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Full Length Research Paper

In vitro clonal propagation of the neem tree (*Azadirachta indica* A. Juss.)

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A study was conducted with root and shoot tip explants of neem to develop an efficient protocol of regeneration. Shoot tips and root tips from 10 - 20 days old seedlings of neem were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations and combinations of BAP (0.0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg l^{-1}) and NAA (0.0 and 0.05 mg l^{-1}). Shoot buds were initiated from the both explants. The highest percentage of regeneration was found from shoot tip but the highest average number of shoots/explants was found from root tip explants. The regenerated shoots were further subcultured and later could be rooted on a medium supplemented with different concentrations and combinations of IBA and IAA and complete plants could be obtained. The rooted plants were transplanted to pots for hardening.

Key words: Neem, root tip, shoot tip, explants, indole-butyric acid, indole-3-acetic acid.

INTRODUCTION

Neem, *Azadirachta indica* A. Juss., a member of the Meliaceae Family, is indigenous to Southern Asia (Akula et al., 2003). The neem tree is a multipurpose timber tree from which high value products are extracted for use as insecticides, fertilizers and multipurpose medicines. In India, neem is popularly known as the village dispensary (Akula et al., 2003). Azadirachtin, a limonoid (tetranotriterpenoid), is the main insecticidal constituent of neem. It occurs in all parts of the neem tree, but is concentrated in the seed kernel. The commercial potential of this tree has renewed worldwide research interest in neem. These trees are now being introduced to other tropical and subtropical regions of the globe, including Australia. More land is being brought under commercial plantations of neem. Hence, the demand for elite clonal planting stock is increasing.

The recent approval of neem as a botanical insecticide in the USA has prompted industrialists and researchers to investigate several aspects of this crop and its products. Neem seeds are the main source of neem oil and azadirachtin, the principle active constituent. A single tree will yield an average of about 20.5 kg of fruit per year (NRC, 1992). Approximately 40% of the dry weight of neem seed kernels consists of oil, which is the source of azadirachtin (Isman et al., 1990). However, comercialization of neem products (oil and azadirachtin) is greatly hindered by the limitation of natural resources (Isman, 1997). Multiplications of shoots are considered to be artificial seeds, similar to zygotic embryos and may likely contain azadirachtin. Thus multiplications of shoots possibly serve as an alternative source of neem oil and azadirachtin production, by passing our dependence on field trees. Neem has been conventionally propagated by seed. Depending on the climatic conditions, seed is set within 5 - 7 years; however, it is recalcitrant in nature and rapidly loses viability. Furthermore, the heterozygous nature of neem due to cross-pollination presents a problem when selecting uniform, high-yielding (azadirachtin) and fast-growing trees from seedlings. Tissue culture methods for genetic improvement, rapid propagation, and clonal propagation of genotypes producing high levels of azadirachtin offer obvious advantages over propagation by seed. Several studies have been undertaken to develop tissue culture protocols for neem. *In vitro* multiplication of neem was achieved by using nodal segments (Drew, 1993; Yasseen, 1994) and leaf explants

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Abbreviations: BAP, 6-benzyl amino purine; NAA, α-naphthalene acetic acid; IBA, indole-butyric acid; IAA, indole-3-acetic acid.

Table 1. Main effect of explants and different concentrations and combinations of hormones on shoot induction.

Treatment	% Shoot initiation	Number of shoots per		Shoot length (cm)		Days required for shoot initiation
		Explants	Regenerated shoot	50 days	70 days	
Explants						
Shoot tip	72.89a	2.18	2.21	0.89	2.23	22.46b
Root tip	51.96b	2.64	2.93	0.48	1.93	30.29a
Hormone						
T_0	0.00	0.00	0.00	0.00	0.00	0.00
T_1	70.63	3.23	2.88	0.98	2.79	29.88
T_2	75.13	2.88	3.25	0.75	2.73	32.00
T_3	79.38	3.36	3.38	0.81	2.77	30.75
T_4	73.13	3.13	3.29	0.93	2.45	31.21
T_5	69.38	2.24	2.87	0.75	2.11	30.13
T_6	69.30	2.00	2.25	0.72	1.71	30.63
$LSD_{0.05}$	5.615	0.9216	0.8608	0.0896	0.5452	2.882

