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In Vitro Clonal Propagation of a Fast Growing Legume Tree-*Acacia mangium* Willd. Employing Cotyledonary Node Explants

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Abstract

An efficient protocol for *in vitro* clonal propagation of *A. mangium* was developed using seedling derived explants. Out of three different explants tested for shoot proliferation, cotyledonary node showed best performance than leaf node and shoot tip explants. MS (Murashige and Skoog's) medium was found best for shoot proliferation and cotyledonary nodes were subsequently cultured on MS medium supplemented with BA and Kn alone or in combination with NAA, IBA and GA_3 at different concentrations. Maximum number of shoots was formed on MS medium containing 4.0 µM BA. For adventitious rooting, *in vitro* proliferated shoots were transferred to full strength MS medium fortified with IBA and NAA singly at different concentrations (0-8.0 µM). Best rooting responses were observed in the medium containing 8.0 µM IBA. Plantlets having well developed root system were transferred to soil and successfully acclimatized with 65% survival rate under *ex vitro* condition.

Keywords: *in vitro* propagation, multipurpose legume tree, plantation forestry, seedling explants

Introduction

A. mangium Willd., belonging to Mimosaceae family, is one of the most important leguminous tree characterized by its fast growth, nitrogen fixing ability, good growth in adverse soil condition and a tendency to grow well in humid and hot climate (Umezawa *et al*., 2008). It is native to northern Queensland of Australia, through Papua New Guinea into the Indonesian provinces of Irian Jaya and Maluku. This tree species is now widely used for timber, pulp and fire wood (Galiana *et al*., 1991). In addition, its bark contains considerable amount of antioxidant phenols (Zhang *et al*., 2010) offering increasing demand of its bark in different industries as a source of active substance for cosmetic and pharmaceutical composition. Taking into account of its different valuable characteristics, such as fast growth, good growth in adverse soil condition, good quality pulp and fuel-wood production etc., this tree species was introduced into Bangladesh from Australia during 1980s (Islam, 2003). Besides, several reports (Amin *et al*., 1995; Hossain *et al*., 1997; Khan *et al*., 2004) recommended this species suitable for afforestation and reforestation in degraded hilly areas, marginal lands and roadside plantations in Bangladesh.

Conventional propagation methods like using seeds, cutting, grafting etc. have limited scope for large scale propagation of *A. mangium* because of poor seed germination and poor rooting ability of cuttings. Compare to conventional propagation methods, *in vitro* clonal propagation is a common technique, which has been extensively applied in large scale multiplication of many important forest tree species (Ahuja, 1993; Bonga *et al*., 1992). Due to having recalcitrance nature of adult tissues, however, most of the investigations on *in vitro* clonal propagation of forest legumes are concentrated on juvenile materials especially on seedling derived explants. There are a few successful reports on *in vitro* clonal propagation of *A. mangium* using seedling derived explants like seedling nodes (Ahmad, 1991; Galiana *et al*., 1991; Saito *et al*., 1993), but no noted reports on its clonal propagation employing cotyledonary node explants have been found yet. This communication, therefore, describes a successful and quick method on large scale *in vitro* clonal propagation of *A. mangium* via cotyledonary node explants.

Materials and methods

Seeds of *A. mangium* were collected from a mature elite tree grown in Rajshahi University campus, Rajshahi, Bangladesh. Seeds were washed thoroughly under running tap water for 15 minutes and then washed with continuous agitation in a few drops Savlon™ containing water for 15 minutes. Washed seeds were pre-treated by immersing them in boiling water for 2-5 minutes followed by soaking in cold water for 20 minutes. The pre-treated seeds were then treated with 0.1% $HgCl_2$ for 5 minutes under laminar air flow cabinet to disinfect them. Finally, seeds were washed 3 to 5 times with sterile distilled water and were placed in culture tubes (25 × 150 mm) containing hormone free MS (Murashige and Skoog, 1962) medium prepared with 3% (w/v) sucrose and 0.8% (w/v) agar (Sigma Chemical Co. USA). The pH of the medium was adjusted

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to 5.7 before autoclaving at 121°C for 20 minutes at 1.2 kg/cm^2 pressure. After successful germination of seeds, three different types of explants viz. cotyledonary node, leaf node and shoot tip (1-1.5 cm in length) were excised from 2-week-old seedlings (Fig. 1A) and cultured on MS basal medium containing 4.0 μM BA (6-Benzyl adenine) alone or in combination with 0.5 μM NAA (α-naphthalene acetic acid) to test the effect of explants on *in vitro* shoot multiplication.

