

## Role of tryptophan on *in vitro* rooting in microshoots of *Azadirachta indica* A. Juss (Neem)

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### Abstract

Development of the root is a major step in the rapid propagation of plants under *in vitro* conditions. In the present study, an efficient and rapid protocol was developed for the *in vitro* regeneration of *Azadirachta indica* through axillary shoot proliferation in nodal segments collected from mature trees. For axillary shoot proliferation nodal explants were inoculated on MS medium supplemented with different concentration of BAP + additives. Maximum 100% axillary shoot proliferation with 3.95 number of shoot regenerated and 3.60 cm length of regenerated shoots was obtained on MS medium supplemented with BAP 8.88  $\mu$ M. *In vitro* regenerated shoots were further multiplied on MS medium supplemented with different concentration of BAP + additives. Best shoot multiplication in terms of 4.80 number of shoot and 3.95 cm length of regenerated shoots was observed on MS medium supplemented with 4.44  $\mu$ M BAP. For *in vitro* rooting, regenerated microshoots were cultured on MS medium supplemented with different concentration of tryptophan + additives. The highest rooting percent (100%), number of roots (10.30) and mean root length (6.33 cm) was observed on MS medium supplemented with 146.89  $\mu$ M tryptophan within 7-8 days of inoculation. The *in vitro* rooted plantlets were successfully hardened and acclimatized in poly house. These plants showed a good survival rate of 95% under field conditions.

**Key words:** *Azadirachta indica*; Micropropagation; BAP; Tryptophan; *In vitro* rooting; microshoots.

### Introduction

*Azadirachta indica* A. Juss (Neem) is a versatile Indian tree of great importance, belonging to the family Meliaceae, is native of India and is widely cultivated in India. Besides being a popular avenue tree with a large crown, the wood of neem has been used as timber for house building, furniture and other domestic and agricultural tools. The timber is reported to work well with hand and machine tools (Tewari 1992). Its wood resembles teak in strength and is more resistant to shock, fungi and insect attack (Thengane 1995). *Azadirachta indica* is renowned for its insecticidal properties and neem seed extracts show great potential as environmentally acceptable bioinsecticides for crop protection (Schmutterer 1990). Neem owes these properties due to the presence of several bioactive compounds, the most prominent is azadirachtin. Azadirachtin is the major bioactive chemical present in neem kernels. Azadirachtin shows antifeedant and growth regulatory activities against a wide range of insects (Ley et al. 1993). Azadirachtin yields are variable and dependent on plant ecotype and environment (Ermel et al. 1986; Singh 1986). The development of simple and rapid methods for the production of uniform neem populations with high azadirachtin yields is desirable.

To meet the economic demand of the neem tree, an efficient propagation technique is required for large quantities and good quality of planting materials. Vegetative propagation of an adult neem tree by conventional methods is difficult (Kaushik 2002). Therefore, it is normally grow from seeds but the seeds are recalcitrant, than they lose viability within 2-3 weeks (Mohan Ram et al. 1996). Propagation by seeds is also undesirable because of the highly heterozygous nature of the plant owing to cross-pollination and enormous heterozygosity. Moreover, the reproductive phase of neem normally begins after 5 years of seed propagation (Koul et al. 1990; Schmutterer 1990), so one has to wait for a long time to obtain seeds and fruits. Due to the difficulty in germination of neem seeds, *in vitro* propagation techniques are important for this plant, as they allow for selection and rapid multiplication of high-value phenotypes (Quraishi et al. 2004).

Auxin has been known to be intimately involved in the process of adventitious root formation (Wiesman et al. 1989; Schmutterer 1990) and the interdependent physiological stages of the rooting process are associated with changes in endogenous auxin concentrations (Gaspar et al. 1997). Auxin has been studied extensively for decades, its main biosynthetic route in plants has been revealed by Mashiguchi et al. (2011) and Won et al. (2011). Auxins are a group of tryptophan derived signals, which are involved in most aspects of plant development (Woodward and Bartel 2005). Synthesis of auxin involves two pathways with tryptophan dependent and tryptophan independent pathway (Woodward and Bartel 2005).

A recent breakthrough in the field of auxin biosynthesis has been the discovery that the major source of auxin (indole-3-acetic acid, IAA) is generated by a remarkably straightforward two step pathway starting with the amino acid tryptophan (Trp) (Stepanova et al. 2008; Tao et al. 2008; Yamada et al. 2009; Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011). Indole-3-acetic acid (IAA), the main auxin produced by plants, is known to be synthesized *de novo* using tryptophan (Trp) as a precursor or using a Trp-independent pathway (Zhao 2010). IAA can be produced via tryptophan (Trp)-independent and Trp-dependent pathways (Chandler 2009; Normanly 2010; Zhao 2010). The Trp-independent auxin biosynthesis pathway is not well characterized, but seems to be operational in plants, although its biological relevance is not clear (Wright et al. 1991; Normanly et al. 1993; Ouyang et al. 2000; Ehlert et al. 2008). The Trp-dependent pathways are better defined and appear to be the developmentally important source of auxin. It has been clearly demonstrated that Trp-dependent auxin biosynthesis is essential for embryogenesis, seedling growth, flower development, vascular pattern formation, and other developmental processes (Cheng et al. 2006; Cheng et al. 2007; Stepanova et al. 2008; Tao et al. 2008).

