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Rapid micropropagation of a tree of arid forestry *Anogeissus acuminata*

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Abstract

Multiple shoots (16 - 20 shoots per explant) were induced from cotyledonary node region of *Anogeissus acuminata* (Roxb. ex DC.) Guill. & Perr. on Murashige and Skoog's (MS) medium containing IAA 0.1 mg l^{-1} + BAP 1.5 mg l^{-1} and ascorbic acid 50 mg l^{-1}, citric acid 25.0 mg l^{-1}, arginine 25 mg l^{-1} and adenine sulphate 25 mg l^{-1}. From the first node of seedling only 4 - 6 shoots per explant were proliferated. Segments of *in vitro* produced shoots were used as explants for further multiplication of shoots upto 16 successive cultures at an interval of 4 week on MS medium with IAA 0.1 mg l^{-1} + BAP 1.0 mg l^{-1} and additives. The original cotyledonary explant was repeatedly subcultured upto 4 times after harvesting crop of shoots, each time. *In vitro* produced shoots were rooted on half strength MS medium containing 0.5 mg l^{-1} IBA. Plantlets were transferred to pots. Other explants (cotyledons, hypocotyl, and leaf) produced callus on medium containing auxins and cytokinins. The calluses differentiated into embryo like structures or roots on MS medium.

Introduction

Anogeissus acuminata (Roxb. ex DC.) Guill. & Perr. is a majestic hardwood tree with a straight cylindrical bole and girth upto 2 m; it belongs to family *Combretaceae*. It grows upto height of 18 m, on the plains of Thar Desert and at foothills of the Aravallis, representing an important component of stressed ecosystem and arid forestry. Propagation of *A. acuminata* in nature is through seed but only 0.5 - 1.0 % of seeds were found to be viable. This is a common problem also in other species of *Anogeissus* (Joshi *et al.* 1991). Due to overexploitation and poor

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Abbreviations: BAP - benzylaminopurine; IBA - indolebutyric acid; NAA - naphtaleneacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid.

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propagation, the population of this plant is declining with alarming rate. Most of the trees are older than 100 years. Conventional methods of cutting, budding, grafting and air layering are not available for these species. Tissue cultures offer alternative methods of propagation for forest trees (Thorpe 1985, Boulay 1985, Chalupa 1988, 1990). *In vitro* regeneration of two tree species of family *Combretaceae* have been already reported (Roy *et al.* 1987, Joshi *et al.* 1991). We report here a method for micropropagation of *Anogeissus acuminata.*

Materials and methods

Fruits of *Anogeissus acuminata* were collected from village Supka (Dist. Nagaur), 250 km north-east of Jodhpur. Viable seeds were collected by dissecting out of the fruit as recently described (Joshi *et al.* 1991). Viable seeds were surface sterilized with 70 % ethanol for 90 s followed by 0.1 % mercuric chloride for 3 min. Surface sterilized seeds were washed 8 - 10 times with sterile water, and inoculated for germination on Murashige and Skoog's (1962) basal medium. Four weeks old *in vitro* grown seedlings were used as source of explants (cotyledons, hypocotyl, leaves, root and cotyledonary and shoot segments each with one node). The explants were cultured on MS medium supplemented with auxins - IAA, NAA or 2,4-D (0.05 - 2.5 mg l^{-1}) and cytokinins - kinetin or BAP (0.05 - 5.0 mg l^{-1}). Ascorbic acid 50 mg l^{-1}, citric acid 25 mg l^{-1}, arginine 25 mg l^{-1}, adenine sulphate 25 mg l^{-1}, saccharose (3.0 %) and agar (0.8 %) were added to culture medium. The pH of medium was adjusted to 5.8 before autoclaving. Each treatment consisted of 15 replicates and each experiment was repeated three times. For induction and maintenance of callus the cultures were kept in the dark, while for induction and multiplication of shoots a 12 h photoperiod (irradiance 35 μmol m^{-2} s^{-1}) was provided at 28 ± 2 °C.

Shoots differentiated *in vitro* were cut into segments each of 1.5 - 2.0 cm and having one node. For further multiplication of shoots these segments were cultured on MS medium containing IAA 0.1 mg l^{-1} + BAP 1.0 mg l^{-1} and additives. Subculture was performed every fourth week. The original cotyledonary explant was repeatedly subcultured after harvesting shoots.

