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Full Length Research Paper

Micropropagation of multipurpose medicinal tree *Acacia auriculiformis*

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Acacia auriculiformis **is a multipurpose leguminous tree of medicinal forestry importance. This plant is gaining importance because of its spermicidal and anti-HIV properties along with safe to use on vaginal epithelium. Clonal propagation of this pharmacologically important tree species via *in vitro* micropropagation is a prerequisite because propagation through seed route can otherwise lead to segregational loss of desirable traits. In the present study, multiple shoot induction was achieved from nodal stem segments of mature trees. Murashige and Skoog (1962) media (MS) fortified with or without phytohormones were evaluated for their suitability to support shoot induction, shoot multiplication and root formation. Highest percentage of multiple shoot induction was achieved on MS + BAP (2 mg/L) + NAA (0.1 mg/L). Elongated shoots showed best rooting response on ½ MS basal, when compared to other treatments imposed. Use of coco peat as hardening medium resulted in maximum survival (75%) during hardening phase. In the present study, simple and efficient *in vitro* micropropagation of the woody cum medicinal tree i.e, *A. auriculiformis* was achieved following direct organogenesis pathway of plant regeneration.**

Key words: *Acacia auriculiformis, in vitro* micropropagation, nodal stem, direct organogenesis.

INTRODUCTION

Acacia auriculiformis is a common (Indian) medicinal plant also called Black Wattle. This plant is known for its pharmacological properties notably anti-helminthic, anti-filarial, microbicidal activity etc. (Mandal et al., 2005; Ghosh et al., 1993). The mode of action of saponins (pharmacological chemicals) isolated from this medicinally important tree was reported to be because of induced membrane damage by generating superoxide anions and initiating lipid peroxidation (Sinhababu et al., 1997). Apart from the medicinal properties, this leguminous tree species also forms a major source of paper pulp, fuel wood and timber. It can thrive not only in poor soils but is also adapted well to areas with extended dry seasons (Anonymous, 1984). A decoction of the root is used to treat aches, pains and sore eyes while an infusion of the bark is used to treat rheumatism aborigines of Australia.

The seeds of this tree are rich source of triterpinoid saponins especially, Acaciaside-A (Ac-A) and Acaciaside-B (Ac-B). Earlier investigations have demonstrated that Ac-B isolate from seed extracts of *A. auriculiformis* possess spermicidal activity (anti-fertility) even at significantlylower concentrations (Pakrashi et al., 1991; Pal et al., 2009) especially safe for vaginal epithelia (Pal et al., 2009) and most importantly inhibits transmission of HIV (Kabir et al., 2008) without any mutagenic effect (Pal et al., 2009). These properties of saponin Ac-B from this tree mark its potential of being a major pharmacological medicine of the future. Bark extracts of *A. auriculiformis* also show significant pesticidal activity (Kaur et al., 2010).

Trees producing high concentrations of desirable metabolites like Ac-B need to be screened and propagated clonally. This is because seed is a product of genetic recombination it can results in segregation of alleles leading to loss of desirable traits (Fenner and Thompson, 2005). On the contrary, clonal multiplication forms an alternative means of propagating elite trees by preserving their desirable characters intact (Mittal et al., 1989). *In vitro* micropropagation using nodal stem segments from mature trees can results in clonal propagation of selected elite trees such as *A. auriculiformis* where other clonal propagation methods were not popular. Alternatively, tissue culture protocols enable *in vitro* exploitation of secondary metabolites (Karuppusamy, 2009). So, keeping these major

Table 1. Morphogenic response of axillary buds of *Acacia auriculiformis* after 2 months of culturing.

Medium	Explants inoculated	Explants survived	Explants response towards			
			Calli	Percentage	Shoots	Percentage
MS	25	18	14	78	4	22
MS+ 1BAP + 0.2 NAA	25	20	15	75	10	50
MS+ 2BAP + 0.1 NAA	25	21	8	38	16	76
MS+ 1BAP	25	16	12	75	7	43.7

Phytohormone concentrations are expresses in mg/L.

Table 2. Rhizogenesis from shoots of *Acacia auriculiformis* after 2 months of culturing in respective rooting medium.

Medium	No. of shoots inoculated	No. of shoots survived	Rooted shoots	Percent rooting
MS	15	12	3	25
½ MS	15	12	7	63.6
MS + 0.2 NAA	15	11	3	27
MS + 0.1 IAA	15	10	4	40
MS + 2BAP + 0.1 NAA (SIM medium)	15	13	0	0

Phytohormone concentrations are expresses in mg/L.

advantages of *in vitro* micropropagation in view, an attempt was made to develop an efficient tissue culture protocol for clonal propagation of desirable mature trees of *A. auriculiformis*.