T_0 = 0.0 mg l^{-1} BAP + 0.0 mg l^{-1} NAA, T_1 = 0.1 mg l^{-1} BAP + 0.05 mg l^{-1} NAA, T_2 = 0.2 mg l^{-1} BAP + NAA 0.05 mg l^{-1}NAA, T_3 = 0.3 mg l^{-1} BAP + 0.05 mg l^{-1} NAA, T_4 = 0.4 mg l^{-1} BAP + 0.05 mg l^{-1} NAA, T_5 = 0.5 mg l^{-1} BAP + 0.05 mg l^{-1} NAA, and T_6 = 0.6 mg l^{-1} BAP + 0.05 mg l^{-1} NAA.

(Eeswara et al., 1998). Plant regeneration in neem was reported from callus derived from leaves (Narayan and Jaiswal, 1985), anthers (Goutam et al., 1993) and cotyledons (Muralidharan and Mascarenhas, 1989). Roots could be an excellent source of explant for rapid multiplication of neem through cyclic and continuous production of seedlings. However, use of roots as explant has rarely reported. In this paper, regeneration efficiency of shoot tip and root tip explants has been discussed for rapid multiplication of neem.

MATERIALS AND METHOD

Immature fruits of *A. indica* were collected from 30 years old tree grown in Bangladesh Agricultural University Campus. Freshly collected immature fruits were washed thoroughly with distilled water and also surface sterilized by 0.1% $HgCl_2$ for 5 min followed by washing with sterile water for five times. Excised cotyledons were then aseptically incubated in flasks containing agar gelled MS medium. After 30 to 40 days root tip and shoot tip of seedlings were dissected out and cultured on MS medium supplemented with various concentrations and combinations of 6-benzyl aminopurine (BAP) and naphthalene acetic acid (NAA) for shoot initiation. The culture vials were transferred to growth room and were allowed to grow in controlled environment. The temperature of the growth room was maintained with in 25±2°C by an air conditioner. A 16 h light period was maintained with light intensity of 2000 lux of the growth and development of culture. The primary shoots from shoot tip and root tip explants cultured on MS medium incorporated with 0.1 - 0.6 mg l^{-1} BAP and 0.05 mg l^{-1} NAA and thereafter regenerated microshoots were excised several times and cultured on different media. For successful rooting, 3 - 4 cm usable shoots excised from multiplication culture were implanted to rooting medium consisting of $^1/_2$ MS medium supplemented with IBA and/or IAA.

Transplantation

One-month-old adequately rooted shoots when reached a height of 4 - 5 cm were brought out from culture room and kept in room temperature for 7 days. Plantlets were then taken out from vial and agar was washed out gently from roots. These plantlets were transplanted in small pots contained compost, soil and sand (1:2:1) and were covered with transparent sheets. They were nurtured under room temperature in presence of sufficient light. An average days nursing under room condition enabled them to adapt in outdoor conditions. In that conditions the plantlets were watered every alternate day.

RESULTS AND DISCUSSION

Shoot proliferation

In this experiment, the effects of explants and different concentrations and combinations of BAP and NAA on shoot proliferation of neem were studied. *In vitro* produced explants (shoot tip and root tip) were used in this experiment.

Shoot induction

Shoot initiation was significantly influenced by explants used in the experiment. Shoot tip explants showed higher shoot initiation (72.89%) than the root tip explants (51.96%) (Table 1). BAP and NAA combinations exhibited significant influence on the percentage of shoot initiation. The highest percentage (79.38) of shoot initiation was observed in T_3 and the lowest percentage (69.30) was found in T_6. No shoot initiation occurred when BAP and NAA were omitted from medium (Table 1).

A significant difference was recorded from the combined effect of explant and BAP on per cent shoot initiation. The highest percentage (94.00) was found in shoot tip explant of T_3 and the lowest percentage (53.75) was observed from root tip explant in T_6 (Table 2). A similar result (from shoot tip explant) was reported by Rahima

Table 2. Combined effect of explants and different concentrations and combinations of hormones on shoot development.

