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, 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; Hussain 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% HgCl₂ 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 x 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...
5.10 ± 0.58 a
0.30 ± 0.15 d
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99.33 ± 0.67 a
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51.67 ± 1.28 d
2.40 ± 0.40 b
80.00 ± 1.61 c
0.70 ± 0.33 cd
80.67 ± 1.33 c
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1.50 ± 0.40 bc
2
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regenerative capacity of cotyledonary node explants of
formulation. Results of this experiment indicated the high
and 0.70 ± 0.33 shoots, respectively on the same media
51.67 ± 1.28% shoot tips formed maximum 1.50 ± 0.40
and produced 5.10 ± 0.58 shoots on MS + 4.0 µM BA.
ary nodes showed the maximum 99.33 ± 0.67% response
by leaf node and shoot tip explants (T ab. 1). Cotyledon
Comparison efficiency irrespective of media type, which followed
cotyledonary nodes were initially cultured on four different basal medium viz. MS, MMS, MMS, 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 GA3 (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. Results 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 istora (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 specific 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, and MMS) 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 |
| Leaf node | BA 4.0 + NAA 0.5 | 89.33 ± 2.33 b | 2.40 ± 0.40 b |
| Shoot tip | 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 |

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.
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 madagascarensis* (Berger and Schaffner, 1995).

Cotyledonal 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 cotyledonal 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 cotyledonal 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 cotyledonal node explants of A. mangium

| Basal medium | Percentage of explant responded (X ± SE) | Number of shoots (X ± SE) | Length of shoots (cm) (X ± SE) |
|--------------|----------------------------------------|---------------------------|-----------------------------|
| MS           | 98.67 ± 0.37 a                         | 3.93 ± 0.37 a             | 1.43 ± 0.18 ab              |
| MMS<sub>1</sub> | 98.33 ± 1.67 a                     | 2.87 ± 0.36 ab            | 0.90 ± 0.13 bc              |
| MMS<sub>2</sub> | 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.

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

| Plant growth regulators (µM) | Number of shoots (X ± SE) | Length of shoots (cm) (X ± SE) |
|-----------------------------|---------------------------|-------------------------------|
| BA | Kn | | |
| 2.0 | 0 | - | 1.25 ± 0.25 ghi | 0.85 ± 0.19 bcd |
| 0.5 | - | - | 1.08 ± 0.26 ghi | 0.83 ± 0.18 bcd |
| 0.0 | - | - | 0.67 ± 0.19 i | 0.59 ± 0.16 cd |
| 0.4 | 2.0 | - | 3.75 ± 0.70 a | 1.00 ± 0.10 bcd |
| 1.0 | - | - | 2.25 ± 0.45 cdghi | 0.94 ± 0.09 bcd |
| 4.0 | 2.0 | - | 1.92 ± 0.45 cdghi | 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 cdghi | 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 abcede | 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, Jayakumar 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 Prosopis cinererea 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 axillary 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. Cotyledonal 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 cotyledonal nodes of Acacia sinuata. In Acacia sevolanica 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...
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 isteria* (Hegazi and Gabr, 2010), *Nyctanthes arbor-tristis* (Siddique et al., 2006), *Pterocarpus marsupium* (Husain et al., 2008) and *Terminalia bellerica* (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 seyal*.

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, fragile 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), *Baubinia variegata* (Mathur and Mukunthakumar, 1996), *Dalbergia latifolia* (Swamy et al., 1992), *D. sissoo* (Joshi et al., 2003; Pradhan et al., 1988a), *Parkinsonia aculeata* (Mathur and Mukunthakumar, 1996), *Phellodendron amurense* (Azad et al., 2005, 2009), *Pterocarpus marsupium* (Husain et al., 2008) and *Syzgium cumini* (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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