MATERIALS AND METHODS

Plant material and surface sterilization

Nodal cuttings (approximately 3 cm length) from 18 months-old trees of *A. auriculiformis* were collected. The explants were first surface sterilized in 70% ethanol for 1 min following which the nodal stem cuttings were immersion in 1% sodium hypochlorite solution (v/v) containing few drops of Tween-20 and shaken for 15 min. Later, they were rinsed thrice (3 min per wash) with sterile distilled water.

Media and culture conditions

Murashige and Skoog (1962) (MS) basal medium was gelled either with 0.8% (w/v) bacteriological agar or 0.3% (w/v) of Phytagel/Gelrite. Sucrose (30 g/L) was used in all experiments as the source of carbohydrate. The pH of the medium was adjusted to 5.8 and the culture media was autoclaved at 121°C for 20 min at 15 lb. Filter sterilized phytohormones were added to the medium before pouring it into jam bottles (capacity: 300 mL volume). For all experiments, cultures were maintained under 16 h photoperiod and 8 h dark. Light intensity of 50 $\mu E/m^2/s$ was provided by white fluorescent tube lights and the room temperature was maintained at 28 ± 2°C.

MS media in combination with or without phytohormones was tried and the best combinations for *in vitro* regeneration are mentioned in Tables 1 and 2. For shoot induction studies, a total of 25 nodal stem segments were inoculated for each of the 4 treatments imposed. On the other hand root induction studies were carried with a total of 15 elongated shoots being inoculated per each of the 5 treatment under study. The results were expressed as percentage of explants responding to the treatment imposed.

Hardening

Rooted plantlets were removed from the culture jam bottles. After thorough washing of the roots under tap water, the plantlets were transferred to three different sterile soils prepared by mixing different constitutients as mentioned in Table 3. These plantlets were transferred into earthen pots with inverted jam bottles on top (in order to reduce evapo-transpiration losses) and kept in incubation room under controlled temperature (28 ± 2°C) for acclimatization.

RESULTS AND DISCUSSION

Shoot induction

The development of primordial shoot from the axil of nodal stem segment (especially in trees) is termed as bud break stage (BB). It took a minimum of one month for the initial response towards shoot proliferation from the inoculated nodal stem segments (Figure 1a). Multiple shoot formation was observed to be highest in MS fortified with 2 mg/L Benzyl amino purine (BAP) and 0.1 mg/L Naphthalene acetic acid (NAA). This composition is named as shoot induction medium (SIM) (Figure 1b). Both BB and multiple shoot induction occurred on SIM media, so there were no separate media compositions for these stages. Multiple shoots developed on nodal

Table 3. Effect of different hardening mediums on plant establishment.

Soil composition	No of plantlets hardened	No. of plants successfully acclimatized (%)	Remarks
Sand + Red soil + FYM (1:1:1)	12	5 (42)	Most of the plants died within the first week of transfer
Coco peat + Vermiculite (1:1)	10	6 (60)	Few plants survived
Coco peat	12	9 (75)	Highest survival recorded. Roots elongated while shoot growth was medium