Explants	Hormone conc. (mg/L)	% Shoot initiation	Number of shoots per		Shoot length (cm)		Days required for shoot initiation
			Explants	Regenerated shoot	50 DAI	70 DAI	
Shoot tip	T_0	0.00	0.00	0.00	0.00	0.00	0.00
	T_1	83.75	3.24	3.09	1.35	3.40	24.25
	T_2	91.25	2.73	2.76	1.03	2.86	28.75
	T_3	94.00	3.50	3.25	0.90	3.03	26.75
	T_4	85.00	2.26	2.20	1.20	2.53	25.75
	T_5	81.25	2.10	2.50	0.90	1.98	26.25
	T_6	75.00	1.50	2.10	0.88	1.80	25.50
Root tip	T_0	0.00	0.00	0.00	0.00	0.00	0.00
	T_1	57.50	3.23	2.72	0.78	2.73	34.25
	T_2	56.25	3.11	3.76	0.47	2.05	35.25
	T_3	67.50	4.00	4.75	0.71	2.51	34.75
	T_4	61.25	3.25	3.48	0.67	2.37	36.75
	T_5	57.50	2.49	3.21	0.55	2.25	34.35
	T_6	53.75	2.49	2.50	0.42	1.61	37.00
	$LSD_{0.05}$	5.615	0.9216	0.8608	0.0896	0.5452	2.882

T_0 = 0.0 mg l^{-1} BAP + 0.0 mg l^{-1} NAA, T_1 = 0.1 mg l^{-1} BAP + 0.05 mg l^{-1} NAA, T_2 = 0.2 mg l^{-1} BAP + NAA 0.05 mg l^{-1}NAA, T_3 = 0.3 mg l^{-1} BAP + 0.05 mg l^{-1} NAA, T_4 = 0.4 mg l^{-1} BAP + 0.05 mg l^{-1} NAA, T_5 = 0.5 mg l^{-1} BAP + 0.05 mg l^{-1} NAA, and T_6 = 0.6 mg l^{-1} BAP + 0.05 mg l^{-1} NAA.

et al. (1998).

Number of shoots/explant

The number of shoots produced per explant varied with the type of explants and different combinations of BAP and NAA. Data were recorded at 50 DAI from explant and 70 DAI from the cultured regenerated shoots. Number of shoots was influenced by explants used in the experiment. Root tip explant showed higher number of shoot (2.64) compared to shoot tip explant (2.18) both at 50 and 70 DAI (Table 1). The effects of BAP and NAA on shoot proliferation were significant. The results showed that the highest number of shoots (3.36/explant at 50 DAI; 3.38/explant at 70 DAI) was proliferated by T_3 and the lowest (2/explant at 50 DAI and 2.25 at 70 DAI) was observed in T_6. The combined effect of explants and different combinations of BAP and NAA on shoot prolife-ration has been presented in Table 2. Among the combinations, T_3 showed the highest shoot (4.0) prolife-ration from root tip explant (Figures 1a and b) followed by 3.5 in shoot tip explant (Figure 1c and d). The lowest (1.5) number of shoots was recorded in T_6 from shoot tip explants. Regenerated shoots from root tip also showed the highest (4.75) initiation in T_3 followed by shoot derived shoots (3.25) (Table 2). Similar number of shoots/root tip was reported by Salvi et al. (2001).

Shoot length

The length of shoots was influenced by explants used in the experiment. Shoot tip explants showed higher length than the root tip explants both at 50 and 70 DAI (Table 1). The effects of different concentrations and combinations of BAP and NAA on shoot length were found significant. The results showed that the highest shoot length (0.98 cm) was found by T_1 at 50 DAI and the lowest was 0.72 cm by T_6. At 70 DAI, the highest shoot length (2.79 cm) was also found by T_1 and the shortest length (1.71 cm) by T_6 (Table 1). From the combined effect of different explants and different concentrations and combinations of BAP and NAA on shoot elongation it was found that, T_1 showed the highest shoot increment (3.35 cm) in shoot tip explant and the lowest (0.42 cm) was found by T_6 in root tip explant at 50 DAI. At 70 DAI, the highest shoot increment (3.40 cm) was observed in T_1 from shoot tip explant while the shortest shoot (1.61 cm) was found in T_6 from root tip explant. In this parameter shoot tip explants performed better than root tip explant.