For standardization of micropropagation protocol, induction of early rooting is major step. For this tryptophan was added for early induction of *in vitro* rooting. Tryptophan is known to be a physiological precursor of auxins in higher plants. The earlier study revealed that tryptophan has more positive effect on plant growth and yield as compared to pure auxins (Zahir et al. 1999). Tryptophan may act as an osmolyte, ion transport regulator, modulates stomatal opening and detoxify harmful effects of heavy metals (Rai 2002). The role of the amino acid tryptophan in stimulating the growth of several species was studied by Russell (1982) who reported that the increase in growth as a result of tryptophan application may be due to its conversion into IAA. Attoa et al. (2002) on *Iberis amara* L., Talaat et al. (2005) on *Chatharanthus roseus* L. and Abou Dahab and Abd El-Aziz (2006) on *Philodendron erubescens* reported that spraying plants with the amino acid tryptophan increased plant growth. There is no report on role of tryptophan on rooting in microshoots.

Earlier, *in vitro* regeneration of *Azadirachta indica* through axillary shoot proliferation has been reported by Arya et al. (1995), Islam et al. (1997), Arya and Arya (1998), Venkateswarlu et al. (1998), Chaturvedi et al. (2004), Quraishi et al. (2004) and Arora et al. (2010). The limited success to develop efficient and rapid micropropagation protocol had been recorded. In the present investigation, making use of earlier study, success has been achieved in development and standardization of micropropagation protocol using axillary shoots proliferation.

In present investigation, the main objective was to study the effect of tryptophan on early rooting of *in vitro* regenerated microshoots and to establish a procedure which can be used routinely to induce early rooting and produce complete plantlets in a short period of time.

## Materials and methods

### Plant materials

The selection of tree is done based on vegetative characters (i.e., general growth, girth of the main stem at breast level, plant height, straightness of the stem and crown diameter) and reproductive characters (i.e., regeneration ability, initiation of leaf fall, initiation of new leaves, initiation of flowering, number of flower, initiation of fruiting, number of fruits/bunch and fruiting period), seed traits 100 seed weight (g), oil percentage, seed viability and azadirachtin percentage. The nodal segments were collected from selected tree, which is naturally growing at Forest Genetics and Tree Breeding Field, Jodhpur (Fig. 1A). In the present investigation semi mature nodal segment was used as explants for culture initiation and regeneration of *in vitro* plantlets.

### Explant preparation and surface sterilization

The nodal segments collected from mature trees were washed under running tap water to remove dirt and superficial impurities. The explants were washed in RO water (Milipore RiOS5) with 2-3 drops of Tween-80 and were shaken for 5-10 min, then rinsed with autoclaved RO water for 3-4 times. In aseptic condition, explants were treated with 0.1% (w/v) Bavestin and 0.1% (w/v) streptomycin for 8-10 min to reduce the chance of fungal and bacterial contamination. After, explants were rinsed with autoclaved RO water for 3-4 times. Finally, the explants were surface sterilization with 0.1% (w/v) HgCl<sub>2</sub> for 6 min. After treatment, they were rinsed with autoclaved RO water for 3-4 times.

### Nutrient media and culture conditions

MS (Murashige and Skoog 1962) medium supplemented with BAP (0.00, 0.44, 2.22, 4.44, 8.88 and 17.75 µM) with sucrose (3%), ascorbic acid (100 mg/l), citric acid (50 mg/l), adenine sulphate (80 mg/l), ammonium sulphate (80 mg/l), cystine (10 mg/l), glutamine (10 mg/l) and proline (10 mg/l) were used for culture initiation and amplification of cultures. Agar-agar was added to medium as 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 ≈1.0 kgf cm<sup>-2</sup> and 121°C for 15 min. The culture initiation and amplification were done aseptically in laminar air flow hood. Culture was incubated in tissue culture racks in aseptic growth room having a temperature of 26±2°C in photoperiod of 16 h light and 8 h dark and 1600 lux intensity light via white cool florescent tube (Philips, India).

### In vitro rooting of microshoots

*In vitro* regenerated microshoots from established cultures (longer than 2-4 cm) were harvested from clumps and transfer singly to medium for rooting. Individual microshoots transferred to MS medium were enriched with tryptophan (0.0, 48.96, 97.92, 146.89, 195.85 and 244.82 µM) + additive ascorbic acid (100 mg/l), citric acid (50 mg/l), adenine sulphate (80 mg/l), ammonium sulphate (80 mg/l), cystine (10 mg/l), glutamine (10 mg/l) and proline (20 mg/l).

The *in vitro* rooted microshoots 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 ½MS salts. The bottles were capped and kept for 2 weeks under growth room conditions for *in vitro* hardening. After 2 weeks, jam bottles containing regenerated plants were kept in poly house near the pad section (RH 80-90% and temperature 28±2°C) in order to harden the plantlets.

