Difficulties Arising During I*n Vitro* Propagation Through Mature Nodal Explant of *Acacia senegal* (L) Willd - An Arid Zone Tree Species

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***Abstract*: The present communication is about factors affecting *in vitro* regeneration of *Acacia senegal* through mature nodal explant. Various problems like leaching, contamination, leaf-fall were experienced during the *in vitro* propagation of *A. senegal.* Pretreatment of mature tree explant with chilled anti-oxidant solution (polyvinyl pyrollidone 150 mg l^{-1}, ascorbic acid 100 mg l^{-1} and citric acid 100 mg l^{-1}) helped to combat the problem of leaching. 70%v/v ethanol treatment was helpful against the excessive contamination and various additives like adenine sulphate, glutamine and ascorbic acid when added in the media were helpful in overcoming the problems of leaf fall and leaching. Growth regulators viz. BAP, Kn, IBA, NAA, 2,4-D, were used for desired morphogenetic responses in *in vitro* cultures.**

***Keywords*: *in vitro* propagation, *Acacia senegal*, problems, solutions.**

Abbreviations: *AA, ascorbic acid; AS, adenine sulphate; PVP, polyvinyl pyrollidone; BAP, 6 – benzylaminopurine; Kn, Kinetin, IBA, indole-3-butyric acid, NAA, -napthalene-acetic acid 2,4-D, 2,4-dichlorophenoxy acetic acid.*

1. Introduction

Acacia senegal (L.) willd., an economically important tree legume yielding gum arabic, meets all the requirements of social forestry. Because of the importance of nitrogen fixing trees and shrubs in programmes of re-forestation and development of derelict soils, there has been interest in including such leguminous tree species in these programmes. New foliage is very useful as forage, dried seeds are used as food by humans and It produces gum arabic, which is used as a food additive, in crafts, and as a cosmetic. The gum is used for soothing mucous membranes of the intestine and to treat inflammed skin. It is also reportedly used as for its astringent properties, to treat bleeding, bronchitis, diarrhea, gonorrhea, leprosy, typhoid fever and upper respiratory tract infections. Investigations were carried out to explore the morphogenetic potential of this woody leguminous tree (Hustache et al., 1986; Badji et al., 1993; Gupta et al., 1994; Kaur et al., 1996). In general the woody taxa are difficult to regenerate under *in vitro* conditions. However, some success has been achieved in recent years to regenerate several species of *Acacia* (Beck and Dunlop, 2001; Xie and Hong, 2001; Vengadesan et al.,2003). Rooting is recalcitrant in *in vitro* regenerated shoots of woody species. Difficulty in root formation has been reported earlier in some legumes (Mukhopadhyay and Bhojwani, 1978; Monteuuis, 2004) and also in some woody species (Gupta and Durzan,1985; Nandwani and Ramawat,1993). Rooting in the *in vitro* regenerated shoots by a dip treatment of auxin has been successfully attempted in *Wrightia tomentosa* (Purohit et al.,1994). In our endeavour to micropropagate *A. senegal* we also faced several problems and in the present communication an effort has been made to highlight the problems faced and their possible solutions. There are no "easy" rules to directly adopt a particular method or procedure *in toto*, as it may or may not work. Each case has to be investigated for its response(s) *ab initio* and various adaptation for procedures may be necessary for standardizing a protocol. The operations are labour intensive and time consuming. In the on-going account, there is an overview of details about several factors to be taken care of, which directly or indirectly affect the *in vitro* propagation of *A.senegal*.

2. Material and Methods

Nodal shoot segments bearing axillary buds, collected from mature 'elite tree', identified by the forest department, were used as explants. The shoots were carried to the laboratory in an ice-box under low temperature. Shoots were washed under running tap water, cut into one or two nodes segments, subsequently they were washed in 2% commercial detergent, extran solution, rinsed thoroughly in sterile distilled water and pretreated with chilled sterile antioxidant solution (PVP 150 mgl^{-1} , AA 100 mgl^{-1} and citric acid 100 mgl^{-1}) for one hour. The explants were then surface sterilized in 0.1% v/w aqueous mercuric chloride. The sterilized nodal explants were then transferred on MS-medium supplemented with growth regulators at various concentrations. Growth regulators used for experimentation were BAP, Kn, IAA, NAA. adenine sulphate and ascorbic acid (10-25 mgl^{-1}) were also added to the medium along with sucrose (20-30 g l^{-1}) and agar 8.0 g l^{-1}.

The cultures were incubated at 30±2°C under 16 hours photoperiod (2500-3000 lux) and 30-40% RH for 4 weeks. Six replicates with two explants per replicate for each treatment were taken and all experiments were repeated thrice. The cultures were regularly sub-cultured on fresh medium . Regular observations were made every week. Data were analysed statistically. Student's t-statistics was used to compare treatment means.