Shoots measuring 2.0 - 3.0 cm were cultured on full, half and 1/4 strength MS medium with IAA, IBA or NAA (0.1 - 2.5 mg l^{-1}) for root induction. Initially cultures were kept in dark for 3 - 5 d for root induction and later kept under irradiance of 15 μmol m^{-2} s^{-1} at 28 ± 2 °C. Plantlets (8 - 10 cm) with actively growing roots were carefully removed from culture vessels and washed thoroughly with tap water to remove adhered nutrient-agar medium, and then treated with 0.05 % *Bavistin* for 3 min, followed by washing with distilled water. Vermiculite and sandy soil (1:4 v/v) were used as potting mixture. Plantlets were hardened at 30 °C, under irradiance of 43 - 57 μmol m^{-2} s^{-1} at a 12 h photoperiod for 15 d and later transferred to field.

Results and discussion

We collected about 300 000 fruits of *Anogeissus acuminata* during all the seasons. Mechanical removal of fruit wall was proved to be the only suitable and quick method to collect and identify viable seed. Only 0.5 -1.0 % of fruits contained viable seeds.

Cotyledonary nodal segments were found to be the best explant for multiple shoot induction, which is similar as in *A. pendula* (Joshi *et al.* 1991). On MS medium containing 0.1 mg l^{-1} IAA + 1.5 mg l^{-1} BAP + additives, 16 - 20 shoots per explant were differentiated (Fig. 1.). Only 4 - 6 shoots differentiated from the first node containing shoot segments. On 2.5 - 5.0 mg l^{-1} of BAP about 30 - 40 shoot primordia were induced from cotyledonary node region, but these did not elongate and remained dwarf. On lower concentration of BAP or Kn (0.1 - 0.5 mg l^{-1}) a limited number of shoots were produced (Table 1). Incorporation of NAA 0.1 mg l^{-1} instead of IAA in the shoot multiplication medium caused callusing of the explant.

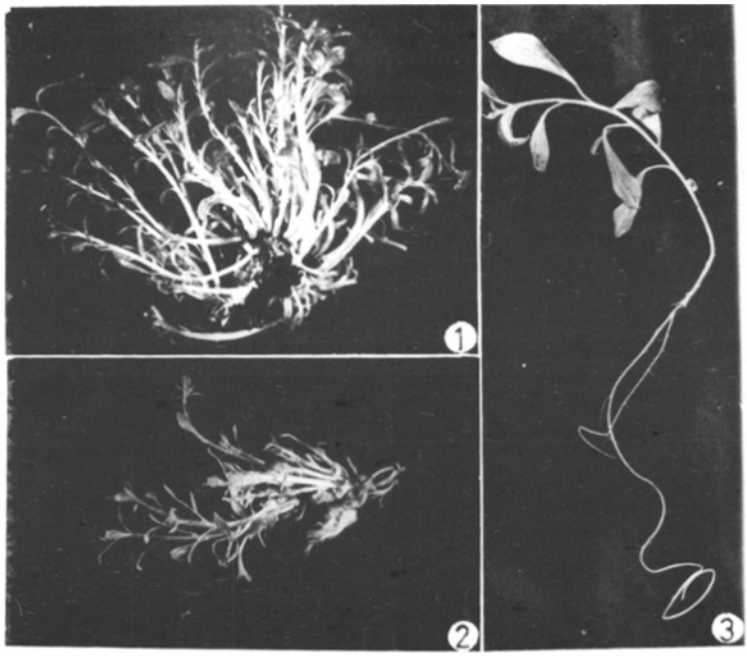

Fig. 1. *In vitro* regeneration of *Anogeissus acuminata*. Multiple shoots from cotyledonary nodal segment on MS medium containing IAA 0.1 mg l^{-1} + BAP 1.5 mg l^{-1} + additives (4 weeks old).
Fig. 2. Multiple shoots produced from subcultured shoot segment on MS medium + IAA 0.1 mg l^{-1} + BAP 1.0 mg l^{-1} + additives.
Fig. 3. Rooted plantlet on half strength MS medium containing IBA 0.5 mg l^{-1}.