### Hardening and acclimatization of in vitro regenerated plantlets

One week after the transfer of *in vitro* raised plantlets to poly house, the caps of jam bottles were gradually opened over a period of 2 weeks and were finally removed. Bottles containing plantlets were 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, plantlets were transferred to poly bags containing a mixture of soil + sand + FYM (1:1:0.5). Such plantlets were kept in the poly house for 3-4 weeks, shifted to agros shade house and then to opened environment (Fig. 1F).

### Experimental design and statistical analysis

The data were analyzed through an analysis of variance using statistical package for social sciences (SPSS 14.0). BAP and tryptophan was taken as an independent (fixed) factor with five levels, whereas axillary shoot bud proliferation, number of shoot regenerated, length of regenerated shoots (cm), root number, root length (cm) and rooting percentage (%) were taken as dependent variables. For the analysis, under the null hypothesis, the dependent variables were not affected significantly by the fixed factors and their interactions. All the significant main effects and their interaction were studied. Duncan Multiple Range Test (DMRT) at  $p \leq 0.05$  was used to compare the means from main effects. Minimum of 4 replicates with 5 samples (one explant) were taken per treatment and the experiments were repeated thrice. Degree of variations was shown by Mean

and standard error. The data given in percentages were subjected to arcsine  $\sqrt{X}$  transformation (Snedecor and Cochran 1967) before statistical analysis.

## Results and discussion

### Axillary shoot bud proliferation

The supply of growth regulators promoted the production of shoots from the axillary buds of nodal explants cultured on MS medium. The difference in shoot induction percentage and number of shoot buds may be due to addition of cytokinins in the medium and culture conditions, as cytokinin promotes chloroplast development, synthesis, increases cell expansion in leaves and increase nutrient sink activity, promotes cell division and organ development. The percent axillary shoot proliferation also indicates explant compatibility to plant growth regulators (PGRs) concentration.

BAP is one of the most commercial cytokinin to causes reinvigoration of mature tissues (old) and causes bud induction, a prerequisite for cloning of mature trees (Bonga and von Aderkas 1992; Zhang et al. 2010; Kumar et al. 2010; Singh and Tiwari 2012; Rathore et al. 2013). In the present investigation, the results showed that the different concentration of BAP has significant effect on axillary bud proliferation percent ( $F_5 = 13.67, p < 0.00$ ), number of shoot regenerated ( $F_5 = 16.24, p < 0.00$ ) and length of regenerated shoots ( $F_5 = 18.99, p < 0.00$ ). The highest axillary bud proliferation percent (100%), number of shoot regenerated (3.95) and length regenerated shoots (3.60 cm) was observed in explants inoculated on MS medium supplemented with  $8.88 \mu\text{M}$  BAP (Table 1) (Fig. 1B). Role of BAP on axillary shoot proliferation was also reported by Venkateswarlu et al. (1998), Arya and Arya (1998) and Quraishi et al. (2004) in *Azadirachta indica*.

Table 1. Effect of cytokinin (BAP) in MS medium on axillary bud proliferation using nodal explants of *Azadirachta indica*.

BAP ( $\mu\text{M}$ )	Axillary bud proliferation (%)	Number of shoot regenerated	Length of regenerated shoots (cm)
0.00	20.00 (26.56) <sup>ad</sup>	$0.35 \pm 0.17^d$	$0.19 \pm 0.09^e$
0.44	45.00 (42.12) <sup>cd</sup>	$1.05 \pm 0.28^d$	$0.99 \pm 0.26^d$
2.22	65.00 (54.22) <sup>c</sup>	$2.00 \pm 0.40^c$	$1.88 \pm 0.40^c$
4.44	90.00 (76.08) <sup>ab</sup>	$3.15 \pm 0.38^{ab}$	$2.75 \pm 0.30^b$
8.88	100.00 (88.72) <sup>a</sup>	$3.95 \pm 0.29^a$	$3.60 \pm 0.11^a$
17.75	65.00 (57.66) <sup>bc</sup>	$2.25 \pm 0.39^{bc}$	$2.02 \pm 0.36^{bc}$
Mean	64.17 (57.56)	$2.13 \pm 0.17$	$1.90 \pm 0.15$
ANOVA (Analysis of variance)			
DF	5	5	5
F - value	13.67	16.24	18.99
P - value	0.00	0.00	0.00

\* Arc sine values in parentheses. Values within the column followed by different letters significantly different at  $p \leq 0.05$  level as determined using Duncan's multiple range test. A value represents mean  $\pm$  standard error. Data recorded after 4 weeks.

