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Determination of the Best Culture Medium and Plant Growth Regulators for Micropropagation of Neem Tree (*Azadirachta indica* A.Juss)

Aminu Mallam Bello[1], Kazem Kamali Aliabad *[1], Afagh Tabandeh Saravi[2], Hamid SodaeiZade[1]

1. Department of Arid Land and Desert Management, Faculty of Natural Resources and Desert Studies, Yazd University, Yazd, Iran
2. Department of Environmental Science, Faculty of Natural Resources and Desert Studies, Yazd University, Yazd, Iran

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ABSTRACT

Azadirachta indica is a tree with high medicinal value that is conventionally propagated by seed while exhibiting heterozygosity. The aim of this research was to determine the best culture media and plant growth regulators for the micropropagation of Neem. Lateral and terminal buds of *A. indica* were sterilized with 0.15% and 0.2% mercuric chloride for 10, 11, 12, and 13 min, followed by washing with sterilized double-distilled water three times. At the proliferation and elongation stage, WPM and MS media were tested with different concentrations of BAP either alone or in combination with 0.01 mg L^{-1} IBA. LS and MS media containing four different levels of IBA (0, 0.5, 1.0, and 2.0 mg L^{-1}) were used for the rooting stage. Pulsing technique with different IBA concentrations was investigated at the rooting stage. Hardening of rooted plantlets was done in potting soil containing peat and perlite (2:1), at 23-24 ℃ prior to transfer into the natural environment. Maximum survival percentage (70.83%) with minimum browning (10.42%) was achieved by sterilizing the explant with 0.15% of mercuric chloride at all times. The longest shoots (3.66 cm) were observed in the media containing BAP (0.5 mg L^{-1}). Furthermore, the highest number of leaves (14.2 leaves per plant) was recorded in MS medium. Additionally, the MS media containing BAP hormone alone at 0.7 mg L^{-1} produced the highest number of shoots (3.6 shoots per treatment). LS medium supplemented with IBA (4.0 mg L^{-1}) using the pulsing technique gave the best result at the rooting stage.

Introduction

Azadirachta indica A. Juss, commonly known as Neem, belongs to the mahogany family Meliaceae. Meliaceae contains about 51 genera and 575 species. The *A. indica* is well known as the Indian Neem or Indian lilac tree. Its genus has only one specie besides Neem called *A. excelsa* that is found everywhere (Benelli et al., 2017). Neem tree, also known as "Tree of the 21st century" in the US; "Nature's drugstore" or "Village dispensary" in India, and "Tree of forty" in Africa, is an evergreen perennial, rapidly growing plant, resistant to high temperature and drought, widespread all over the Asian countries and a source of countless beneficial products (Patel et al., 2016). Many products like medicines, cosmetics, insecticides, and fertilizers, can be obtained from Neem; hence known as the multipurpose tree. US and many European countries have accepted Neem as a harmless insecticide for use in organic production. The most crucial bioactive compound of Neem is Azadirachtin (Kuravadi et al., 2015), which is an active plant-based bio-insecticide that acts by disrupting the growth and development of harmful insects through deterring their feeding (Chaudhary et al., 2017),

* Corresponding Author, Email: kkamali@yazd.ac.ir
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and concentrates in the seed than other parts of the tree (Rupani and Chavez 2018).

The content of azadirachtin varies due to meiosis and cross-pollination. The tree is also biologically heterozygous, which makes it challenging to select a uniform fast-growing tree with high azadirachtin content. The natural propagation of Neem is by seed, which takes around three to five years before the tree starts bearing harvestable seeds (Suresh et al., 2019). The seeds also have very short viability during storage (Pandey and Pati, 2017). Through *in vitro* propagation, the trees with superior genotype and high Azadirachtin contents can be selected and produced homogeneously throughout the year.

A good deal of research has been reported so far on the tissue culture of Neem. Regeneration of the tree through callus production (Gautam et al., 1993; Shrikhande et al., 1993; Houllou et al., 2015; Phukan et al., 2017) shows clonal variation; hence the aim of homogenous clonal production is not achieved. The direct regeneration (Quraishi et al., 2004; Srinidhi et al., 2008; Arora et al., 2011) failed to report the effects of culture media and the effect of cytokinin (BAP) alone or in combination with auxin (IBA) in the regeneration of the tree. The aim of the present study was to determine the effect of different culture media and plant growth regulators on the micropropagation of the Neem tree.

