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Clonal Multiplication of Multipurpose Desert Tree *Azadirachta indica* - Neem

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Abstract: *Azadirachta indica* A. Juss (Neem) is an untapped natural resource as far as its genetic potential is concerned. It is an important semi-arid tree, which provides fuel wood, shelter, shadow, foliage and a large canopy. It is planted in wind-breaks to protect food crops from the desert winds. It is used in shelterbelt plantations to reduce wind velocity, and helps in ecological restoration and regeneration of mined wastelands. The maximum industrial utilization of neem is for its seed oil, which contains Azadirachtin. Neem trees show considerable variability in the azadirachtin content in seeds irrespective of the habitat. The seeds lose viability within two weeks. Propagation by seed is also undesirable because of the highly heterozygous nature of the plant due to cross-pollination. *In vitro* regeneration of *Azadirachta indica* was achieved through axillary shoot proliferation in nodal segments collected from mature trees. The season and types of explants showed direct influence on bud break. Nodal explants collected during April gave the highest shoot bud sprouting percentage (100%), number of off shoot regeneration (5.65 ± 0.11) and growth of regenerated shoots (5.02 ± 0.27) as obtained on a medium containing 9.00 μM/l BAP + 0.25 μM/l NAA. These *in vitro* regenerated shoots further multiplied on MS medium + 9.00 μM/l BAP + 0.25 μM/l NAA, which resulted in the highest number of off shoots regenerated (9.35 ± 0.55) and growth of regenerated shoots (7.13 ± 0.54). Regenerated shoots were successfully *in vitro* rooted on MS medium + 4.92 μM/l IBA with 100% rooting percentage, 9.50 ± 1.16 root numbers and 5.13 ± 0.18 root length. The *in vitro* rooted plantlets were successfully hardened and acclimatized in a poly house. These plants showed a good survival rate of 95% under field conditions.

Key Words: *Azadirachta indica*, Azadirachtin, *In vitro* culture, Micropropagation

1. Introduction

Neem (*Azadirachta indica*) is a versatile Indian tree of great importance. Neem is an untapped natural resource as far as its genetic potential is concerned. *Azadirachta indica* is one of the most valuable arid zone trees belonging to the family *Meliaceae*. A native of dry forest areas of India and the subcontinent, it is widely cultivated in the arid, nutrient-deficient regions of India and Africa. Neem tree is known to increase soil fertility due to its water holding capacity coupled to its unique property of calcium mining, which changes the acidic soil into neutral. The tree is resistant to high temperatures and drought and has been employed for afforestation of dry localities, reforesting bare ravines and checking soil erosion (Gill *et al.*, 1996). *Azadirachta indica* A. Juss is renowned for its insecticidal properties and Neem seed extracts show great potential as environmentally acceptable bioinsecticides for crop protection (Jacobson, 1988; Schmutterer, 1990) due to the presence of Azadirachtin compound. Vegetative propagation of an adult Neem tree by conventional methods is difficult (Kaushik, 2002). Therefore, it is normally grown from seeds but the seeds are of recalcitrant type; they lose viability within 2-3 weeks (Mohan *et al*., 1996). Propagation by seeds is also undesirable because of the highly heterozygous nature of the plant owing to cross-pollination which brings enormous heterozygosity.

In the present investigation, the main objective was to establish a procedure which can be used routinely to produce complete micropropagated plantlets from nodal explants of mature trees of Neem.

2. Materials and Methods

Plant Materials: four types of nodal explants *i.e.* mature, semi-mature, young and shoot tips were used for the establishment of aseptic cultures. *In vitro* shoots proliferated as a result of axillary bud break were used to regenerate plants.

2.1. Explants preparation and surface sterilization

Under aseptic condition, explants were treated with 0.1% (w/v) Bavestin (BASF India Ltd. Mumbai, India) and 0.1% (w/v) streptomycin for 8-10 min to reduce the chance of fungal contamination. After treatment these explants were rinsed with autoclaved water for 3-4 times and treated with 0.1% (w/v) $HgCl_2$ and streptomycin for 6 min for surface sterilization. After treatment, explants were again rinsed with autoclaved water 3-4 times.