The role of BAP on axillary bud proliferation percent from mature nodal explants has been well documented in number of woody tree species like *Salvedora persica*, (Phulwaria et al. 2011) and *Terminalia catappa* (Phulwaria et al. 2012). BAP may reinvigorate the mature tissues and stimulate the synthesis of endogenous cytokinin, which eventually promotes better shoot bud proliferation (Rai et al. 2010). BAP is very effective for reinvigoration and shoot bud induction from mature tissues in *Pinus radiata* (Zhang et al. 2010), *Terminalia bellirica* (Phulwaria et al. 2012), *Caralluma edulis* (Patel et al. 2014) possibly due to stability of BAP and as it is easily metabolized by plant tissue (Letham and Palni 1983).

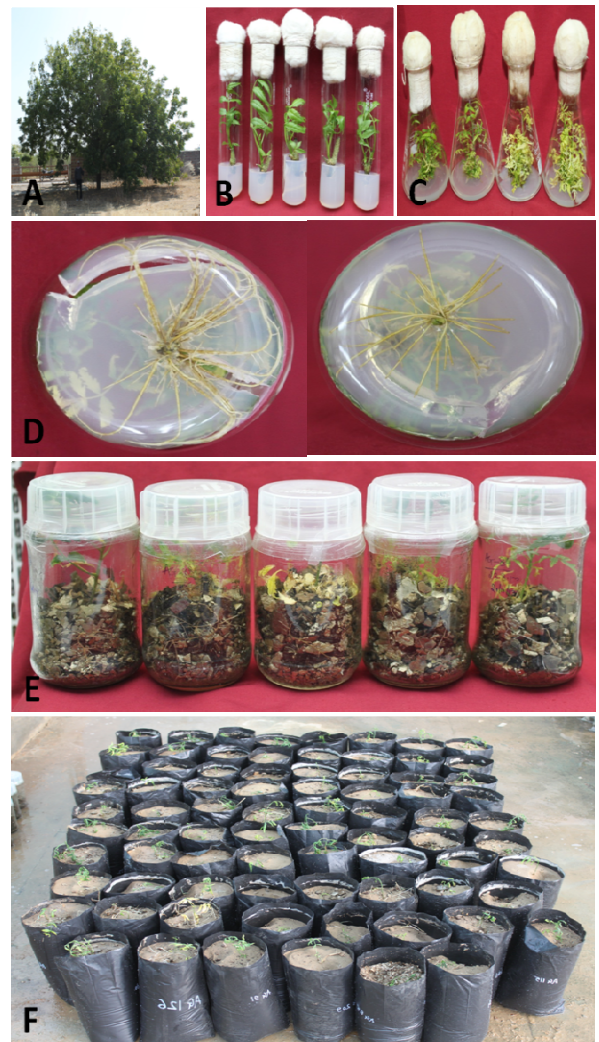


Figure 1. *In vitro* micropropagation of *Azadirachta indica* (Neem). (A) *Azadirachta indica* tree in natural conditions. (B) Axillary shoots proliferation on MS +  $8.88 \mu\text{M}$  BAP + additives. (C) *In vitro* shoot multiplication on MS +  $4.44 \mu\text{M}$  BAP + additives. (D) *In vitro* rooting on MS medium +  $146.89 \mu\text{M}$  tryptophan + additives. (E) Root development during hardening of plantlets. (F) Tissue culture raised plants of *Azadirachta indica*.

### Shoot multiplication

Shoot multiplication is the major criterion for successful commercial micropropagation and the multiplication rate achieved at this stage determines the feasibility of *in vitro* propagation of a given plant species. The rate of multiplication is affected by numerous factors, such as plant growth regulator, physiological status of plant material, culture medium and culture environment. In tissue culture one of the main functions of cytokinin is to release axillary buds from suppression by apical dominance thus initiating shoot proliferation. *In vitro* raised shoots were further multiplied by transferring them to comparatively lower concentration of cytokinin (e.g., BAP) because initial high cytokinin incorporated in the medium for shoot bud induction, may accumulate in the tissues which may suppress further growth and multiplication of cultures (Malik et al. 2005).

In the present study, the results showed that the different concentration of BAP has significant effect on number of shoot regenerated ( $F_5 = 10.19, p < 0.00$ ) and length of regenerated shoots ( $F_5 = 10.35, p < 0.00$ ). The highest number of shoot regenerated (4.80) and length of regenerated shoots (3.95 cm) was observed on MS medium

supplemented with 4.44  $\mu\text{M}$  BAP (Table 2) (Fig. 1C). Significant role of BAP in shoot multiplication was also reported by Quraishi et al. (2004) in *Azadirachta indica*. The promoter effect of BAP on shoot multiplication has been reported earlier in other medicinally important plant species such as *Zingiber officinale* (Balachandran et al. 1990), *Piper* spp. (Bhat et al. 1995), *Houttuynia cordata* (Handique and Bora 1999) and *Curcuma longa* (Rahman et al. 2004).

Table 2. Effect of cytokinin (BAP) in MS medium on *in vitro* shoot multiplication of *Azadirachta indica*.