To test the effect of basal medium on *in vitro* shoot multiplication, cotyledonary nodes were initially cultured on four different basal medium viz. MS, MMS_1, MMS_2 and WPM (Woody plant medium) (Lloyd and McCown, 1980) supplemented with 4.0 μM BA. In addition, excised cotyledonary nodes of 2-week-old seedlings were cultured on MS medium containing various concentrations (2.0-8.0 μM) of BA and Kn (6-furfurylamino purine). Different concentrations (0.5-2.0 μM) of NAA, IBA (Indole-3-butyric acid) and GA_3 (Gibberellic acid) were combined with 4.0 μM BA to test their shoot induction efficiency.

Microshoots of 1-3 cm length were prepared from usable shoots by snipping off the basal leaves and cultured them individually in 25 × 150 mm culture tubes with 15-20 ml of full strength MS medium supplemented with NAA or IBA (2.0-8.0 μM).

The rooted plantlets were transferred on to the small plastic pots containing sterilized soil mix (garden soil and compost in 1:1 ratio). Transferred plantlets were hardened in growth chamber condition for 25 days and then transferred to outdoor condition. The total number of plants transferred to the pots and the number of surviving plants in the outdoor condition were recorded.

All the cultures were maintained at 25 ± 2°C under a 16h light and 8h dark cycle with the light intensity of 2000-3000 lux provided by cool-white fluorescent tubes (36 W). Data were recorded after 8 weeks of culture except for rooting experiment when the data were recorded after 4 weeks of incubation. In all the experiments, 12-15 explants were used and each experiment was repeated three times. Mean and standard error were calculated for all numerical data. The mean data of each treatment were compared by using Duncan's Multiple Range Test (DMRT) at P=0.05%.

Results and discussion

Cotyledonary node showed the best shoot proliferation efficiency irrespective of media type, which followed by leaf node and shoot tip explants (Tab. 1). Cotyledonary nodes showed the maximum 99.33 ± 0.67% response and produced 5.10 ± 0.58 shoots on MS + 4.0 μM BA (Fig. 1B) while the highest 80.67 ± 1.33% leaf nodes and 51.67 ± 1.28% shoot tips formed maximum 1.50 ± 0.40 and 0.70 ± 0.33 shoots, respectively on the same media formulation. Results of this experiment indicated the high regenerative capacity of cotyledonary node explants of *A. mangium* than leaf node and shoot tip explants. Similar results were noted in a range of legume species, such as *A. senegal* (Khalafalla and Daffalla, 2008), *A. sinuata* (Vengadesan *et al.*, 2002b), *Clitoria ternatea* (Barik *et al.,* 2007), *Colutea istria* (Hegazi and Gabar, 2010), *Dalbergia sissoo* (Pradhan *et al.*, 1998a), *D. latifolia* (Pradhan *et al.*, 1998b), *Pterocarpus marsupium* (Anis *et al.,* 2005), *P. santalinus* (Rajeswari and Paliwal, 2008) and *Peltophorum pterocarpum* (Uddin *et al.*, 2005). On the contrary of these findings, Amin *et al.* (1992) in carambola, Ara *et al.* (1991) in *Sesbania grandiflora,* Loh and Rao (1989) in guava and Rahman and Blake (1988) in jackfruit observed increased shoot proliferation from the seedling explants rather than cotyledonary nodes. Debergh and Read (1990) suggested that differential responses of different explants of the same plant are more species specific while Lane (1978) proposed the endogenous hormone level in buds of different regions of the stem as a reason of differential responses of different explants of the same plant. In case of *A. mangium*, further investigations, based on the present findings, should be carried out to find out the specific reason behind the differential responses of different explants.