3. Observations and Results

Physiological heterogeneity of explants related to phenology and endogenous rhythms is one of the major problems with *in vitro* propagation of woody plants which lead to lack of repeatability of data. So it was thought desirable to find out the ideal time suitable for producing a large number of propagules from axillary buds which could be used to raise uniform plantation of this species. In nature the plant produces fresh axillary buds in the months of March-April as well as September-October. Axillary buds from all the months of the year were innoculated after surface sterilization on a shoot induction medium i.e. MS + BAP (4.0 mgl^{-1}) + NAA (0.5 mgl^{-1}) and other additives. It was observed that after four weeks of incubation under controlled environmental conditions about 12-15 shoot buds sprouted from explants sampled in the months of March and April and 10-12 shoot buds developed from the explants sampled in October and November (Table-1)

Another aspect studied was the incidence of contamination. Though around 10-12 shoot buds initiated in explants sampled in the months of September to November (post rainy season months), the contamination was very high as compared to samples taken in the months of March to May. Hence, the ideal months for sampling explants and achieving rapid multiplication of this genotype was found to be March to May, as the shoot buds produced in these months were very healthy and growing vigorously which facilitated their recycling for further production of numerous shoots.

To overcome the problem of heavy infection 70% v/v ethyl alcohol treatment was also given to the stem explants along with the 0.1% w/v aqueous mercuric chloride treatment.

Table 1 : Effect of time of sampling on *in vitro* bud proliferation from nodal explants of *Acacia senegal*

Media	:	MS + Sucrose (3%) + BAP (4.0 mg/l) + NAA (0.5 mg/l) + AS (25.0 mg/l) + AA (10.0 mg/l) + Glutamine (146.0 mg/l).
Inoculum	:	Mature nodal stem explant
Incubation	:	At 32 ± 2°C in 16 h photoperiod (2500-3000 lx) for 4 weeks.
January	2.33±0.55	10%
February	4.67±0.86	9%
March	9.83±0.79	7%
April	10.33±0.84	4%
May	8.0±0.66	2%
June	4.5±1.10	18%
July	3.17±1.23	48%

August	2.5±0.57	48%
September	4.17±0.79	46%
October	8.33±1.08	20%
November	9.17±0.79	17%
December	4.67±1.08	12%

* Data expressed as Mean±SE from 6 replicates

Whether the spines, size and orientation of explant hold any influence on induction of shoot buds, was investigated. After a series of experiments, it was concluded that maximal shoot bud proliferation was observed when explants with two nodes and with intact spines (Fig. 1a) were placed at an angle on the MS - medium enriched with BAP 4.0 mgl^{-1} NAA 0.5 mgl^{-1}, AS 25.0 mgl^{-1} , AA 10 mgl^{-1} and glutamine 146.0 mgl^{-1}.

Additives like adenine sulphate, ascorbic acid and glutamine played an important role in combating several problems in the *in vitro* cultures of *A.senegal.* Problem of leaching was very pronounced in the cultures of *A.senegal*. To overcome this problem care was taken right from bringing the explant from the fields. The explant was brought in an ice-box, pre-treated in chilled sterile anti-oxidant solution of AA 50 mgl^{-1} + citric acid 100 mgl^{-1} and PVP 100 mgl^{-1} for one hour and finally by adding ascorbic acid 10 mgl^{-1} in the culture media, the problem of leaching was overcome completely.

Another very significant problem faced in the cultures was the problem of leaf fall (Fig. 1b). Both adenine sulphate and glutamine played an important role in combating this problem. Adenine sulphate has a weak cytokinin effect and is found to re-inforce the effects of other cytokinins. Murashige (1974) also showed the effect of adenine sulphate on bud proliferation of various genera of orchids. Besides adenine sulphate, glutamine which is an amino acid involved in purine biosynthesis also played an important role in control of leaf-fall problem in the cultures of *A. senegal*. Glutamine at a concentration of 146.0 mgl^{-1} and adenine sulphate 25.0 mgl^{-1} were most effective in controlling the leaf-fall problem and showed synergistic effects on shoot proliferation and the quality of shoots also improved considerably (Fig. 1c). Thus, adenine sulphate 25.0 mgl^{-1} and glutamine 146.0 mgl^{-1} became constant additives in the shoot induction and shoot development media.