When pieces of *in vitro* differentiated shoots were used as explants, 6 - 8 shoots developed from nodal region of each segment on MS medium containing 0.1 mg l^{-1}

IAA + 1.0 mg l^{-1} BAP and additives. Differentiated shoot segments of upto 16 generations were multiplied without showing any decline in growth. The original cotyledonary explant could be repeatedly subcultured on fresh culture medium 4 times. Each time a fresh crop of shoots (Fig.2) was harvested. This method may be useful for semi-automation for large scale production of plants as it avoids isolation and culture of explant. The original explant adapts to the culture conditions and proliferate rapidly. Sharma and Chatuverdi (1988) and Aitken-Christie and Jones (1987) recommended this technique for large scale propagation of other woody species. Subculturing on fresh medium within a period of 4 - 5 weeks was found essential for healthy growth. Delay in subculturing resulted in decline of shoot growth and increased mortality. Probably, depletion of nutrients and overcrowding in vessels stressed the cultures. The stressed tissues produced toxic gases and factors which caused death of the cultures similarly as in *Tecomella undulata* (Rathore *et al.* 1991) cultures. Incorporation of additives like ascorbic acid, citric acid, arginine and adenine sulphate supported shoot growth and increased number of shoots. Ascorbic acid and citric acid are known for their potency to decrease browning and to increase differentiation in cultures (Krikorian 1988, Joy *et al.* 1988).

Table 1. Effect of cytokinins on the multiple shoot induction from the cotyledonary nodal segment of *A. acuminata* on MS medium with 0.1 mg l^{-1} IAA, 50 mg l^{-1} ascorbic acid, 25 mg l^{-1} citric acid, 25 mg l^{-1} arginine and 25 mg l^{-1} adenine sulphate. Results were obtained after four weeks, each treatment consists of 15 replicates and was repeated three times.

Cytokinin [mg l^{-1}]	Shoot number per explant	Shoot length [cm]	Remark
Control	1.4 ± 0.5	2.2 ± 0.4	roots induced from explant
Kinetin			
0.1	1.3 ± 0.4	1.9 ± 0.4	callus and roots from explant
0.5	2.1 ± 0.7	2.3 ± 0.5	callus from cut base
1.0	3.8 ± 0.6	2.5 ± 0.6	thin shoot, callus, broad leaf
1.5	4.6 ± 0.7	2.7 ± 0.6	thin shoot, long internode
2.0	6.3 ± 0.9	2.6 ± 0.7	fleshy shoot, broad leaf
2.5	7.9 ± 1.4	2.4 ± 0.5	fleshy shoot, normal leaf
5.0	11.5 ± 3.2	1.8 ± 0.6	fleshy shoot, small leaf
BAP			
0.1	1.9 ± 0.6	2.0 ± 0.5	roots and callus from explant base
0.5	5.9 ± 1.3	2.2 ± 0.7	roots and callus from explant base
1.0	11.4 ± 1.8	2.4 ± 0.8	little callus, fleshy shoots
1.5	17.9 ± 2.4	2.5 ± 0.8	no callus, fleshy shoots
2.0	20.5 ± 3.1	2.1 ± 0.6	no callus, fleshy shoots
2.5	24.8 ± 4.8	1.6 ± 0.4	condensed internode, small leaf
5.0	33.4 ± 6.7	1.0 ± 0.5	condensed internode, small leaf

All types of explants produced callus on MS medium containing 0.5 - 1.0 mg l^{-1} NAA + 0.1 mg l^{-1} kinetin or 0.5 mg .l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin. Callus cultures

initiated from cotyledons and leaves were compact, green, and organised. These cultures differentiated into embryo like structures or roots on full and half strength MS medium with 0.5 - 2.5 mg l^{-1} BAP. The embryoid like structures proliferated in rossette fashion and showed extensive dichotomy. Callus cultures initiated from root segments were soft, granular and creamish. These differentiated only into roots, on all combinations of auxins and cytokinins used. Callus culture could be multiplied on MS medium containing 0.5 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin, or 0.25 mg l^{-1} 2,4-D + 0.1 mg l^{-1} kinetin.

Table 2. Effect of MS medium concentration and auxins (IAA, IBA or NAA) on root induction from differentiated shoots of *A. acuminata*.

Treatment, auxin (0.5 mg l^{-1})	Root number per shoot	Root length [cm]	Shoot length [cm]
MS	1.36 ± 0.49	2.20 ± 0.17	2.86 ± 0.23
MS + IAA	1.41 ± 0.50	2.37 ± 0.26	3.10 ± 0.44
MS + IBA	1.54 ± 0.76	2.85 ± 0.38	3.12 ± 0.47
MS + NAA	1.60 ± 0.81	1.96 ± 0.40	2.74 ± 0.32
MS/2	1.65 ± 0.48	2.75 ± 0.20	3.50 ± 0.22
MS/2 + IAA	1.80 ± 0.40	2.97 ± 0.41	3.72 ± 0.33
MS/2 + IBA	3.65 ± 0.68	4.87 ± 0.51	4.88 ± 0.51
MS/2 + NAA	5.58 ± 0.70	2.50 ± 0.49	4.46 ± 0.24
MS/4	1.37 ± 0.50	2.70 ± 0.31	2.76 ± 0.22
MS/4 + IAA	1.69 ± 0.47	3.27 ± 0.46	2.85 ± 0.18
MS/4 + IBA	1.88 ± 0.68	3.42 ± 0.25	3.46 ± 0.31
MS/4 + NAA	2.71 ± 0.82	2.16 ± 0.20	3.20 ± 0.27