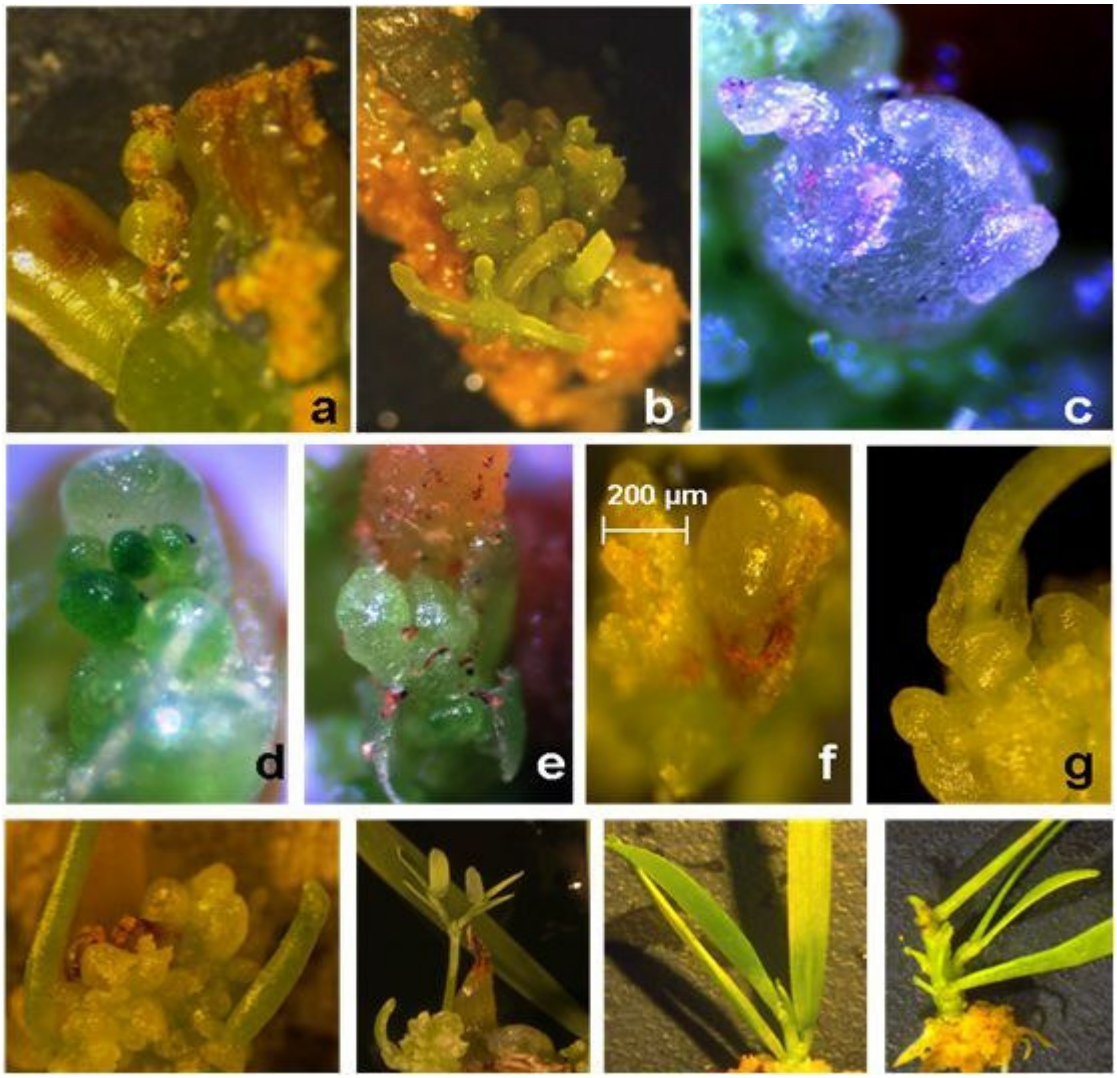

Figure 1. (a) Shoot induction or bud break stage from the center of 2-3 cm long 'Y' shaped nodal segments of A. auriculiformis. b) Multiple shoot induction stage in shoot induction medium (SIM). c) Top view of primordial shoot formation. d) Close view of primordial leaves and shoot initials @ 2 MAI. e and f) Side view of shoot induction revealing primordial leaves concealing the hidden shot apex. g) Shoot/leaf elongating from cup like aperture. h) Direct organogenesis pathway leading to the development of multiple shoot initials along with elongating shoots. i) Pair of pinnate leaves on elongated shoot. Also cluster of shoot initials along with newly elongating shoot can be seen. j) Well formed shoot with phyllodes. Note that the early formed pinnate leaves were lost and only phyllodes were left. k) Occurrence of multiple roots at the base of elongated shoot in root induction medium that is, ½ MS basal. Note the development of callus at the shoot cut end from where multiple roots originate.

explants in 20 to 30 days after the occurrence of BB. Thus, it took a minimum of 2 months after inoculation (MAI) (1 month for BB+ 1 month for multiple shoot induction) from nodal stem segments till the occurrence of multiple shoots. Shukor et al. (2000) reported shoot initiation occurred from nodal explants of *A. auriculiformis* even at low concentrations of BAP (0.1 to 0.5 mg/L). In concurrence to our observations, they also reported that the gestation period for *in vitro* shoot initiation was longer in *A. auriculiformis* when compared to other *Acacia species* especially *A. excelsa* and *A. crassicarpa.*

Though slight callusing was observed at cut ends of explants inoculated into all the concentrations tried, presence or absence of callus did not seem to have any effect on BB and differentiation of multiple shoots. However, we considered that, an explant developed callus only when ≥ 50% of the explant developed callus tissue (Figure 1b, j and k; Table 1). None of the shoots originated from callus. The superficial callus observed on nodal explants is white to yellow in colour, powdery and non-embryogenic in nature. It is well known that superficial callus formation on nodal stem segments is a common feature observed during *in vitro* regeneration of *A. auriculiformis* (Zhang et al., 1995; Mittal et al., 1989). Different stages of shoot initiation and morphogenesis were shown in Figure 1c to g. A maximum of 7 shoot initials were generated per nodal segment after a period of 3 MAI into SIM (Figure 1h and i).