Materials and Methods

Explant collection and sterilization treatments

This research was conducted at the Biotechnology and Plant Tissue Culture Laboratory at Yazd University, Yazd, Iran. A one-year-old Neem tree was collected from the state Government Greenhouse, Bandar Abbas, the provincial center of Hormozgan province, Iran, and was utilized as the explant in the greenhouse. The axillary and terminal buds of *A. indica* were used as the explant. The explants were washed with tap water and one drop of detergent at first, followed by thorough washing with double distilled water to remove any trace of detergent. Then the explants were surface sterilized with two types of treatments with aqueous mercuric chloride solution (0.15% and 0.20%) both at different durations (10 min, 11 min, 12 min, and 13 min) followed by washing three times with the sterilized distilled water to avoid accumulation of phenolic compounds. The explants were then inoculated in MS medium containing 0.1 mg L^{-1} BAP and 0.01 mg L^{-1} IBA and maintained in 16:8 hr light:dark ratio at 25±2 ˚C temperature; 2000-3000 lux light intensity. All the sterilization and inoculation activities were carried out under a laminar hood, frequently disinfecting the working area with 70% alcohol.

Elongation and proliferation treatments

The established cultures were sub-cultured after every 28 days to obtain sufficient plant material for shoot elongation and proliferation treatments in MS medium containing 0.7 mg L^{-1} BAP. Then the explants were cultured in two different media (MS and WPM). Each medium was supplemented with eight different concentrations of BAP (0.0, 0.1, 0.3, 05, 0.7, 0.9, 1.1, and 1.3 mg L^{-1}) alone or in combination with 0.01 mg L^{-1} IBA. After 28 days of culture, the data on the number of leaves, length and the number of shoots were recorded.

Rooting treatments

The *in vitro* regenerated shoots were prepared in 2 cm, and used as an explant for rooting treatment. Different levels of auxin (IBA) were used at this stage. LS and MS media were both supplemented with different IBA concentrations (0.0, 0.5, 1.0, and 2.0) mg L^{-1} for root induction. At this stage, another treatment of pulsing technique was also tested. Pulsing technique is a method in which 2 cm explants were inoculated in a liquid media solution containing 0.0, 2.0, 4.0, 6.0, 8.0, 10.0 mg L^{-1} of IBA concentrations in a dark condition for three days, and was transferred to free hormone LS medium still in dark for four days in order to get the maximum root induction. The cultures were transferred to 16:8 hr light:dark cycles for another two weeks. In the above treatments the control was sterilized in double-distilled water only. In both cases the number of roots and length of the roots were recorded.

Hardening and acclimatization of plantlets

After 30 days of culture on rooting media, the shoots that successfully formed roots were carefully taken out from the bottles and dipped in tepid water to remove the attached agar. After the media residues were removed, the plantlets were carefully planted in pots containing sterilized mixture of peat and perlite (2:1). The plants were kept in a greenhouse, with a maintained temperature of about 23-24 ˚C. The pots were covered after planting to ensure that the plant did not lose its 100% humidity once. So the *in vitro* established plantlets lost its humidity stepwise and survived by carrying out the following activities subsequently: After 48 h, two holes were created, two other bigger holes were

created after 96 h, and the cover was removed after one week of planting in the soil. The plants were sprayed with 1/3 MS solution after two weeks, and irrigated with 1/3 MS solution after 20 days of planting in the soil. During this period, the plants were watered at an interval of 2-3 days to maintain moisture in the root trainers. Then the plants were transferred to the shade where there was no direct sunlight.

Statistical analysis

All data collected were first subjected to the Kolmogorov Smirnov normality test. If the data were normally distributed, then the analysis of variance followed. Data were subjected to one-way analysis of variance (ANOVA) using a full factorial experiment based on completely randomized design (CRD). SPSS software was used for all data analysis. Each treatment consisted of five replications (culture vials), and each replication contained three explants. Data for each stage was analyzed by a CRD. If ANOVA indicated statistically significant differences, the means were separated using Duncan's multiple range test (DMRT) at $p<0.05$, where the treatments were two only, means were separated using the t-test.

Results

Sterilization stage

In the presence of $HgCl_2$, at any concentration, no bacterial contamination was observed for all the duration intervals used in this study. Fungal contamination revealed no significant differences for both duration treatment, the concentration of mercury chloride and the interaction between the time and mercury chloride concentration (Table 1). The highest survival percentage of (70.83%) was observed in the treatment sterilized with 0.15% of $HgCl_2$, while only 43.75% survival rate was observed in 0.2% of $HgCl_2$ treatment (Fig. 1a and 2a). The highest browning rate was observed in 0.2% $HgCl_2$ (39.58%) while 0.15% $HgCl_2$ showed only 10.42% (Fig. 1b and 2b).