Days required for shoot initiation

Cultured explants were observed in regular basis for data collection on days required for shoot regeneration. The explants showed significantly different effects on days required for shoot induction. Table 1 clearly shows that shoot tip explants performed better and needed least number of days (22.46) for shooting. The root tip explants needed relatively higher number of days (30.29) for shoot regeneration. Days required for shoot induction was significantly influenced by BAP and NAA. The least num-ber of days required for shoot induction was 29.88 in T_1.

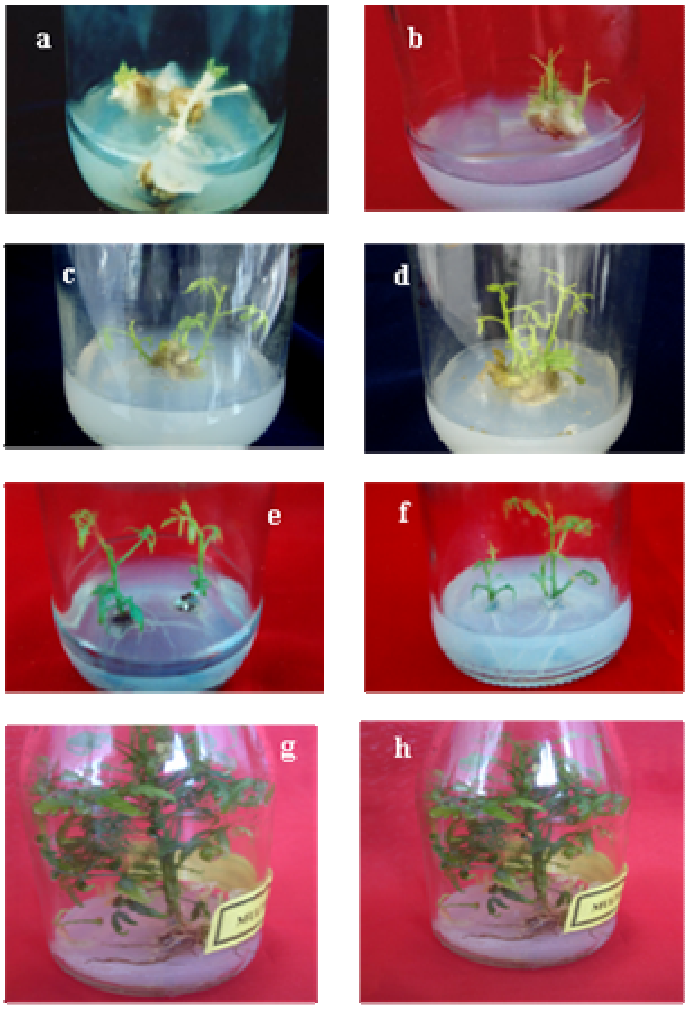

Figure 1. Plant regeneration in *A. indica*. **a.** Multiple shoots on MS + 0.3 mg l^{-1} BAP + 0.05 mg l^{-1} NAA from root tip explant at 50 DAI. **b.** Multiple shoots on MS + 0.3 mg l^{-1} BAP + 0.05 mg l^{-1} NAA from root derived shoot at 70 DAI. **c.** Multiple shoots on MS + 0.3 mg l^{-1} BAP + 0.05 mg l^{-1} NAA from shoot tip explant at 50 DAI. **d.** Multiple shoots on MS + 0.3 mg l^{-1} BAP + 0.05 mg l^{-1} NAA from shoot derived shoot at 70 DAI. **e.** Root development from shoot tip derived shoot on MS + 0.05 mg l^{-1} IBA + 0.05 mg l^{-1} IAA at 60 DAI. **f.** Root development from root tip derived shoot on MS + 0.05 mg l^{-1} IBA + 0.05 mg l^{-1} IAA at 60 DAI. **g.** Root development from shoot tip derived shoot on MS + 0.05 mg l^{-1} IBA + 0.05 mg l^{-1} IAA at 90 DAI. **h.** Root development from root tip derived shoot on MS + 0.05 mg l^{-1} IBA + 0.05 mg l^{-1} IAA at 90 DAI.

No shoots regenerated when the medium lacked of BAP and NAA (Table 1). The combined effect of explants and different concentrations of BAP and NAA on days required for shoot initiation has been presented in Table 2. Among the concentrations, T_1 needed the least days (24.25) for shoot formation from shoot tip explant. In case of root tip explants, the minimum days required for shoot initiation was 34 with the same concentration of BAP and NAA (Table 2).