The full strength MS medium affected shoot proliferation from cotyledonary node explants significantly than other three media (WPM, MMS_1 and MMS_2) tested. Maximum 98.67 ± 0.37 % explants produced highest 3.93 ± 0.37 shoots on MS medium. Although explants produced longest shoots (1.63 ± 0.34 cm) in WPM medium, but both percentage of response and multiplication rate were lower in this medium than full strength MS medium. This study revealed that full strength MS medium was preferred for axillary shoot proliferation from cotyledonary nodes of *A. mangium* while WPM showed a little effect in terms of shoot proliferation. Full strength MS medium has been proved best for axillary shoot proliferation in many other *Acacia* species, including *A. albida* (Ruredzo and Hanson, 1993), *A. catechu* (Kaur *et al.*, 1998), *A. mearnsii* (Huang *et al.*, 1994), *A. nilotica* (Abbas *et al.*, 2010), *A. salicina, A. saligna* and *A. sclerosperma* (Jones *et al.*, 1990). Similar results were also observed in some other woody

Tab. 1. Effects of three different explants, cultured on MS medium containing 4.0 μM BA alone or in combination with 0.5 μM NAA, on axillary shoot proliferation of *A. mangium*

Type of explant	Plant growth regulators (μM)	Percentage of explant responded (X ± SE)	Number of shoots (X ± SE)
Cotyledonary node	BA 4.0	99.33 ± 0.67 a	5.10 ± 0.58 a
	BA 4.0 + NAA 0.5	89.33 ± 2.33 b	2.40 ± 0.40 b
Leaf node	BA 4.0	80.67 ± 1.33 c	1.50 ± 0.40 bc
	BA 4.0 + NAA 0.5	80.00 ± 1.61 c	1.20 ± 0.29 cd
Shoot tip	BA 4.0	51.67 ± 1.28 d	0.70 ± 0.33 cd
	BA 4.0 + NAA 0.5	42.67 ± 2.33 e	0.30 ± 0.15 d

Note: Values represent means ± standard error of 20 explants per treatment in three repeated experiments. Means followed by the same letters are not significantly different by Duncan's multiple Range Test at 0.05 % probability level