Table 1. Effects of different concentrations of $HgCl_2$ and time, on the sterilization of explants for the survival of the Neem tree

Traits	S.O.V	df	MS
Browning	$HgCl_2$	1	0.510^{***}
	Time	3	0.007^{ns}
	$HgCl_2$×Time	3	0.045^{ns}
	Error	16	0.021
Fungi contamination	$HgCl_2$	1	0.003^{ns}
	Time	3	0.093^{ns}
	$HgCl_2$×Time	3	0.037^{ns}
	Error	16	0.057
Healthy	$HgCl_2$	1	0.440^{*}
	Time	3	0.114^{ns}
	$HgCl_2$×Time	3	0.135^{ns}
	Error	16	0.086

Values with ***, *, ns means differ significantly at ($p < 0.01$), ($p < 0.05$), No significant difference at ($p < 0.05$) respectively.

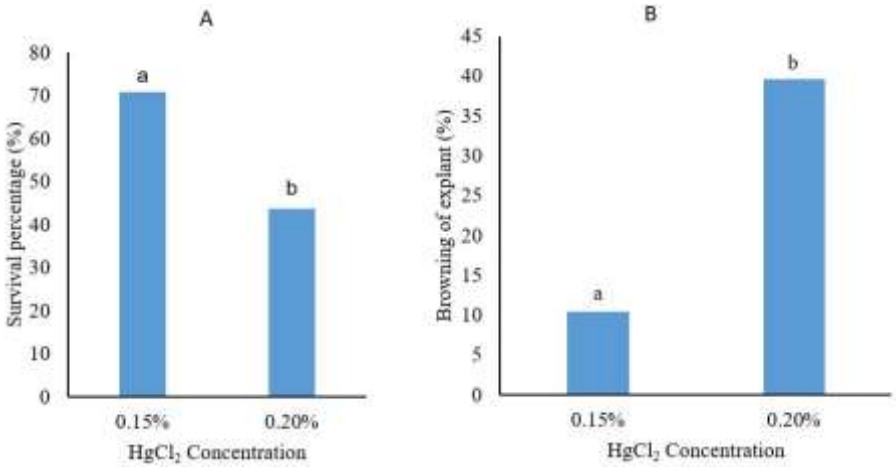

Fig. 1. Effect of different concentrations of $HgCl_2$ on a) Survival percentage b) Browning percentage of Neem tree explants. Columns with different letters are significantly different from each other at $p< 0.05$

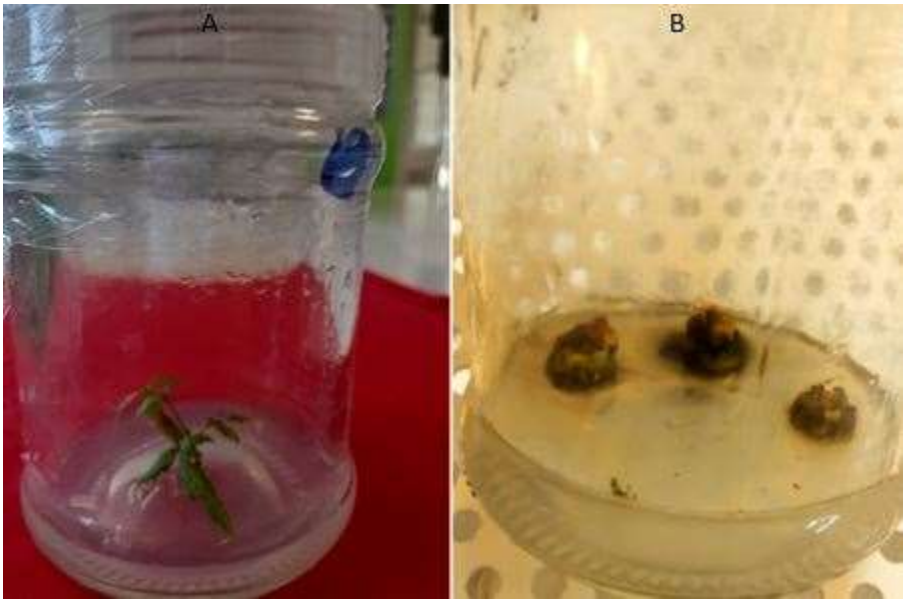

Fig. 2. a. Established explant in 0.15% of $HgCl_2$ b. Browning of explant mostly on woody part of the plant

Elongation and proliferation stage

The highest number of leaves (7.387) was obtained in MS medium (Fig. 3a), without IBA (0.0 mg L^{-1}) having the highest mean of 6.938 (Fig. 3b). The mean separation of the interactive effect between media, BAP, and IBA in terms of the number of leaves showed that MS medium with 0.5 mg L^{-1} BAP, 0.0 mg L^{-1} IBA is the best treatment for producing highest number of leaves in the tissue culture of the Neem tree with the highest mean of 14.2 (Fig. 3c).