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2.2. Nutrient media and culture conditions

Murashige and Skoog's (MS: Murashige and Skoog, 1962) medium was supplemented with different concentrations of BAP (6-Benzylamino purine) and Kinetin (6-Furfurylaminopurine) and in combination with NAA (Naphthalene acetic acid). 3% sucrose with additives (567.76 µM/l ascorbic acid, 260.24 µM/l citric acid and 484.38 µM/l adenine sulphate) was used in the medium for culture initiation. Agar-agar (Hi-media, India) was added to media as a gelling agent at the concentration of 0.6% (w/v). The pH of the medium was adjusted to 5.8 ± 0.2 with 1N NaOH and the medium was autoclaved at 15 psi and 121°C for 15 min. Culture initiation and multiplication were done aseptically in a laminar air flow hood. Cultures were incubated in tissue culture racks in an aseptic growth room having a temperature of 26 ± 2°C in 16 hours light and 8 hours dark photoperiod and 1600 lux intensity light via white cool florescent tubes (Philips, India).

2.3. Multiplication of micro-shoots

MS medium, supplemented with BAP + NAA and additives (567.76 µM/l ascorbic acid, 260.24 µM/l citric acid and 484.38 µM/l adenine sulphate), was used for multiplication of microshoots.

2.4. *In vitro* rooting of micro-shoots

In vitro regenerated micro-shoots were harvested from clumps and transferred to a medium for rooting. Individual micro-shoots were transferred to MS medium enriched with IBA + additive (86.85 µM/l Proline and 48.96 µM/l Tryptophan) with sucrose (3%). The *in vitro* rooted micro-shoots were removed from culture vessels and washed with autoclave RO water to remove adhered nutrient agar. These were carefully transferred to jam bottles containing autoclaved vermiculite moistened with ½ MS salts. The bottles were kept for 2 weeks for *in vitro* hardening under growth room conditions. After 2 weeks, jam bottles containing regenerated plants were kept in a poly house initially near the pad section (RH 80 - 90% and temperature 28 ± 2°C) in order to harden the plantlets.

2.5. Hardening and acclimatization of regenerated plantlets

After one week of transfer of *in vitro* raised plantlets to the poly house, the caps of jam bottles were gradually opened over a period of 2 weeks and finally removed. Bottles containing plantlets were then shifted from the pad section towards the fan section to provide growing conditions of low humidity (50 - 60%) and high temperature (30 ± 2°C). After 4 weeks, acclimatized plantlets were transferred to poly bags containing a mixture of soil + sand + FYM (2:1:0.5). Such plantlets were kept in the poly house for 3 - 4 weeks and then shifted to an agro-shade house and later to the open environment.

Table 1. Effect of cytokinin (BAP) and auxin (NAA 0.25 µM/l) on shoot bud proliferation of *Azadirachta indica*.

BAP (µM/l)	Shoot bud Sprouting %	Number of offshoots regenerated	Growth of regenerated shoots (cm)
0	25.00 (29.73)c	1.40±0.24d	0.82±0.14c
0.5	40.00 (38.95)c	2.50±0.19cd	2.05±0.25b
2.25	55.00 (47.89)c	3.09±0.49c	2.81±0.28b
4.5	80.00 (66.59)b	4.63±0.34ab	3.99±0.01a
9	100.00 (88.72)a	5.65±0.11a	5.02±0.27a
18	80.00 (69.75)b	4.44±0.48b	3.98±0.47a
Mean	63.33 (56.94)	4.20±0.21	3.67±0.19
F- value	15.09*	14.39*	16.18*

2.6. Experimental design and statistical analyses

Data were analysed using a statistical package for social sciences (SPSS 8.0). Duncan Multiple Range Test (DMRT) P = 0.05 was used to compare the means from main effects. A minimum of 4 replicates with 5 samples (one explant) were taken per treatment and with repetition of experiments thrice. Degree of variation was shown by mean and standard error. Data given in percentages were subjected to arcsine $\sqrt{X}$) transformation (Snedecor and Cochran, 1967) before statistical analysis.

3. Results and Discussion

In the present investigation, axenic cultures of *Azadirachta indica* were established from nodal segments derived from mature trees. The explants harvested during April were found most suitable for culture establishment. One way analysis of variance (ANOVA) showed that BAP + NAA had a highly significant effect on shoot bud sprouting percentage (F_5 = 15.09, p<0.05), number of off shoots regenerated (F_5 = 14.39, p<0.05) and growth of regenerated shoots (F_5 = 16.18, p<0.05). In **Table 1** it is shown that the different concentration of BAP in combination with NAA had a significant effect on shoot bud sprouting percentage, number of off shoots regenerated and growth of regenerated shoots. The highest shoot bud sprouting percentage (100%), number of off shoot regenerated (5.65 ± 0.11) and growth of regenerated shoots (5.02 ± 0.27) were obtained with the treatment of 9.00 µM/l BAP + 0.25 µM/l NAA. These *in vitro* regenerated micro shoots were further amplified on BAP + NAA. **Table 2** shows that BAP + NAA had a highly significant effect on number of off shoots regenerated (F_5 = 35.30, p<0.05) and growth of regenerated shoots (F_5 = 24.68, p<0.05).