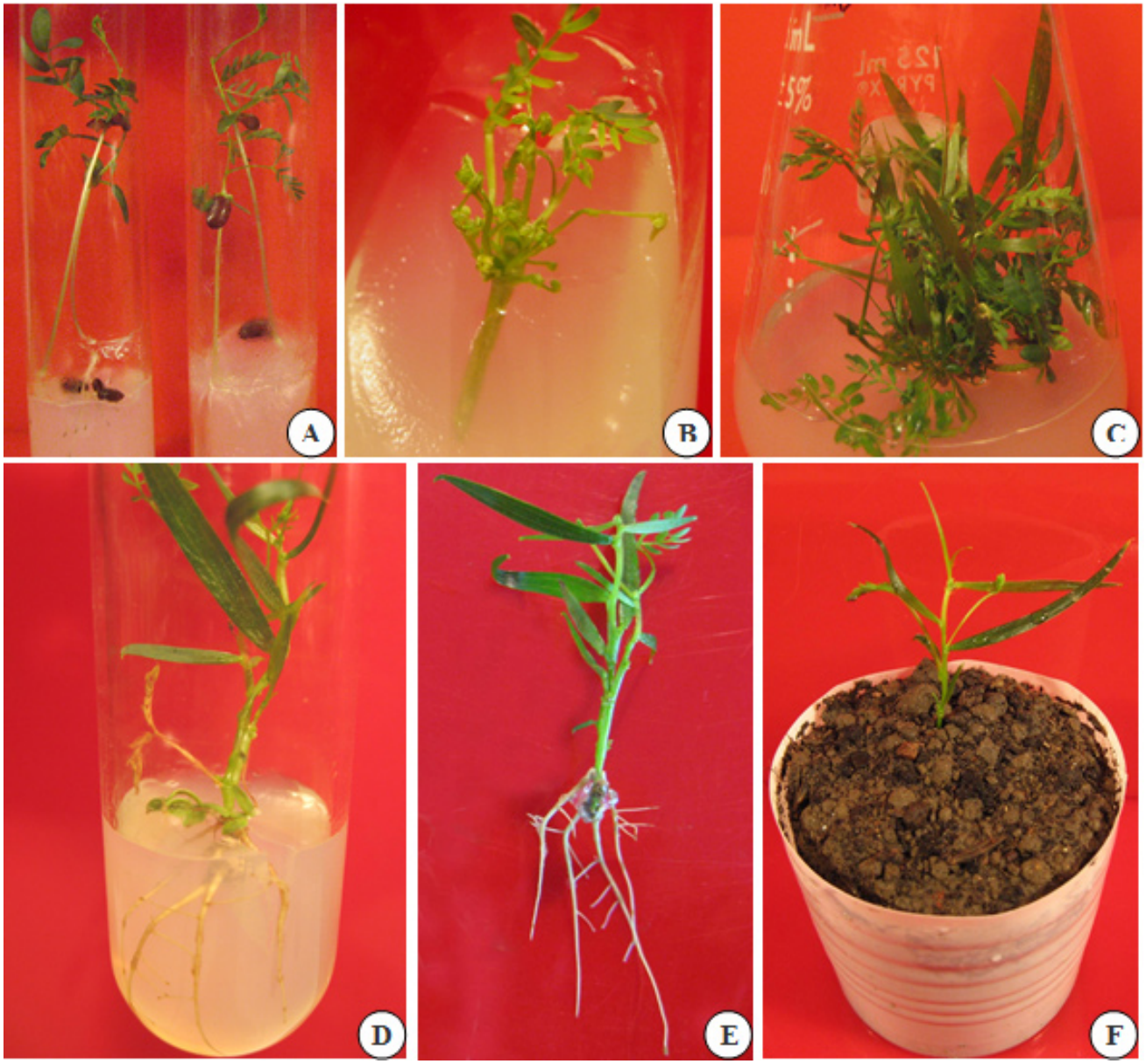

Fig. 1. *In vitro* propagation of *A. mangium* Willd. from cotyledonary node explants. A. Two-week old aseptically germinated seedlings; B. Multiple shoot formation from cotyledonary node explant after 4 weeks of culture on MS + 4.0 μM BA; C. *In vitro* proliferated shoots from cotyledonary node explant after 8 weeks of culture on MS + 4.0 μM BA; D. Rooted plantlets ready for transplantation; E. Acclimatized plantlets after 7 days of transplantation onto soil mix

trees, like *Colutea istria* (Hegazi and Gabr, 2010), *Dalbergia latifolia* (Swamy *et al.,* 1992), *Lagerstromia parviflora* (Tiwari *et al.,* 2002), *Pterocarpus marsupium* (Hussain *et al.*, 2008), *P. santalinus* (Rajeswari and Paliwal, 2008), *Sterculia urens* (Hussain *et al.,* 2007) and *Swartzia madegascariensis* (Berger and Schaffner, 1995).

Cotyledonary node explants cultured on MS medium devoid of growth regulator produced 0.92 ± 0.19 shoots with 0.64 ± 0.14 cm lengths, which may be due to the presence of endogenous cytokinin in cotyledonary nodes, suggested by Rajeswari and Paliwal (2008) in *Pterocarpus santalinus*. However, the addition of exogenous cytokinin to MS medium induced shoot multiplication rate remarkably, indicating the requirement of exogenous cytokinin supply in the medium for better axillary shoot proliferation. Out of two different cytokinin-BA and Kn, best shoot proliferation was observed on medium containing BA. Highest number of shoots (4.08 ± 0.60) and longest shoots (1.20 ± 0.15 cm) were formed on 4.0 μM BA containing medium (Fig. 1. C) while 3.25 ± 0.43 shoots with maximum 1.16 ± 0.18 cm length were found in medium containing 6.0 μM Kn. The results indicated that BA was superior to Kn for axillary shoot proliferation from cotyledonary nodes of *A. mangium*. The superiority of BA over Kn has also been reported in *in vitro* propagation of other species of *Acacia* (Badji *et al.,* 1993; Beck *et al.,* 1998; Dewan *et al.,* 1992;