The highest shoot length (3.549 cm) was obtained in MS medium (Fig. 4a and 7a), and the results showed that 0.5 mg L^{-1} of BAP is the best concentration for shoot elongation of the tree with the highest mean of (3.66 cm) per plant (Fig. 4b).

The result showed that MS medium with 2.188 maximum number of shoots was better than WPM medium (Fig. 5b and 7b). Although the ANOVA test resulted in no significant difference between the treatments and their interactions, the Duncan multiple range test classified their means in terms of number of shoots. The mean separation of the overall interactive effects of both Media, BAP, and IBA in terms of number of shoots showed that MS medium incorporated with 0.7 mg L^{-1} BAP, without IBA is the best treatment for producing the highest number of shoots of about 3.6 shoots per culture vials in the tissue culture of the Neem tree (Fig. 5a) (Table 2).

Table 2. The effect of Media and different concentrations of BAP and IBA on *in vitro* Number of Leaves, Shoot length and Number of shoots of Neem tree (*A. indica*)

Traits	S.O.V	df	MS
Number of Leaves	Media	1	195.806^{***}
	BAP	7	23.342^{ns}
	IBA	1	68.906^{*}
	Media×BAP	7	20.892^{ns}
	Media×IBA	1	28.056^{ns}
	BAP×IBA	7	18.592^{ns}
	Media×BAP×IBA	7	75.513^{***}
	Error	128	14.931
Shoot length (cm)	Media	1	43.786^{***}
	BAP	7	6.387^{*}
	IBA	1	0.248^{ns}
	Media×BAP	7	4.466^{ns}
	Media×IBA	1	0.915^{ns}
	BAP×IBA	7	2.631^{ns}
	Media×BAP×IBA	7	2.124^{ns}
	Error	128	2.377
Number of Shoots	Media	1	11.025^{*}
	BAP	7	2.143^{ns}
	IBA	1	2.025^{ns}

Traits	S.O.V	df	MS
	Media×BAP	7	1.354^{ns}
	Media×IBA	1	2.500^{ns}
	BAP×IBA	7	2.782^{ns}
	Media×BAP×IBA	7	1.886^{ns}
	Error	128	1.378

Values with *** and * means differ significantly at ($p < 0.01$) and ($p < 0.05$) respectively, while ns means No significant difference ($p < 0.05$).