BAP ( $\mu\text{M}$ )	Number of shoot regenerated	Length of regenerated shoots (cm)
0.00	1.86 $\pm$ 0.14 <sup>d</sup>	1.01 $\pm$ 0.07 <sup>d</sup>
0.44	2.50 $\pm$ 0.15 <sup>cd</sup>	2.32 $\pm$ 0.16 <sup>c</sup>
2.22	3.23 $\pm$ 0.32 <sup>bc</sup>	2.97 $\pm$ 0.35 <sup>bc</sup>
4.44	4.80 $\pm$ 0.33 <sup>a</sup>	3.95 $\pm$ 0.21 <sup>a</sup>
8.88	4.05 $\pm$ 0.34 <sup>ab</sup>	3.79 $\pm$ 0.28 <sup>ab</sup>
17.75	3.75 $\pm$ 0.19 <sup>b</sup>	3.43 $\pm$ 0.35 <sup>ab</sup>
Mean	3.66 $\pm$ 0.15	3.22 $\pm$ 0.15
ANOVA (Analysis of variance)		
df	5	5
F - value	10.19	10.35
P - value	0.00	0.00

Values within the column followed by different letters significantly different at  $p \leq 0.05$  level as determined using Duncan's multiple range test. A value represents mean  $\pm$  standard error. Data recorded after 6 weeks.

#### *In vitro* rooting

Rooting is the most crucial step particularly for the shoots originated from mature plants. Success of the protocol also depends on frequency of root induction, quality and quantity (e.g., number roots and root length) of roots. In the present study we had tried to induce endogenous level of auxin. For this tryptophan was tried in MS medium for *in vitro* root induction. The role of tryptophan is well known, it has an indirect role on the growth via its influence on auxin synthesis for root induction. Tryptophan is one of the essential amino acid that can increase root growth and increase the level of endogenous auxin (e.g., IAA), which is helpful in rooting of plants. In the present investigation, the data revealed that when tryptophan gradually increased from 48.96  $\mu\text{M}$  to 146.89  $\mu\text{M}$  in the MS medium, it increases rooting potential (i.e., induction of rooting, number of root and root length) of *in vitro* regenerated shoots from 5% to 100%. The results showed that the different concentration of tryptophan in MS medium significantly affected rooting percent ( $F_5 = 22.62$ ,  $p < 0.00$ ), number of roots ( $F_5 = 17.41$ ,  $p < 0.00$ ) and root length ( $F_5 = 10.65$ ,  $p < 0.00$ ). The highest rooting percent (100%), number of roots (10.84) and root length (6.66 cm) were observed in MS medium supplemented with 146.89  $\mu\text{M}$  tryptophan within 7-8 days of experiment (Table 3) (Fig. 1D). During hardening of *in vitro* regenerated plantlets, tryptophan increase number and length of regenerated roots (Fig. 1E). It is the first report for obtaining *in vitro* rooting using tryptophan in MS medium.

Phillips (1971) reported that alternative routes of IAA synthesis exist in plants, all starting from tryptophan. Thus, when tryptophan is supplied to some plant tissues, IAA is formed and it helps the induction and growth of roots. Addition of tryptophan is helpful for the differentiation of embryogenic callus (Bano et al. 2005). Also the effect of the amino acid tryptophan has been reported by Shahsari (2011). This author reported that rice plantlet regeneration frequency was significantly increased when an appropriate level of tryptophan was added to culture media, the optimum being 100-200  $\mu\text{M}$ . The positive effect of tryptophan was reported on the tissue culture system of rice by Siriwardana and Nabors (1983) and Chowdhry et al. (1993). Talaat et al.

(2005) reported that photosynthetic pigments (chlorophyll a, b and carotenoids) in the leaves were increased as a result of application of tryptophan in *Catharanthus roseus* L.

In the present study, we observed that tryptophan induces early rooting with increase in rooting potential (number of roots and root length). It also increases growth of shoot and more number of roots as compared to auxin with reduction in cost. It was also observed that tryptophan increases growth and development of root during hardening.

Table 3. Effect of tryptophan concentration in MS medium on *in vitro* rooting of *Azadirachta indica*.

Tryptophan ( $\mu\text{M}$ )	Rooting (%)	Number of roots	Root length (cm)
0.00	5.00 (7.60) <sup>c</sup>	2.00 $\pm$ 0.00 <sup>d</sup>	1.00 $\pm$ 0.00 <sup>c</sup>
48.96	25.00 (26.58) <sup>c</sup>	5.42 $\pm$ 0.83 <sup>c</sup>	3.49 $\pm$ 0.49 <sup>b</sup>
97.92	95.00 (82.40) <sup>a</sup>	7.55 $\pm$ 0.50 <sup>b</sup>	4.11 $\pm$ 0.37 <sup>b</sup>
146.89	100.00 (88.72) <sup>a</sup>	10.84 $\pm$ 0.73 <sup>a</sup>	6.66 $\pm$ 0.56 <sup>a</sup>
195.85	60.00 (51.34) <sup>b</sup>	5.00 $\pm$ 0.45 <sup>c</sup>	4.01 $\pm$ 0.50 <sup>b</sup>
244.82	50.00 (45.28) <sup>b</sup>	4.79 $\pm$ 0.33 <sup>c</sup>	2.79 $\pm$ 0.42 <sup>b</sup>
Mean	55.83 (50.32)	6.63	4.12
ANOVA (Analysis of variance)			
df	5	5	5
F - value	22.62	17.41	10.65
P - value	0.00	0.00	0.00

<sup>a</sup> Arc sine values in parentheses. Values within the column followed by different letters significantly different at  $p \leq 0.05$  level as determined using Duncan's multiple range test. A value represents mean  $\pm$  standard error. Data recorded after 4 weeks.