Tab. 2. Effects of different basal media containing 4 µM BA on *in vitro* shoot multiplication from cotyledonary node explants of *A. mangium*

Basal medium[q]	Percentage of explant responded ($\bar{X}$ ± SE)	Number of shoots ($\bar{X}$ ± SE)	Length of shoots (cm) ($\bar{X}$ ± SE)
MS	98.67 ± 0.37 a	3.93 ± 0.37 a	1.43 ± 0.18 ab
MMS_1	98.33 ± 1.67 a	2.87 ± 0.36 ab	0.90 ± 0.13 bc
MMS_2	63.33 ± 1.69 c	2.13 ± 0.55 b	0.61 ± 0.18 c
WPM	78.33 ± 2.41 b	2.87 ± 0.52 ab	1.63 ± 0.34 a

Note: Values represent means ± standard error of 20 explants per treatment in three repeated experiments. Means followed by the same letters are not significantly different by Duncan's multiple Range Test at 0.05% probability level. [q]MS = Full strength MS medium; MMS_1 = MS with ½ strength of major salts only; MMS_2 = MS with ½ strength of both major and minor salts

Tab. 3. Effects of plant growth regulators on axillary shoot proliferation from cotyledonary node explants of *A. mangium*

Plant growth regulators (µM)				Number of shoots ($\bar{X}$ ± SE)	Length of shoots (cm) ($\bar{X}$ ± SE)
BA	Kn				
0	0			0.92 ± 0.19 hi	0.64 ± 0.14 bcd
2.0	-			1.42 ± 0.19 ghi	0.86 ± 0.10 bcd
4.0	-			4.08 ± 0.60 a	1.20 ± 0.15 b
6.0	-			1.67 ± 0.43 fghi	0.63 ± 0.19 bcd
8.0	-			2.42 ± 0.29 bcdefg	1.05 ± 0.14 bcd
-	2.0			1.17 ± 0.21 ghi	0.80 ± 0.16 bcd
-	4.0			1.58 ± 0.23 fghi	0.85 ± 0.16 bcd
-	6.0			3.25 ± 0.43 abcd	1.16 ± 0.18 bc
-	8.0			2.08 ± 0.34 defgh	0.83 ± 0.12 bcd
BA	NAA	IBA	GA_3		
2.0	0.5	-	-	1.25 ± 0.25 ghi	0.85 ± 0.19 bcd
2.0	1.0	-	-	1.08 ± 0.26 ghi	0.83 ± 0.18 bcd
2.0	2.0	-	-	0.67 ± 0.19 i	0.59 ± 0.16 cd
4.0	0.5	-	-	3.75 ± 0.70 a	1.00 ± 0.10 bcd
4.0	1.0	-	-	2.25 ± 0.45 cdefgh	0.94 ± 0.09 bcd
4.0	2.0	-	-	1.92 ± 0.43 efghi	0.66 ± 0.13 bcd
2.0	-	0.5	-	1.08 ± 0.26 ghi	0.66 ± 0.15 bcd
2.0	-	1.0	-	0.92 ± 0.23 hi	0.49 ± 0.18 d
2.0	-	2.0	-	0.67 ± 0.19 i	0.48 ± 0.13 d
4.0	-	0.5	-	3.50 ± 0.67 abc	0.86 ± 0.14 bcd
4.0	-	1.0	-	2.25 ± 0.46 cdefgh	0.79 ± 0.14 bcd
4.0	-	2.0	-	1.67 ± 0.41 fghi	0.71 ± 0.15 bcd
2.0	-	-	0.5	1.38 ± 0.32 ghi	1.72 ± 0.32 a
2.0	-	-	1.0	1.13 ± 0.23 ghi	0.73 ± 0.18 bcd
2.0	-	-	2.0	0.88 ± 0.23 hi	0.65 ± 0.18 bcd
4.0	-	-	0.5	2.88 ± 0.61 abcdef	1.82 ± 0.17 a
4.0	-	-	1.0	3.63 ± 0.65 ab	2.09 ± 0.30 a
4.0	-	-	2.0	3.13 ± 0.55 abcde	0.96 ± 0.21 bcd