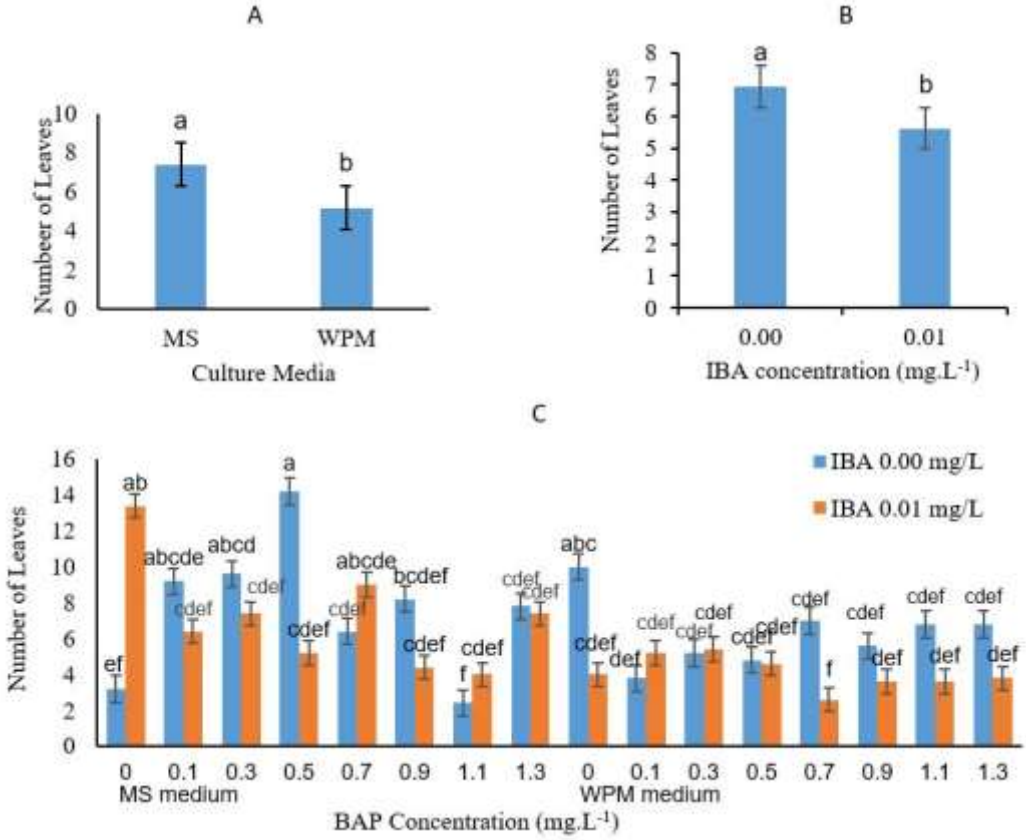

Fig. 3. a. Effect of culture media on the number of leaves of Neem tree b. Different concentrations of IBA on *in vitro* number of leaves c. Interactive effects of Media, BAP and IBA concentrations on in vitro umber of leaves Columns with different letters are significantly different from each other at $p < 0.05$. Bars represent SE

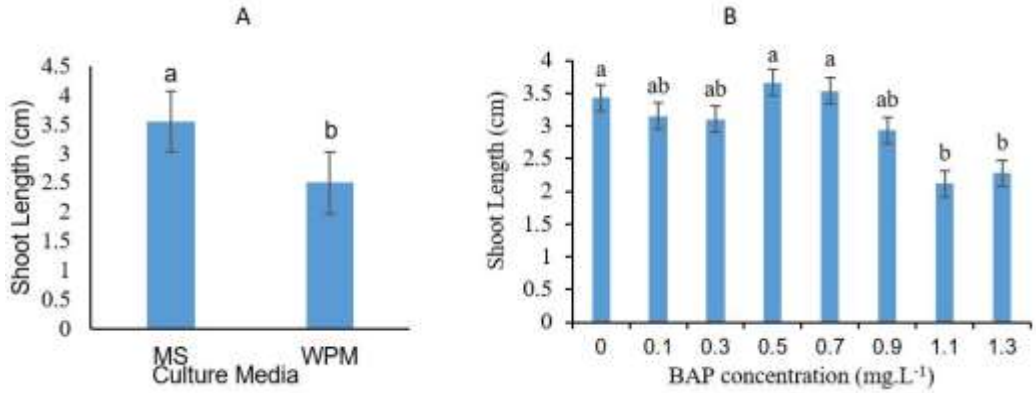

Fig. 4. a Effect of different culture media on in vitro Shoot length b. Effect of different concentration of BAP on in vitro Shoot length. Columns with different letters are significantly different from each other at $p < 0.05$. Bars represent SE

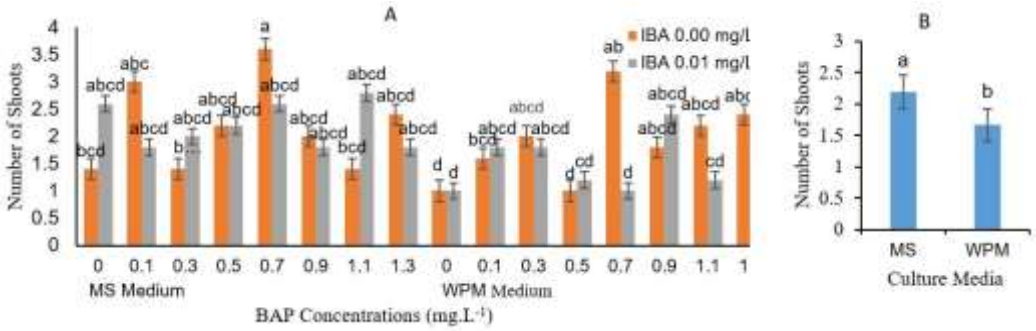

Fig. 5. a. Interactive effects of Media, BAP and IBA concentrations on *in vitro* number of Shoots b. Effect of different culture media on in vitro number of shoots. Columns with different letters are significantly different from each other at $p< 0.05$. Bars represent SE

Rooting stage

Solid media incorporated with 1.0 mg L^{-1} of IBA produced the longest root of about 2.4 cm while the shortest root of about 0.5cm was observed in the control treatment (Fig. 6a). The maximum number of roots was induced in a pulsing technique (Liquid medium) in LS medium containing 4.0 mg L^{-1} IBA (10 roots per plant), while the minimum number of roots was recorded in the control of IBA (0.0 mg L^{-1}) with 0.6 roots per plant (Fig. 6b). The pulsing technique containing 4.0 mg L^{-1} of IBA induced the longest root of (10 cm) while the minimum root length was recorded in the control of IBA (0.0 mg L^{-1}) with 0.5 cm root length (Fig. 6c and 7c).