#### Conclusion

Rooting under *in vitro* conditions is a major step in the development of a protocol for propagation of plants. In the present finding, tryptophan plays an important role in the achieving of early rooting. This may be helpful in difficult to root species.

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#### References

- Abou Dahab TAM, Abd El-Aziz NG (2006) Physiological effect of diphenylamine and tryptophan on the growth and chemical constituents of *Philodendron erubescens* plants. *World Journal of Agricultural Sciences*, 2(1):75-81.
- Arora K, Sharma M, Srivastava J, Ranade AS, Sharma AK (2010) Rapid *in vitro* cloning of a 40-year-old tree of *Azadirachta indica* A. Juss. (Neem) employing nodal stem segments. *Agroforestry Systems*, 78(1):53-63. doi: 10.1007/s10457-009-9230-1
- Arya S, Arya ID (1998) Micropropagation of neem (*Azadirachta indica*) by adventitious organogenesis. *Annals of Forestry*, 6(2):123-129.
- Arya S, Rathore TS, Arya ID (1995) *In vitro* propagation of Neem from seedlings and mature trees. *Indian Journal of Plant Genetics Resources*, 8(2):247-252.
- Attoa GE, Wahba HE, Farahat AA (2002) Effect of some amino acids and sulphur fertilization on growth and chemical composition of *Iberis amara* L. plants. *Egyptian Journal of Horticulture*, 29:17-37.