Rooting

The regenerated shoots were then subcultured on half strength MS medium supplemented with different levels of IBA (0.0, 0.05, 0.1 and 0.2 mg l^{-1}) and IAA (0.00, 0.05, 0.3 and 0.4 mg l^{-1}) in order to allow root formation. Root development varied with combinations of IBA and/or IAA (Figures 1e, f, g and h).

Percentage of rooting

Roots were developed from the shoots and the percentage was calculated. There was no significant difference between the two types of explants as was revealed from the data recorded on 30 DAI. The effects of different concentrations of IBA and/or IAA on root initiation were significant (Table 3). The highest percentage of rooting (85.88) was achieved with IBA at 0.05 + IAA at 0.05 mg l^{-1} and the lowest percentage (79.87) was found with IBA at 0.1 mg l^{-1}. The combined effect of explants and hormone was also highly significant. The higher percentage of rooting (94.25) was observed with IBA at 0.5 mg l^{-1} and IAA at 0.05 mg l^{-1} in shoot tip explants followed by 93.53 in root tip explants and the lowest rooting percentage (79.25) was found with IBA at 0.1 mg l^{-1} in shoot tip explant followed by 80.50 in root tip explant (Table 4). Rooting shoots from various explants of neem was reported to be poor (40%) (Salvi et al., 2001). We found 94.25% rooting. However similar rooting frequency was also reported by Rahima et al. (1998).

Number of roots/shoot

There was no significant variation on explants for the number of roots/shoot. But numerically root tip explants produced higher number of roots (5.0) than shoot tip explants (4.82) at 60 DAI (Table 3). A significant difference was recorded on the number of roots/shoot due to the effect of IBA and/or IAA. The highest number of roots/shoot (3.75) was found in the combination of IBA at 0.05 mg l^{-1} and IAA at 0.05 mg l^{-1} at 30 DAI followed by 9.88 at 60 DAI. The lowest number of roots (1.88) was obtained with 0.3 mg l^{-1} IAA at 30 DAI followed by 3.13 at 60 DAI (Table 3). Number of roots/shoot was significantly influenced by the combined effect of explant and hormones. The highest number of roots/shoot (4.25) was recorded from root tip derived shoots with the combination of 0.05 mg l^{-1} IBA and 0.05 mg l^{-1} IAA, which was followed by shoot tip explants (4.00) with the same concentration of IBA and IAA at 60 DAI. The lowest number of roots/shoot (1.75) was recorded both from root tip and shoot tip explants with the treatment of 0.3 mg l^{-1} IAA at 30 DAI. At 60 DAI, the highest number of roots (10.25) was observed from root tip explant with IBA at 0.05 mgl^{-1} and IAA at 0.05 mgl^{-1} followed by 9.50 from

Table 3. Main effect of explants and different concentrations and combinations of hormones on root development.

Treatment	% Root induction	Roots/shoot		Root length (cm)		Days required for root initiation
		30 DAI	60 DAI	30 DAI	60 DAI	
Explants						
Shoot tip	69.50	2.32	4.82	1.92	4.61	12.39
Root tip	70.21	2.39	5.00	1.94	4.54	12.71
Hormone						
0.00	0.00	0.00	0.00	0.00	0.00	0.00
IBA 0.1	79.87	2.76	5.25	2.35	4.37	13.50
IBA 0.2	82.12	2.48	4.72	2.55	4.55	15.62
IAA 0.3	80.13	1.88	3.13	1.58	3.63	15.47
IAA 0.4	81.00	2.00	3.52	1.52	3.58	16.23
IBA 0.05 + IAA 0.05	85.88	3.75	9.88	2.74	7.32	14.38
IBA 0.1 + IAA 0.05	83.13	3.63	7.88	2.76	8.55	12.75
LSD at 0.05%	3.138	0.8467	0.7170	0.1002	0.1953	2.013

Table 4. Combined effect of explants and different concentrations and combinations of hormones on root development.