Acclimatization

The plantlets gradually lost their humidity, and were well acclimatized to the *in vivo* condition with approximately 80% survival frequency (50/63). All the established plants were similar to each other, and no variation among them was detected (Fig. 7d).

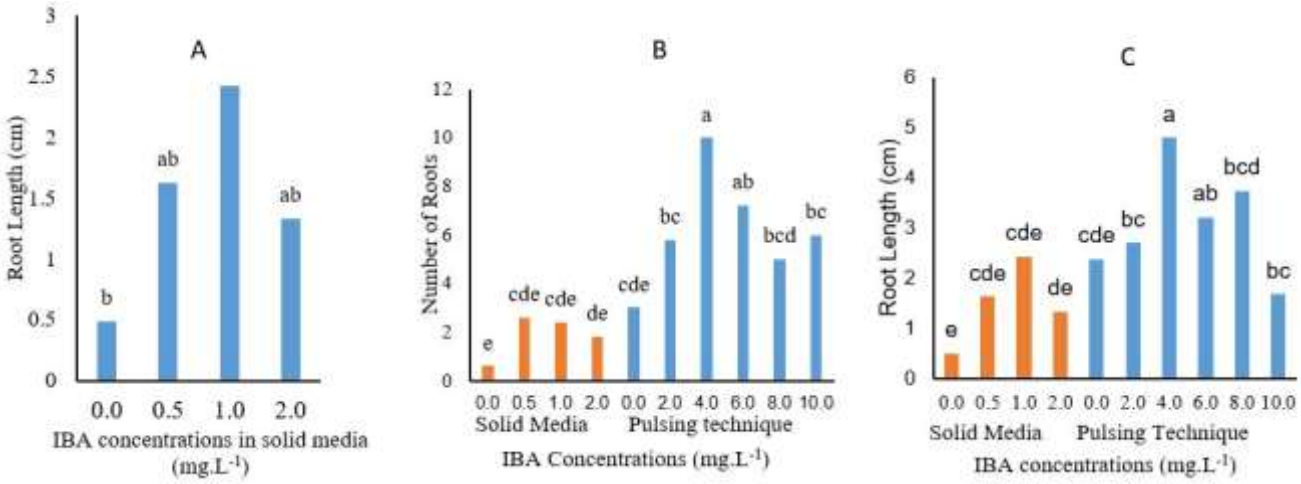

Fig. 6. a. Effect of different concentrations of IBA on *in vitro* root Length of the Neem tree b. Interactive effects of IBA concentrations in solid media and pulsing technique on number of roots. c. Interactive effects of IBA concentrations in solid media and pulsing technique on root length. Columns with different letters are significantly different from each other at $p< 0.05$ (Duncan's multiple range test)

