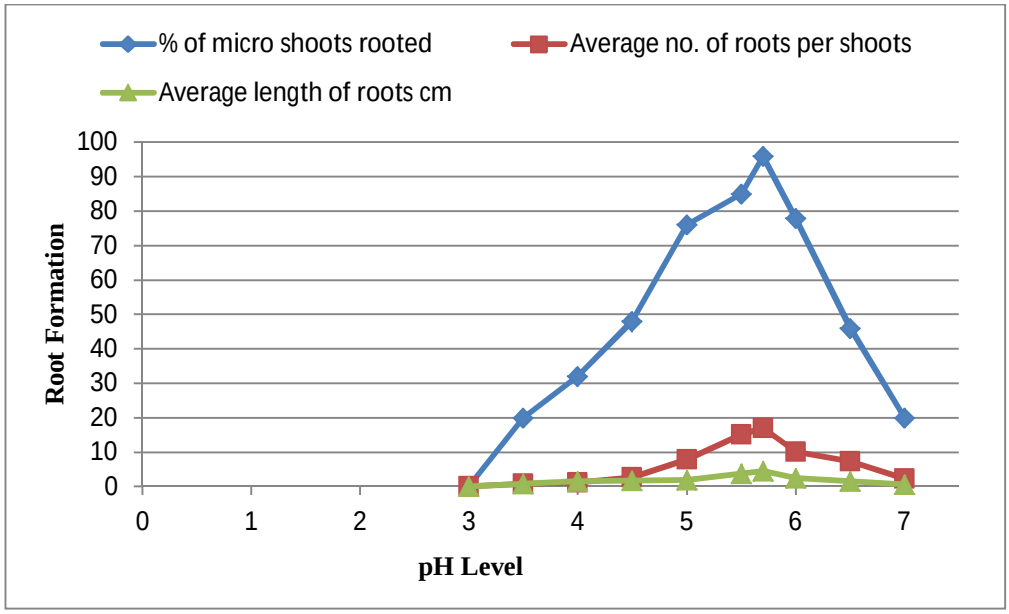


Fig 6: Effect of different pH level on rooting of invitro differentiated shoots.

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IV. DISCUSSION

In the present investigation, callus proliferation from the leaves and seed explant of neem was achieved. The explants were inoculated on MS media supplemented with different concentrations and combinations of 2, 4-D, Kn, NAA and BAP. Both explants showed a variable response of callus proliferation while explant from seed showed the highest callusing response (42.5±0.97%) on MS medium supplemented with 0.5 mg/l of each 2, 4-D and BAP.

Leaf explants showed least callusing response as only about 22.3±0.92% explants were proliferated on MS medium supplemented with 0.3 mg/L 2, 4-D; 0.3 mg/l Kn and 0.3 mg/l NAA. Cultivation of woody plant species under *in vitro* conditions was not successful before 1970. Morphogenesis and tissue culture of the neem was first reported in 1970s (Rangaswamy and Promila, 1972; Sanyal *et al*., 1981). Now number of reports on role of various growth factors in differentiation processes in neem tissue cultures has been published (Ramesh and Padhya, 1990; Rao *et al.*, 1988; Schulz, 1984). Muralidharan and Mascarenhas (1989) were probably first to describe an embryo like structures in cultures of cotyledon segments of neem. At the time of callus culture, the developmental stage of embryos is an important determinant of their morphogenic response (Custers and Bergervoet, 1990). The callus growth from different explants of neem was remarkably affected by type of plant growth regulator and concentration.

However, callus was developed on surface of explants cultured on MS medium supplemented with varying concentrations of either NAA, Kn, 2, 4-D and BAP separately or in combinations. 2, 4-D alone or in combinations with BAP and NAA produced the highest callusing proliferation percentage and callus fresh weight when compared to similar concentrations of other auxins. In plants, the callusing response is genotype specific and each explant has its own callusing potential that can be optimized by changing the concentration and type of plant growth regulators in media.

Many researchers have published reports on callus induction in neem by using different explants. Khalafalla *et al*., (2007) reported that leaf explants on MS basal medium supplemented with 1.0 mg/l of IBA promoted fast growth and produced the highest callusing percentage (92.6%). Schulz (1984) also described callus initiation from young neem leaves and petioles. According to Veeresham *et al*., (1998) modified MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l Kn was found the most suitable media for the flower and leaf callus as 82% and 77.7% callus was produced. The developed callus was light green that turned brown upon subculturing.

Callus formation from neem anthers was achieved through the addition of 21.5 µM NAA and 11.9 µM KIN in MS basal medium. Plant regeneration was best on MS medium with 4.4 µM BA with the addition of 0.25 mg/1 silver nitrate enhancing shoots elongation. Rooting (85%) was achieved on half strength MS medium with 5.7 µM IAA alone, or in combination with 0.046 µM Kn (Dhillon *et al*., 2005). Balaji *et al*., (2003) also established callus cultures from neem petals on MS medium supplemented with 1.0 mg/l NAA, 0.5 mg/L Kn and 3.0% sucrose. Khalafalla *et al*., (2007) found that the increasing sucrose concentration in medium decreased callus induction. The leaf explants were callused in MS medium containing 10 g/l sucrose; the amount of callusing and its appearance were notably better than MS media containing 20 or 30 g/l sucrose.