Root induction was difficult in the *in vitro* regenerated shoots of *A. senegal.* The ability of plant tissues to form roots depends upon interactions of different endogenous and exogenous factors. *In vitro* raised shoots require well defined set of conditions for rooting. An important factor which affects rhizogenesis is mineral salt concentration. Sometimes, shoots fail to regenerate roots on high salt media regardless of the type of hormones present. In the present study also this aspect was taken care of and it was found that ½ MS was found best for root initiation (Table 2) and for further development and elongation the salt concentration was further reduced to one-fourth concentration. In case of *A. senegal* response was positive when a pulse treatment of IBA 150 ppm solution was given to the *in vitro* regenerated shoots for 45 minutes .Size of the regenerated shoots played an important role in rhizogenesis shoots of about 4-5 cm height were found best for rooting. Another factor which influenced rhizogenesis was the gelling agent. Gelling was done by pure agar and also by phytagel (sigma). Phytagel which is clear, colorless highly pure and high strength gelling agent and is composed of glucuronic acid, rhamnose and glucose proved beneficial in root formation and elongation (Fig. 1d, e). Phytagel was used at a concentration 2.0 g/l for gelling the rooting media. Temperature also played an important role in root quality. High temperature range 32-34°C was found optimal and high relative humidity 60-80% facilitated good rooting. An initial dark period of 24 to 36 hours was essential for root initiation. To conclude ½ MS fortified with NAA 2.5 mgl^{-1} and IBA 2.5 mgl^{-1} was best for root initiation and further elongation and development of roots took place on ¼ MS supplemented with low concentration of IBA (0.1-0.5 mgl^{-1}).

Table 2: Effect of concentrations of inorganic mineral nutrients on root initiation in *in vitro* regenerated shoots

MS medium salt strength	Percentage root initiation
Plain sucrose + agar +vitamins	NIL
MS- one fourth strength	34%
MS- half strength	65%
MS- three fourth strength	22%
MS- full strength	14%
MS- double strength	11%

NAA 2.5mgl^{-1}+ IBA 2.5mgl^{-1} were present in all sets of rooting experiments mentioned above.

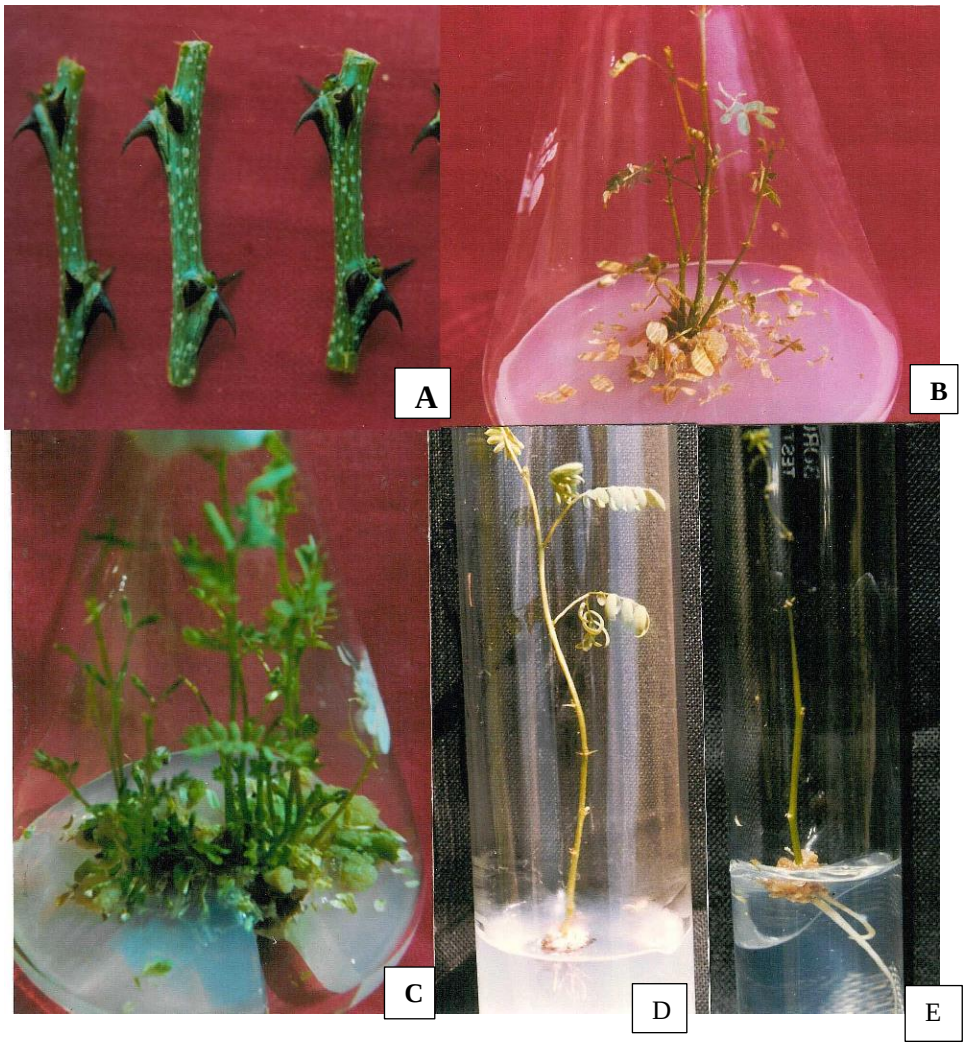

Figure 1

Thus, from the ongoing account, it is evident that there are factors which affect the *in vitro* regeneration of any plant system and which are to be taken care of, and the present communication will be definitely of great help in this direction.

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