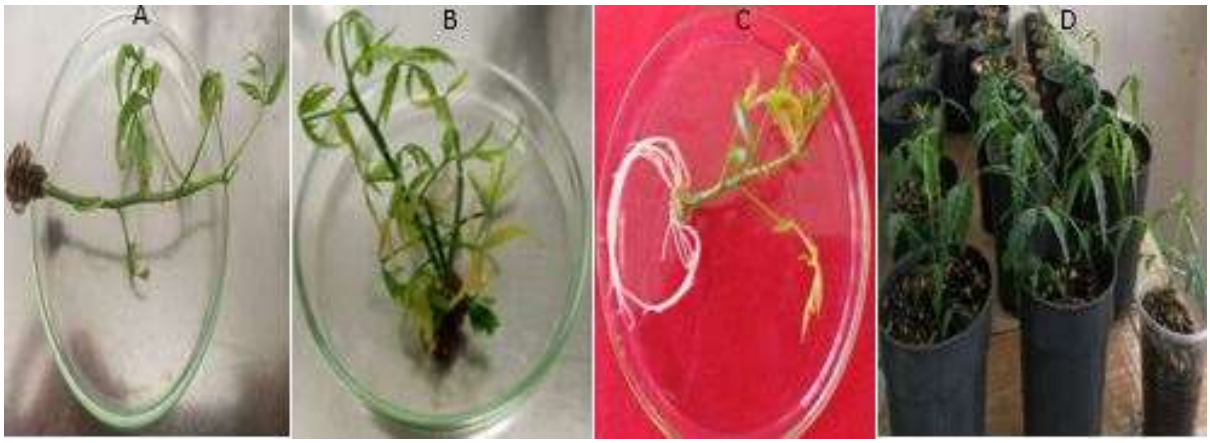

Fig. 7. a. The elongated shoots in IBA free MS Medium containing 0.5 mg L^{-1} BAP. b. The proliferated shoots in MS medium + 0.7 mg L^{-1} without IBA c. Rooted Shoot in LS medium + 4.0 mg L^{-1} IBA in pulsing technique. d. Acclimatized plants in a peat and perlite soil

Discussion

Contamination is the major obstacle encountered during the establishment of cultures mostly by bacteria, fungi, and browning, especially in wooden cultivars. The main problem is obtaining healthy shoots in Neem. In this study, the $HgCl_2$ was used to sterilize the newly developed shoots of the explant obtained from the greenhouse during March to April, in which the 1.5% $HgCl_2$ gave the best result for sterilizing the explant with the time interval from ten to twelve mins. This result is in agreement with the findings by Chaturvedi et al. (2004), who also observed that Neem is hardly contaminated by bacteria and mostly by fungi when sterilized with $HgCl_2$ at this concentration (1.5% $HgCl_2$).

At the elongation and proliferation stage, BAP alone was found to be effective for both increasing the number of shoots and elongating the size of formatted shoots. Kumar and Jakhar (2018) reported that the most prominent influence of bud breaks and shoot multiplication had been found with cytokinins. Furthermore, among the cytokinins also Bennett et al. (1994) reported that BAP is considered as the most effective for the stimulation of axillary shoot proliferation. Many researchers do not identify the effects of cytokinin: auxin ratio with BAP and IBA combination rather BAP and IAA (Singh and Chaturvedi 2009) and 2,4 D only (Rout 2005). Concentrations below a range of 0.7 mg L^{-1} only show growth stimulation while higher concentrations inhibit plant growth; this shows that the inhibitory effects of BAP appear at this higher concentration.

Root induction was observed at all levels of IBA except in the control treatment of MS medium without IBA. Maximum rooting was observed in the pulsing technique with 95% frequency. The longest and the maximum number of roots were also obtained in LS medium through pulsing technique supplemented with 4.0 mg. L^{-1} IBA. Rooting of the regenerated shoots of this tree is usually achieved with 70-100% success by using auxin. There was no reported result that investigated the effect of LS medium in root induction stage of the Neem tree, which is found better than full strength MS in the present study. Joshi (1996) and Venkateswarlu et al. (1998) reported the use of IAA at 2 to 3 mg L^{-1} to be the best auxin concentration for rooting of Neem. In the study of Islam et al. (1997) IBA (0.5 mg L^{-1}) was the most effective hormone for rooting of Neem. This result shows that the *Azadirachta* genus seems to be responsive to IBA as reported by Cha-um et al. (2003). The result of the present study is in close agreement with the findings of Quraishi et al. (2004), where they found 1.0 mg L^{-1} IBA, is effective in root induction. Gautam et al. (1993) and Houllou et al. (2015) also reported similar results.

Conclusion

This study depicted that superior genotype with high Azadirachtin content of Neem tree can be selected as an explant and homogenously propagated *in vitro* by sterilizing the lateral and terminal buds with 0.15% of $HgCl_2$. However, our results showed that using axillary buds as an explant is better than apical buds. The obtained results showed that mass multiplication of the explants can be done using 0.7 mg L^{-1} BAP in MS medium, and then elongating the plantlets to 2 cm by using 0.5 mg L^{-1} BAP in MS medium. For root induction, 4.0 mg L^{-1} of IBA can be used through the pulsing technique, and finally transferring the rooted shoots to the soil.

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References

Arora K, Sharma M, Srivastava J, Ranade SA, Sharma AK. 2011. In vitro cloning ofAzadirachta indica from root explants. Biologia Plantarum 55(1), 164-168.

Benelli G, Buttazzoni L, Canale A, D'Andrea A, Del Serrone P, Delrio G, Foxi C, Mariani S, Savini G, Vadivalagan C, Murugan K, Toniolo C, Nicoletti M, Serafini M. 2017. Bluetongue outbreaks: Looking for effective control strategies against Culicoides vectors. Research in Veterinary Science 115, 263-270.