From various auxins (IAA, IBA and NAA) incorporated in different concentrations into full, half and one fourth strength MS medium 0.5 mg l^{-1} IBA incorporated in half strength MS medium was the best for rooting of isolated shoots (Fig. 3). Root induction was visible within a week and about 95% of shoots rooted within three weeks. On IAA or NAA containing medium the shoots rooted but callusing occurred from the bases of shoots. The roots developed were weak and fibrous on MS/2 + NAA (0.5 - 1.0 mg l^{-1}). Shoot and root growth was poor on one fourth MS medium containing IAA, IBA or NAA (Table 2). Initial incubation in darkness for 3 - 5 d favoured root initiation. Treatments supporting callusing as described by Gaspar and Coumans (1987) must be avoided. Plantlets with actively growing roots were the most suitable for pot transfer. Before transfer to the field, it was necessary to harden the plants at elevated temperature and reduced air humidity for two weeks. We transferred about 60 plantlets out of which 51 survived in the field.

Present protocol for micropropagation of *A. acuminata* is highly reproducible and high frequency of shoot and root induction could be obtained. From a single *in vitro* grown seedling, 500 - 600 plantlets could be obtained within 11 - 12 weeks. This method can be useful for making available propagules of the plants for arid zone afforestation.

References

Aitken-Christie, J., Jones, C.: Towards automation: Radiata pine shoot hedges *in vitro*. - Plant Cell Tissue Organ Cult. **8**: 185-196, 1987.

Boulay, M.: Some practical aspects and applications of the micropropagation of forest trees. - In: Proceedings of the International Symposium on "*In vitro* Propagation of Forest Tree Species". Pp. 51-81. A.R.F., Bologna 1985.

Chalupa, V.: Large scale micropropagation of *Quercus robur* L. using adenine-type cytokinins and thidiazuron to stimulate shoot proliferation. - Biol. Plant. **30**: 414-421, 1988.

Chalupa, V.: Micropropagation of hornbeam (*Carpinus betulus* L.) and ash (*Fraxinus excelsior* L.). - Biol. Plant. **32**: 332-338, 1990.

Gaspar, T., Coumans, M.: Root formation. - In: Bonga, J.M., Durzan, D.J. (ed.): Cell and Tissue Culture in Forestry. Volume 2. Pp. 202-217. Martinus Nijhoff Publishers, Dordrecht - Boston - Lancaster 1987.

Joshi, R., Shekhawat, N.S., Rathore, T.S.: Micropropagation of *Anogeissus pendula* Edgew. - an arid forest tree. - Indian J. exp. Biol. **29**: 615-618, 1991.

Joy, R.W., Patel, K.R., Thorpe, T.A.: Ascorbic acid enhancement of organogenesis in tobacco callus. - Plant Cell Tissue Organ Cult. **13**: 219-228, 1988.

Krikorian, A.D: Plant tissue culture: Perceptions and realities. - Proc. indian Acad. Sci. Plant Sci. **98**: 425-464, 1988.

Murashige, T. Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Rathore, T.S., Singh, R.P., Shekhawat, N.S.: Clonal propagation of desert teak (*Tecomella undulata*) through tissue culture. - Plant Sci. **15**: 217-222, 1991.

Roy, S.K., Pal, R.K., Das, S,K.: Propagation of a timber tree, *Terminalia bellerica* Roxb. by tissue culture. - Bangladesh J. Bot. **16**: 125-130, 1987.

Sharma, A.K., Chaturvedi, H.C.: Micropropagation of *Bougaivillea* × *Buttiana* Scarlet queen variegated by shoot tip culture. - Indian J. exp. Biol. **26**: 285-288, 1988.

Thorpe, T.A.: Application of tissue culture technology to forest tree improvement. - Forest. Chronicle **61**: 436-438, 1985.

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