Note: Values represent means ± standard error of 20 explants per treatment. Means followed by the same letters are not significantly different by Duncan's multiple Range Test at 0.05% probability level

Galiana *et al.,* 1991; Junior *et al.,* 2004; Khalafalla and Daffalla, 2008; Mittal *et al.,* 1989; Nandwani, 1995; Rout *et al.,* 2008; Singh *et al.,* 1993; Vengadesan *et al.,* 2002b). In addition, Jeyakumar and Jayabalan (2002) in *Psoralea corylifolia*, Husain *et al.* (2008) in *Pterocarpus marsupium*, Pradhan *et al.* (1998a) in *Dalbergia sissoo*, Rajeswari and Paliwal (2008) in *Pterocarpus santalinus*, Shyamkumar *et al.* (2003) in *Terminalia chebula*, Widiyanto *et al.* (2008) in *Albizia falcataria* also observed the superiority of BA over Kn on axillary shoot proliferation from seedling explants. On the contrary, Nandwani and Ramawat (1993) in *Prosopsis cinerarea* and Kumar (1992) in *Bauhinia purpurea* found Kn as superior cytokinin to BA in *in vitro* shoot multiplication. The differential effects of BA and Kn on *in vitro* axillay shoot proliferation might be due to the different mode of action of BA and Kn during shoot development (Widiyanto *et al.,* 2008).

Inclusion of NAA or IBA along with BA had no significant effect on both proliferation and elongation of axillary shoots. Average number of shoots and average length of shoots both were markedly reduced in all auxin-cytokinin combinations. Cotyledonary nodes produced highest 3.75 ± 0.70 shoots on medium containing 4.0 µM BA + 0.5 µM NAA and the length of shoots was 1.00 ± 0.10 cm in that medium. The results revealed that exogenous auxin was not essential to initiate shoot bud formation, which also indicated the antagonistic effect of NAA or IBA with BA on *in vitro* shoot proliferation of *A. mangium*. Vengadesan *et al.* (2002b) also observed that auxins (NAA, IBA and IAA) along with BA were not effective for shoot proliferation from cotyledonary nodes of *Acacia sinuata*. In *Acacia senegal* similar results were reported by Khalafalla and Daffalla (2008). This finding is also in agreement with Mallikarjuna and Rajendrudu (2009) in *Holarrhena antidysenterica,* Hussain *et al.* (2007) in *Sterculia urens*. Garland and Stoltz (1981) demonstrated that

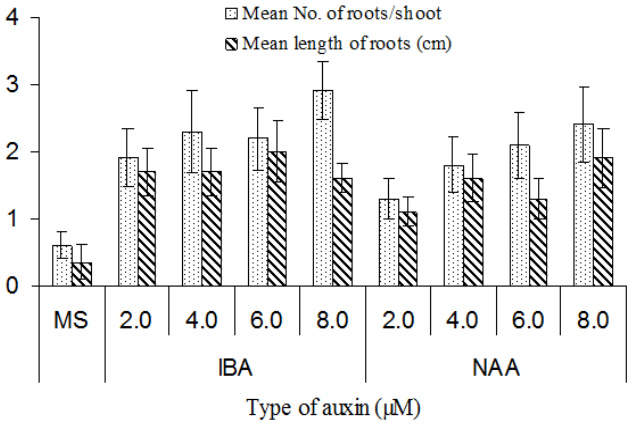

Fig. 2. Effects of auxins (IBA and NAA) on adventitious rooting of *in vitro* derived microshoots of *A. mangium*