Bennett I, McComb J, Tonkin C, McDavid D. 1994. Alternating cytokinins in multiplication media stimulates in vitro shoot growth and rooting of Eucalyptus globulus Labill. Annals of Botany 74(1), 53-58.

Cha-um S, Mosaleeyanon K, Kirdmanee C, Supaibulwatana K. 2003. A more efficient transplanting system for Thai neem (*Azadirachta siamensis* Val.,) by reducing relative humidity. ScienceAsia 29, 189-196.

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(1), 501-506.

Chaudhary S, Kanwar RK, Sehgal A, Cahill DM, Barrow CJ, Sehgal R, Kanwar JR. 2017. Progress on *Azadirachta indica* Based Biopesticides in Replacing Synthetic Toxic Pesticides. Frontiers in plant science 8, 610-610.

Gautam VK, Nanda K, Gupta SC. 1993. Development of shoots and roots in anther-derived callus of *Azadirachta indica* A. Juss.—a medicinal tree. Plant cell, tissue and organ culture 34(1), 13-18.

Houllou LM, Souza RAd, Santos ECPd, Silva JJPd, Barbosa MR, Sauvé JPG, Harand W. 2015. Clonal propagation of neem (*Azadirachta indica* A. Juss.) via direct and indirect in vitro regeneration. Revista Árvore 39(3), 439-445.

Conflict of Interest
The authors indicate no conflict of interest for this work.

Islam R, Hoque A, Khalekuzzaman M, Joarder O. 1997. Micropropagation of *Azadirachta indica* A.Juss. from explants of mature plants. Plant Tissue Culture 7, 41-46.

Joshi M. 1996. In vitro propagation of Azadirachta indica A. Juss (neem) by shoot proliferation. Indian Journal of Experimental Biology 34, 480-485.

Kumar R, Jakhar M. 2018. Factor Affecting Tissue Culture Experiments Of Woody Plant: A Review. Journal of Cell & Tissue Research. 18(2), 6467-6471.

Kuravadi NA, Yenagi V, Rangiah K, Mahesh H, Rajamani A, Shirke MD, Russiachand H, Loganathan RM, Lingu CS, Siddappa S. 2015. Comprehensive analyses of genomes, transcriptomes and metabolites of neem tree. PeerJ 3, e1066.

Pandey V, Pati A. 2017. Limitation of Improvement in Germination by Osmopriming of Differentially Aged Non-Orthodox Neem (Azadirachta indica) Seeds. Biochemistry and Physiology 6(217), 2.

Patel SM, Venkata KCN, Bhattacharyya P, Sethi G, Bishayee A. 2016. Potential of neem (*Azadirachta indica* L.) for prevention and treatment of oncologic diseases. In: Seminars in Cancer Biology 40, 100-115.

Phukan H, Kumar R, Mitra PK. 2017. Plant Regeneration by Somatic Embryogenesis in *Azadirachta indica* A. Juss.(Neem). International Research Journal of Engineering and Technology 4, 3212-3217.

Quraishi A, Koche V, Sharma P, Mishra S. 2004. In vitro clonal propagation of neem (*Azadirachta indica*). Plant cell, tissue and organ culture 78(3), 281-284.

Rout GR. 2005. In vitro somatic embryogenesis in callus cultures of *Azadirachta indica* A. Juss.—a multipurpose tree. Journal of Forest Research 10(4), 263-267.

Rupani R, Chavez A 2018. Medicinal plants with traditional use: Ethnobotany in the Indian subcontinent. Clinics in Dermatology, 36(3),

306-309.

Shrikhande M, Thengane S, Mascarenhas A.1993. Somatic embryogenesis and plant regeneration in *Azadirachta indica* A. Juss. In Vitro Cellular & Developmental Biology-Plant 29(1), 38-42.

Singh M, Chaturvedi R. 2009. An efficient protocol for cyclic somatic embryogenesis in Neem (*Azadirachta indica* A. Juss.). In: Proceedings of World Academy of Science Engineering and Technology656-658.

Srinidhi HV, Gill RIS, Sidhu DS. 2008. Micropropagation of Adult and Juvenile Neem (*Azadirachta indica* A. Juss). Journal of Crop Improvement 21(2), 221-232.

Suresh KS, Suresh P, Kudre TG. 2019. Prospective ecofuel feedstocks for sustainable production. In: Advances in Eco-Fuels for a Sustainable Environment. India, Elsevier.89-117.

Venkateswarlu B, Katyal J, Choudhuri J, Mukhopadhyay K. 1998. Micro-propagation of plus neem (*Azadirachta indica* A. Juss) and evaluation of field transferred plants. Indian forester 124(7), 537-543.