Su *et al*., (1997) initiated embryogenic callus from cotyledons or hypocotyls on MS medium containing 50 g/l sucrose, 1.0 mg/l BAP, 0.5 mg/l NAA and 1.0 g/l casein hydrolysate. In another study, neem callus induction was obtained at a low sucrose concentration compared with high concentrations (Wewetzer, 1998). Shrikhande *et al*., (1993) described the development of globular and shiny masses of callus from immature cotyledon on MS medium supplemented with 1.0 mg/l BA, 0.5 mg/l NAA, 1.0% w/v CH and 5.0% sucrose. Sanyal *et al*., (1981) also reported that 5% sucrose gave the best callus initiation and growth when neem stem sections were inoculated on MS basal medium supplemented with different combinations of IAA (0.1-0.5 mg/l), BAP (0.1-0.5 mg/l) and sucrose (5%). Chaturvedi *et al*., (2003) obtained the best callus proliferation response from anthers on MS medium supplemented with 9% sucrose, 1.0 µM 2, 4-D, 1.0 µM NAA and 5.0 µM BAP. The percentage of anthers callused and amount of callusing were markedly better than 3.0% or 12.0% sucrose.

In the present investigation, Shoot regeneration was achieved by culturing the callus culture on MS media supplemented with different hormone concentrations of cytokinin (BAP and Kn) and different combination of BAP+NAA and BAP+IBA. Among the all experimental combination 1.0+0.5 (BAP+NAA) and 1.0+0.5 mg/l (BAP+IBA) showed better performance in neem shoot regeneration. The average number of shoots in BAP+NAA (1.0+0.5 mg/l) was 15.5 and BAP+IBA (1.0+0.5 mg/l) was 6.4 respectively. The best shoot development and elongation was observed on MS medium with 1.0+0.5 mg/l (BAP+NAA) and BAP 1.0 mg/l showed maximum (74%) respectively. Rooting (95%) occurred on MS medium with BAP+IBA (1.0+0.5 mg/l) combinations showed maximum production and all plantlets survived 30 days after acclimatization (Foan and Othman 2006).

The plant tissue culture studies carried in present work will help in future on micropropagtion of candidate plus trees and also during genetic engineering work related to crop improvement and to meet the renewable energy demand.

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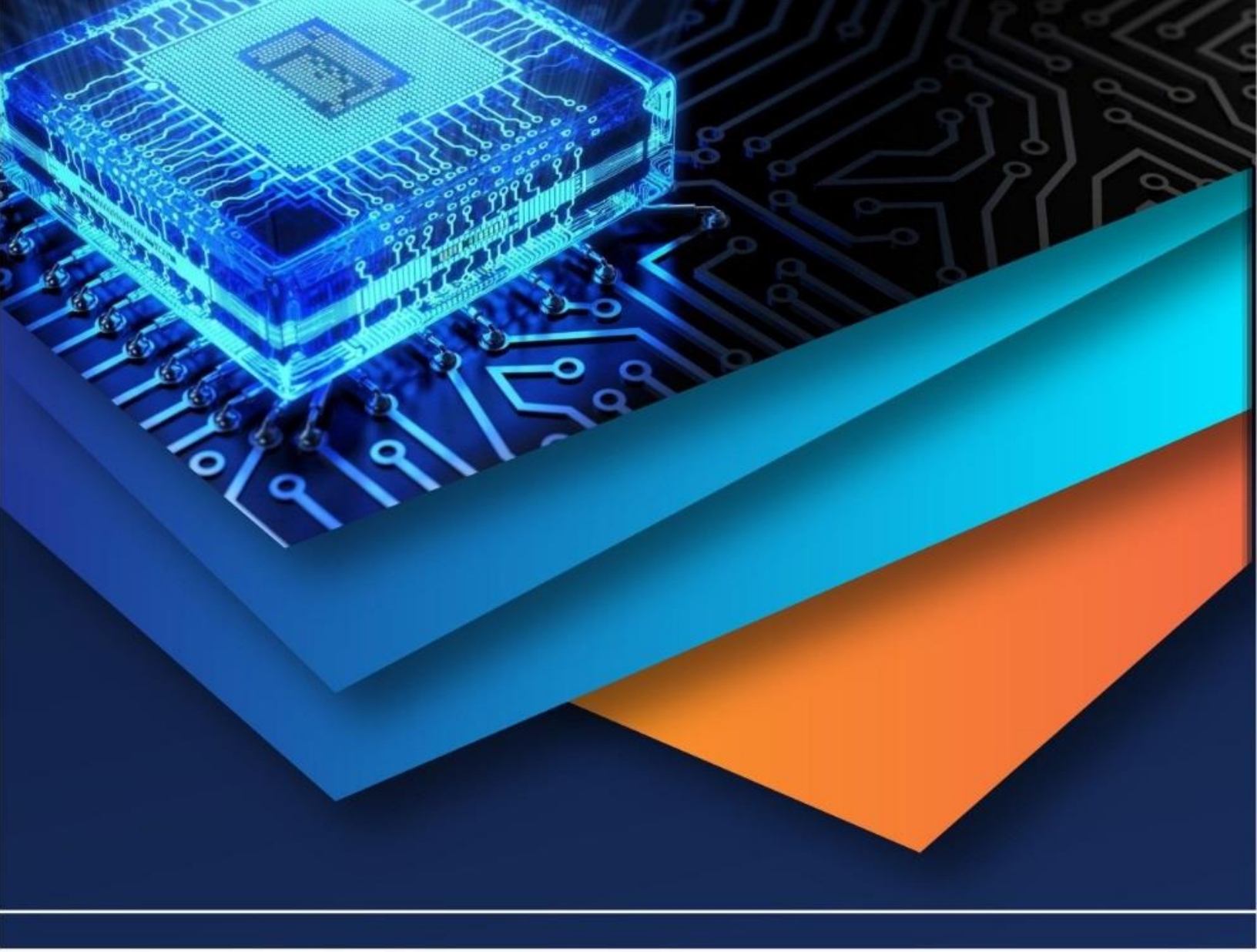
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