- Balachandran SM, Bhat SR, Chandal KPS (1990) *In vitro* clonal multiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiber officinale* Rosc.). *Plant Cell Reports*, 8(9):521-524. doi: 10.1007/BF00820200
- Bano S, Jabeen M, Rahim F, Ilahi I (2005) Callus induction and regeneration in seed explants of rice (*Oryza sativa* cv. Swat-II). *Pakistan Journal of Botany*, 37(3):829-836.
- Bhat SR, Chandal KPS, Malik SK (1995) Plant regeneration from various explants of cultivated *Piper* species. *Plant Cell Reports*, 14(6):398-402. doi: 10.1007/BF00238605
- Bonga JM, von Aderkas P (1992) *In vitro* cultures of trees. Dordrecht: Kluwer Academic Publishers. 236p.
- Chandler JW (2009) Local auxin production: a small contribution to a big field. *BioEssays*, 31(1):60-70. doi: 10.1002/bies.080146
- Chaturvedi R, Razdan MK, Bhojwani SS (2004) *In vitro* clonal propagation of an adult tree of neem (*Azadirachta indica* A. Juss.) by forced axillary branching. *Plant Science*, 166(2):501-506. doi: 10.1016/j.plantsci.2003.10.021
- Cheng Y, Dai X, Zhao Y (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. *Genes & Development*, 20(13):1790-1799. doi: 10.1101/gad.1415106
- Cheng Y, Dai X, Zhao Y (2007) Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. *The Plant Cell*, 19(8):2430-2439. doi: 10.1105/tpc.107.053009
- Chowdhry CN, Tyagi AK, Maheshwari N, Maheshwari SC (1993) Effect of L-proline and L-tryptophan on somatic embryogenesis and plantlet regeneration of rice (*Oryza sativa* L. cv. Pusa 169). *Plant Cell, Tissue and Organ Culture*, 32(3):357-361. doi: 10.1007/BF00042300
- Ehlert B, Schöttler MA, Tischendorf G, Ludwig-Müller J, Bock R (2008) The paramutated *SULFUREA* locus of tomato is involved in auxin biosynthesis. *Journal of Experimental Botany*, 59(13):3635-3647. doi: 10.1093/jxb/ern213
- Ermel K, Pahlich E, Schmutterer H (1986) Azadirachtin content of Neem kernels from different geographical locations, its dependence on temperature, relative humidity and light. In: *Proceedings of the Third International Neem Conference*, GTZ, Eschborn.
- Gaspar T, Kevers C, Hausman JF (1997) Indissociable chief factors in the inductive phase of adventitious rooting. In: Altman A, Waisel M (ed) *Biology of root formation and development*. New York: Plenum Press. p.55-63.
- Handique PJ, Bora P (1999) *In vitro* regeneration of a medicinal plant - *Houttuynia cordata* Thunb. from nodal explants. *Current Science*, 76(9):1245-1247.
- Islam R, Hoque A, Khalekuzzaman M, Joarder OI (1997) Micropropagation of *Azadirachta indica* A. Juss from explants of mature plants. *Plant Tissue Culture*, 7(1):41-46.
- Kaushik N (2002) Determination of azadirachtin and fatty acid methyl esters of *Azadirachta indica* seeds by HPLC and GLC. *Analytical and Bioanalytical Chemistry*, 374(7-8):1199-1204. doi: 10.1007/s00216-002-1638-7
- Koul O, Isman MB, Ketkar CM (1990) Properties and uses of neem, *Azadirachta indica*. *Canadian Journal of Botany*, 68(1):1-11. doi: 10.1139/b90-001
- Kumar A, Aggarwal D, Gupta P, Reddy MS (2010) Factors affecting *in vitro* propagation and field establishment of *Chlorophytum borivilianum*. *Biologia Plantarum*, 54(4):601-606. doi: 10.1007/s10535-010-0109-9
- Letham DS, Palni LMS (1983) The biosynthesis and metabolism of cytokinins. *Annual Review of Plant Physiology*, 34:163-197. doi: 10.1146/annurev.pp.34.060183.001115
- Ley SV, Denholm AA, Wood A (1993) The chemistry of azadirachtin. *Natural Product Reports*, 10:109-157. doi: 10.1039/NP9931000109
- Malik SK, Chaudhury R, Kalia RK (2005) Rapid *in vitro* multiplication and conservation of *Garcinia indica*: a tropical medicinal tree species. *Scientia Horticulturae*, 106(4):539-553. doi: 10.1016/j.scienta.2005.05.002
- Mashiguchi K, Tanaka K, Sakai T, Sugawara S, Kawaide H, Natsume M, Hanada A, Yaeno T, Shirasu K, Yao H, McSteen P, Zhao Y, Hayashi K, Kamiya Y, Kasahara H (2011) The main auxin biosynthesis pathway in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 108(45):18512-18517. doi: 10.1073/pnas.1108434108
- Mohan Ram HY, Nair MNB (1996) Botany. In: Randhawa NS, Parmar BS (ed) *Neem*. New Delhi: New Age International (P) Ltd. p.6-26.
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3):473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x
- Normanly J (2010) Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. *Cold Spring Harbor Perspectives in Biology*, 2(1):a001594. doi: 10.1101/cshperspect.a001594
- Normanly J, Cohen JD, Fink GR (1993) *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. *Proceedings of the National Academy of Sciences of the United States of America*, 90(21):10355-10359.
- Ouyang J, Shao X, Li J (2000) Indole-3-glycerol phosphate, a branchpoint of indole-3-acetic acid biosynthesis from the tryptophan biosynthetic pathway in *Arabidopsis thaliana*. *The Plant Journal*, 24(3):327-333. doi: 10.1046/j.1365-313x.2000.00883.x
- Patel AK, Phulwaria M, Rai MK, Gupta AK, Shekhawat S, Shekhawat NS (2014) *In vitro* propagation and *ex vitro* rooting of *Caralluma edulis* (Edgew.) Benth. & Hook. f.: an endemic and endangered edible plant species of the Thar Desert. *Scientia Horticulturae*, 165:175-180. doi: 10.1016/j.scienta.2013.10.039
- Phillips IDJ (1971) *Introduction to the biochemistry and physiology of plant growth hormones*. New York and London: McGraw Hill. 173p.
- Phulwaria M, Ram K, Gahlot P, Shekhawat NS (2011) Micropropagation of *Salvadora persica*-a tree of arid horticulture and forestry. *New Forests*, 42(3):317-327. doi: 10.1007/s11056-011-9254-z