Repeated subculturing for every 3-week interval in SIM promoted multiple shoot induction and elongation instead of fortnight interval. Though the base of explants is covered in yellow superficial callus, it is interesting to note that none of the multiple shoots originated from callus. After 3 MAI in SIM, the shoot elongated to 2 cm with a minimum of 2 pairs of phyllodes (Figure 1g to j). Up to 76% of nodal segments responded to shoot induction in SIM. Mittal et al. (1989) reported similar shoot induction percentage with Gamborg's B5 medium while Zhang et al. (1995) could report up to 36% shoot induction on MS medium.

Rooting

These elongated shoots (2 to 3 cm length) are aseptically separated and inoculation into different rooting media's (Table 2). Half strength MS basal without supplemented phytohormones showed best rooting response (63%) when compared to all other treatments evaluated in the present study (Figure 1k). Repeated sub culturing in rooting medium resulted in improved shoot development but deteriorated roots health and growth. It was observed that once the root initiation occurs, further subculturing lead to deterioration or death of roots. Prevention of subculturing after root induction phase encouraged further elongation and formation of new roots. Thus, preventing further subculturing after root induction encouraged elongation and formation of new roots.

Compared to earlier reports of Zhang et al. (1995) and Mittal et al. (1989), here we reported better rooting performance which was upto 63%. For unknown reasons, the use of Phytagel or Gelrite improved the shoot and root formation as well as their development when compared to the routine gelling agent namely bacteriological agar (0.8%). In our laboratory, it was observed that, *A. auriculiformis* nodal segments are easy to the establish, respond slowly with multiple shoots, hard survivors even in the absence of frequent sub culturing when compared to similar explants from other forestry trees such as *Eucalyptus camaldulensis* and *E. tereticornis*. None of the shoots, rooted even after 2 MAI into SIM. Instead, they continued developing into multiple shoots. Mittal et al. (1989) reported that the frequency of rhizogenesis was higher with NAA than Indole-3-acetic acid (IAA).

Hardening

Hardening is a slow and controlled processes of exposing or habituating the *in vitro* grown plantlets to outside environment. Hardening of rooted plants was 100% in coco peat. Hardening for 2 weeks under reduced evapo-transpiration losses improved the survival percentage (Table 3). Inverted jam bottles or transparent plastic cups when used to cover the rooted plants during hardening phase improved the initial survival in coco peat. Hardening conditions for *A. auriculiformis* were not reported by earlier (Mittal et al., 1989; Zhang et al., 1995).

Absence of origin of shoot or rhizogenesis from callus phase showed that the in vitro development of A. auriculiformis occurred through direct organogenesis pathway. Different *in vitro* regenerating protocols for *A. auriculiformis* were reported earlier, using various sources of explants such as cotyledons (Das et al., 1993), axillary buds from seedlings (Mittal et al., 1989), nodal stem segments from mature trees (Prasenjit and Roy, 2005; Ranga Rao and Prasad, 1991; Zhang et al., 1995). Our studies are in concurrence with the earlier observations on the occurrence of superficial callus that developed on the explant's surface as well as near the cut ends. The micropropagation method described here is a complete and efficient regeneration protocol unlike the earlier reports (Mittal et al., 1989; Zhang et al., 1995) which fall short in studying one or the other steps of micropropagation such as explant source, rooting percentage achieved and survival of micropropagated plants during hardening process.

The pharmaceutical importance of *A. auriculiformis* is growing at a fast pace in the modern world especially the triterpene glycoside fraction (Acaciaside-B) having virucidal and spermicidal activities which are already patented (Kabir et al., 2008). Having an important role in forestry and wood related industries, the tissue culture method described here can help in large scale clonal

micropropagation as well as enable genetic transformation of the desirable trees of Acacia auriculiformis without segregational loss of parental traits.

Conclusion

Our study reports the development of an efficient *in vitro* micropropagation technique which can cater the needs of clonally propagation of desirable/elite mature trees of *A. auriculiformis* with commercial importance. Best response for *in vitro* shoot induction and multiplication of *A. auriculiformis* was recorded on MS + 2 mg/L BAP + 0.1 mg/L NAA. Rooting in ½ MS followed by hardening in 100% coco peat resulted in better morphogenesis and survival, than other combinations studied. We could successfully micropropagate this important woody legume and multipurpose medicinal tree via direct organogenesis pathway of plant regeneration which could have the advantage of short time of culturing under *in vitro* conditions thereby reducing the chances to form somaclonal variants.

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