in a number of cases, cytokinins alone are enough for optimal shoot multiplication as also indicated by the works of Amin and Jaiswal (1993) and Aliou *et al.* (2006). Many researchers suggested that incorporation of low level auxin with BA enhanced shoot induction in different tree species, including *Acacia catechu* (Kaur *et al.,* 1998), *Acacia seyal* (Al-wasel, 2000), *Acacia tortilis* (Nandwani, 1995), *Aegle marmelos* (Nayak *et al.*, 2007), *Colutea istria* (Hegazi and Gabr, 2010), *Nyctanthes arbor-tristis* (Siddique *et al.*, 2006), *Pterocarpus marsupium* (Husain *et al.*, 2008) and *Terminalia belerica* (Rathore *et al.,* 2008).

The effect of GA_3 in combination with BA revealed that GA_3 had no stimulatory effect on the shoot multiplication although shoot elongation was somewhat enhanced by the addition of GA_3. Shoot length was increased maximum 0.89 cm upon the addition of 1.0 μM GA_3 to the medium containing 4.0 μM BA. When GA_3 was incorporated at higher concentration than 1.0 μM, explants showed no elongation of shoots rather reduction in shoot number. Similar observation was recorded by Bhuyan *et al.* (1997) in *Murraya koenigii*, who observed maximum 0.6 cm elongation upon the addition of 0.4 mg/l (1.15 μM) GA_3 to the medium containing 5.0 mg/l (22.2 μM) BA. Vengadesan *et al.* (2000, 2002a, 2002b and 2003) also demonstrated that GA_3 at low level could enhance shoot elongation in *Acacia sinuata*.

Microshoots attained 2-4 cm in length were cultured on auxin free medium and poor rooting was observed in auxin free MS medium. Rooting frequency was enhanced considerably when either IBA or NAA at different concentrations were added to MS medium. The use of IBA in the culture medium remarkably influenced the rate of root induction (Fig. 1D) than NAA. Among the different concentrations of IBA tested, maximum number of roots per shoot and longest roots having considerable lateral roots were obtained with 8.0 μM IBA (Fig. 1E). NAA was found to be less effective than IBA regarding rooting of microshoots. However, the roots produced by NAA were thin, delicate and hairy in nature. In this study, IBA was proved to be best auxin as comparable to NAA with regard to all rooting parameters. Documented literature shows that IBA has been found suitable for rooting in a number of tree species like *Acacia mearnsii* (Beck *et al.*, 1998), *Acacia nilotica* (Dhabhai *et al.*, 2010), *Acacia senegal* (Khalafalla and Daffalla, 2008), *Acacia tortilis* (Ali, 2009; Nandwani, 1995), *Bauhinia variegata* (Mathur and Mukunthakumar, 1996), *Dalbergia latifolia* (Swamy *et al.*, 1992), *D. sissoo* (Joshi *et al.*, 2003; Pradhan *et al.*, 1998a), *Parkinsonia aculeata* (Mathur and Mukunthakumar, 1996), *Phellodendron amurense* (Azad *et. al.*, 2005, 2009), *Pterocarpus marsupium* (Husain *et al.*, 2008) and *Syzygium cuminii* (Yadav *et al.*, 1990).

After successful rooting of microshoots, attempts were taken to establish regenerated plantlets onto soil. Plantlets had been transferred to small plastic pots containing soil mix (garden soil: compost, 1:1) and maintained under humid *ex vitro* condition in the growth room (Fig. 1F). The *in vitro* derived plantlets acclimated better under *ex vitro* condition when they were maintained in growth room for 25 days before transferring them to outdoor condition. Finally, 65% transplanted plantlets were survived and acclimated well under *ex vitro* condition after 25 days of transplantation.

Conclusions

The described strategy demonstrates an efficient system of *in vitro* clonal propagation of *A. mangium* via cotyledonary node explants, which could play a significant role in large scale plantlet production all around the year, as well as in wide plantation and in conservation of this plant's genetic resources.

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