Table 2. Effect of cytokinin (BAP) and auxin (NAA) on shoot multiplication of *Azadirachta indica*.

BAP (µM/l)	NAA (µM/l)	Number of offshoots regenerated	Growth of regenerated shoots (cm)
0	0	1.73±0.19d	1.06±0.15d
0.5	0.25	3.10±0.41c	2.36±0.35c
2.25	0.25	3.69±0.43bc	3.14±0.33bc
4.5	0.25	4.95±0.28b	4.18±0.20b
9	0.25	9.35±0.55a	7.13±0.54a
18	0.25	4.83±0.51b	4.11±0.48b
Mean		5.12±0.31	4.09±0.26
F- value		35.30*	24.68*

Table 3. Effect of IBA on *in vitro* rooting of *Azadirachta indica*.

IBA (µM/l)	Rooting %	Number of Roots	Mean root length (cm)
0	35.00 (36.06)d	2.14±0.14c	1.04±0.17d
0.49	45.00 (41.83)cd	2.67±0.29bc	2.12±0.43cd
2.46	70.00 (60.26)bc	3.21±0.24bc	2.65±0.43bc
3.94	85.00 (69.76)ab	5.00±0.48b	3.44±0.41b
4.92	100.00 (88.72)a	9.50±1.16a	5.13±0.18a
9.84	70.00 (57.39)bv	4.79±0.48b	3.09±0.39bc
Mean	67.50 (59.00)	5.26±0.43	3.31±0.20
F- value	7.42*	13.16*	13.60*

The different concentrations of BAP in combination with NAA had a significant effect on number of off shoots regenerated and growth of regenerated shoots. Highest number of off shoot regeneration (9.35 ± 0.55) and growth of regenerated shoots (7.13 ± 0.54) were obtained on medium treated with 9.00 µM/l BAP + 0.25 µM/l NAA. For rooting of *in vitro* regenerated shoots, IBA was found to be the most suitable for root induction. The individual *in vitro* shoots regenerated were tested for their ability to form roots. The rooting treatment consisted of MS medium supplemented with IBA. One way analysis of variance (ANOVA) showed that the IBA treatment resulted in highly significant affect on rooting % (F_5 = 7.42, P<0.05), root number (F_5 = 13.16, P<0.05), and mean root length (F_5 = 13.60, P<0.05). The results showed that different concentrations of IBA significantly affect the plant regeneration parameters, rooting %, root per shoots, and mean root length. The optimal rooting of *in vitro* shoots was obtained on the medium with 4.92 µM/l IBA resulting in 100% rooting percentage, 9.50 ± 1.16 root number and 5.13 ± 0.18 root length (**Table 3**). Plantlets with well developed shoot and root systems were transferred to soil after hardening and acclimatization. Establishment of aseptic cultures of Neem was not possible round the year since significant seasonal fluctuation affected the explant viability in culture.

Season of collection of explants is one of the important factors in the establishment and growth of *in vitro* cultures (Siril and Dhar, 1997). Explants collected during March-April responded best in culture as during this period of the year, not only maximum bud-break leading to regeneration of shoots was obtained, but the infection percentage was also low. To be more specific, mid-April was the best time for explant collection for obtaining maximum bud-break which is about 80% with minimum infection, *i.e.*, 20%. Similar results were obtained by Chaturvedi *et al*. (2004) in Neem. BAP was found to be most effective cytokinin in inducing multiple shoot formation. Similar findings have been reported by Arya *et al*. (1995), Chaturvedi *et al*. (2004) and Quraishi *et al*. (2004). IBA has also been reported suitable for rooting in neem by several workers (Eeswara *et al*., 1998; Salvi *et al*., 2001; Quraishi *et al*., 2004). The present work exhibits superiority over *in vitro* shoot multiplication rate and *in vitro* rooting without callus formation which was a bottle neck in all earlier work reported.

4. Conclusion

A reproducible protocol is developed for the regeneration of complete plants from nodal segments containing axillary buds. The Neem plant growth and development was remarkably affected by growth regulator type and concentration as described in the protocol.

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