- Phulwaria M, Ram K, Harish, Gupta AK, Shekhawat NS (2012) Micropropagation of mature *Terminalia catappa* (Indian Almond), a medicinally important forest tree. *Journal of Forest Research*, 17(2):202-207. doi: 10.1007/s10310-011-0295-0
- Quraishi A, Koche V, Sharma P, Mishra SK (2004) *In vitro* clonal propagation of neem (*Azadirachta indica*). *Plant Cell, Tissue and Organ Culture*, 78(3):281-284. doi: 10.1023/B:TICU.0000025647.58548.3d
- Rahman MM, Amin MN, Jahan HS, Ahmad R (2004) *In vitro* regeneration of plantlets of *Curcuma longa* Linn. a valuable spice plant in Bangladesh. *Asian Journal of Plant Sciences*, 3(3):306-309. doi: 10.3923/ajps.2004.306.309
- Rai M.K, Asthana P, Jaiswal VS, Jaiswal U (2010) Biotechnological advances in guava (*Psidium guajava* L.): recent developments and prospects for further research. *Trees*, 24(1):1-12. doi: 10.1007/s00468-009-0384-2
- Rai VK (2002) Role of amino acid in plant responses to stresses. *Biologia Plantarum*, 45(4):481-487. doi: 10.1023/A:1022308229759
- Rathore MS, Rathore MS, Shekhawat NS (2013) *Ex vivo* implications of phytohormones on various *in vitro* responses in *Leptadenia reticulata* (Retz.) Wight. & Arn. - an endangered plant. *Environmental and Experimental Botany*, 86:86-93. doi: 10.1016/j.envexpbot.2010.05.009
- Russell RS (1982) *Plant root systems*. 1<sup>st</sup> Edition. New York: McGraw-Hill Inc. 298p.
- Schmutterer H (1990) Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. *Annual Review of Entomology*, 35:271-297. doi: 10.1146/annurev.en.35.010190.001415
- Shahsari E (2011) Impact of tryptophan and glutamine on the tissue culture of upland rice. *Plant, Soil and Environment*, 57(1):7-10.
- Singh J, Tiwari KN (2012) *In vitro* plant regeneration from decapitated embryonic axes of *Clitoria ternatea* L. - an important medicinal plant. *Industrial Crops and Products*, 35(1):224-229. doi: 10.1016/j.indcrop.2011.07.008
- Singh RP (1986) Comparison of antifeedant efficacy and extract yields from different parts and ecotypes of neem (*Azadirachta indica* A. Juss) trees. *Proceedings of the Third International Neem Conference*, GTZ, Eschborn.
- Siriwardana S, Nabors MW (1983) Tryptophan enhancement of somatic embryogenesis in rice. *Plant Physiology*, 73(1):142-146. doi: 10.1104/pp.73.1.142
- Snedecor GW, Cochran WG (1967) *Statistical methods*. Iowa-USA: The Iowa State University Press. 593p.
- Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, Dolezal K, Schlereth A, Jürgens G, Alonso JM (2008) TAA1 - mediated auxin biosynthesis is essential for hormone crosstalk and plant development. *Cell*, 133(1):177-191. doi: 10.1016/j.cell.2008.01.047
- Stepanova AN, Yun J, Robles LM, Novak O, He W, Guo H, Ljung K, Alonso JM (2011) The *Arabidopsis* YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. *The Plant Cell*, 23(11):3961-3973. doi: 10.1105/tpc.111.088047
- Talaat IM, Bekheta MA, Mahgoub MH (2005) Physiological response of periwinkle plants (*Catharanthus roseus* L.) to tryptophan and putrescine. *International Journal of Agriculture & Biology*, 7(2):210-213.
- Tao Y, Ferrer JL, Ljung K, Pojer F, Hong F, Long JA, Li L, Moreno JE, Bowman ME, Ivans LJ, Cheng Y, Lim J, Zhao Y, Ballaré CL, Sandberg G, Noel JP, Chory J (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell*, 133(1):164-176. doi: 10.1016/j.cell.2008.01.049
- Tewari DN (1992) *Monograph on neem (Azadirachta indica A. Juss)*. Dehradun: International Book Distributors. 279p.
- Thengane S (1995) Somatic embryogenesis in neem (*Azadirachta indica*). In: Jain SM, Gupta P, Newton R (ed) *Somatic embryogenesis in woody plants*. v.2. Dordrecht: Kluwer Academic Publishers. p.357-374.
- Venkateswarlu B, Katyal JC, Choudhuri J, Mukhopadhyay (1998) Micropropagation of plus neem (*Azadirachta indica*) and evaluation of field transferred plants. *Indian Forester*, 124(7):537-543.
- Wiesman Z, Riov J, Epstein E (1989) Characterization and rooting ability of indole-3-butyric acid conjugates formed during rooting of mung bean cuttings. *Plant Physiology*, 91(3):1080-1084. doi: 10.1104/pp.91.3.1080
- Won C, Shen X, Mashiguchi K, Zheng Z, Dai X, Cheng Y, Kasahara H, Kamiya Y, Chory J, Zhao Y (2011) Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF *ARABIDOPSIS* and YUCCAs in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 108(45):18518-18523. doi: 10.1073/pnas.1108436108
- Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. *Annals of Botany*, 95(5):707-735. doi: 10.1093/aob/mci083
- Wright AD, Sampson MB, Neuffer MG, Michalczyk L, Slovin JP, Cohen JD (1991) Indole-3-acetic acid biosynthesis in the mutant maize orange pericarp, a tryptophan auxotroph. *Science*, 254(5034):998-1000. doi: 10.1126/science.254.5034.998
- Yamada M, Greenham K, Prigge MJ, Jensen PJ, Estelle M (2009) The *TRANSPORT INHIBITOR RESPONSE2* gene is required for auxin synthesis and diverse aspects of plant development. *Plant Physiology*, 151(1):168-179. doi: 10.1104/pp.109.138859
- Zahir AZ, Malik MAR, Arshad M (1999) Effect of auxins on the growth and yield of rice. *Pakistan Journal of Agricultural Sciences*, 36(3-4):3-4.
- Zhang H, Horgan KJ, Reynolds PH, Jameson PE (2010) 6-benzyladenine metabolism during reinvigoration of mature *Pinus radiata* buds *in vitro*. *Tree Physiology*, 30(4):514-526. doi: 10.1093/treephys/tpp130
- Zhao Y (2010) Auxin biosynthesis and its role in plant development. *Annual Review of Plant Biology*, 61:49-64. doi: 10.1146/annurev-arplant-042809-112308