Explants	Hormone concentration (mgL^{-1})	% Root induction	Root length (cm)		Roots/plantlet		Days required for root initiation
			30 DAI	60 DAI	30 DAI	60 DAI	
Shoot tip	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	IBA 0.1	79.25	2.38	4.31	2.50	4.75	13.48
	IBA 0.2	87.72	2.49	4.54	2.75	4.50	14.73
	IAA 0.3	80.20	1.61	3.53	1.75	3.25	17.10
	IAA 0.4	80.73	1.48	3.50	2.00	3.50	15.4 0
	IBA 0.05 + IAA 0.05	94.25	2.72	7.61	4.00	9.50	13.72
	IBA 0.1 + IAA 0.05	84.25	2.73	8.74	3.25	8.25	12.75
Root tip	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	IBA 0.1	80.50	2.33	4.44	3.00	5.75	13.51
	IBA 0.2	84.25	2.61	4.55	2.25	5.00	16.45
	IAA 0.3	80.00	1.55	3.73	1.75	3.45	14.20
	IAA 0.4	81.26	1.55	3.65	2.25	3.50	17.24
	IBA 0.05 + IAA 0.05	93.53	2.75	7.03	4.25	10.25	15.30
	IBA 0.1 + IAA 0.05	82.11	2.79	8.37	3.25	7.50	12.75
	LSD at 0.05%	3.138	0.847	0.717	0.10	0.195	2.013

shoot tip explant with the same concentrations of IBA and IAA. The lowest (3.25) was found in shoot tip explant followed by 3.45 in root tip explant with IBA at 0.3 mgl^{-1}.

Root length

No significant variation on the root length was observed among the plantlets. Data were recorded at 30 and 60 DAI and the results have been presented in Table 3. The effects of IBA and/or IAA on root increment were found significant. The result showed that, IBA at 0.1 mg l^{-1} and IAA at 0.05 mg l^{-1} gave the highest root length (2.76 cm) at 30 DAI and the lowest was 1.52 cm with the IAA concentration of 0.4 mg l^{-1}. After 60 days the highest length of root (8.55 cm) was obtained in the combinations of IBA at 0.1 mg l^{-1} and IAA at 0.05 mg l^{-1}. The lowest (3.58 cm) root length was obtained with 0.4 mg l^{-1} IAA alone. No rooting took place in hormone free media (Table 3). The combined effect of explants and different

concentrations of IBA and/or IAA on root increment have been presented in Table 4. Among the hormone concentrations, IBA at 0.1 mgl^{-1} + IAA at 0.05 mgl^{-1} showed the highest root length (2.73 cm) from shoot tip explant followed by 2.79 cm long root form root tip explant at 30 DAI. At 60 DAI, the highest root length (8.74 cm) was observed from shoot tip explant with the same concentration of IBA and IAA followed by 8.3 cm from root tip explant. The lowest (1.48 cm at 30 DAI and 3.5 cm at 60 DAI) root length was found in shoot tip explants with IAA at 0.4 mg l^{-1} IAA followed by 1.55 cm at 30 DAI and 3.65 cm at 60 DAI in root tip explant with the same concentrations of IAA (Table 4).

Days required for root initiation

There was no statistical difference between the number of days required for rooting of shoots derived from shoots and roots. But numerically, shoot tip derived shoots were more responsive to root initiation (Table 3). The effects of IBA and IAA on days required for rooting was significant. Minimum number of days (12.75) for rooting was recorded with IBA at 0.1 mg l^{-1} + IAA at 0.05 mg l^{-1} (Table 3). The combined effect of explant and hormone was significant on days required for rooting. Least days (12.75) was needed for the combination of IBA at 0.1 mg l^{-1} and IAA at 0.05 mg l^{-1} irrespective of the explant type (Table 4).

Conclusion

The present experiments have shown that it is possible to induce shoot differentiation and complete plantlet development from root tip and shoot tip explants of neem. Though shoot tip explants showed better performance in case of percent shoot induction and shoot length with minimum days but root tip explants produce the highest number of shoots that provided large number of plantlets in a short time. Also root derived shoots produced the highest number of roots per plantlet. So, root tip explants are best for multiplication of neem. Among hormone concentrations, T_3 is best for shoot induction and multiplication and IBA at 0.05 mg l^{-1} + IAA at 0.05 mg l^{-1} are best for rooting.

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