Ann Sci For (1995) 52, 183-189
© Elsevier/INRA

Original article

In vitro micropropagation of a tree legume adapted to arid lands *Acacia tortilis* subsp *raddiana*

D Nandwani

Department of Botany, ML Sukhadia University, Udaipur-313001, India

(Received 25 April 1993; accepted 3 August 1994)

Summary — A procedure is described for micropropagation of *Acacia tortilis* subsp *raddiana* (Forsk) Hayne (Mimosoideae) using embryonic explants. Shoot bud formation from cotyledonary nodes was observed on Murashige and Skoog (MS) medium containing various doses of cytokinins (2.5–5.0 mg/l) *viz* 6-benzyl adenine (BA) and kinetin, with or without incorporation of auxin (0.1–0.25 mg/l) *viz* 1-naphthalene acetic acid (NAA) and indole acetic acid (IAA). Multiple shoot regeneration (13–15) was achieved on MS medium supplemented with NAA (0.1 mg/l) and BA (5.0 mg/l). Incorporation of auxin, *eg*, IAA and NAA promoted callusing in the explants. Shoot bud formation was restricted to the cotyledonary node and proximal region of the cotyledons. Scanning electron microscopy showed that shoot bud induction was visible after 5 d of culture. No regeneration of shoot buds from the hypocotyl explants was observed with any treatment used. Half-strength MS medium containing indole butyric acid (3.0 mg/l) was found suitable for rooting in regenerated shoots. *In vitro* developed plantlets were transferred into soil.

***Acacia tortilis* / mimosoideae / forestry / micropropagation / plantlet**

Résumé — Micropropagation *in vitro* d'une Légumineuse de zone aride : *Acacia tortilis* subsp *raddiana*. *Une méthode a été développée pour la multiplication de pousses et la formation de plants* in vitro *à partir d'explants d'embryons d'*Acacia tortilis *(Forsk) Hayne, ssp* raddiana *(Mimosoïdées). Cette Légumineuse est un arbre important adapté aux conditions arides, élevé en particulier pour la stabilisation des dunes et les programmes de reboisement dans différentes zones arides du monde. Les graines d'*A tortilis, *récoltées sur des génotypes sélectionnés par le CAZRI, ont été mises à germer* in vitro *sur un milieu de Murashige et Skoog (MS), sans régulateurs de croissance. Différents types d'explants (hypocotyles, cotylédons, axes embryonnaires [= nœuds cotylédonaires]) ont été excisés à partir de semis d'une semaine. Différents régulateurs de croissance ont été incorporés au milieu MS pour les études de régénération. L'induction de pousses à partir d'explants de nœuds cotylédonaires a été observée dès le 5e j de culture, comme en témoignent les observations faites en microscopie électronique à balayage. La régénération de pousses est favorisée par l'addition dans le milieu de concentrations élevées de cytokinines (2,5 à 5 mg.l^{-1}) avec ou sans auxine (0,1 à 0,25 mg.l^{-1}) (tableau I). Jusqu'à 13 pousses peuvent se différencier à partir d'explants élevés sur milieu de MS additionné d'ANA (0,1 mg.l^{-1}) et de BA (5 mg.l^{-1}). La croissance et la multiplication des pousses est stimulée par des repi-*

quages à 4 sem d'intervalle. Sur les explants se forment à la fois des pousses axillaires et des pousses adventives. Il n'y a en revanche eu aucune régénération à partir d'hypocotyles ou de cotylédons, et cela quels que soient les traitements. Des pousses feuillées, bien allongées, ont été obtenues à partir du milieu de multiplication après 10 à 12 sem de culture. Elles se sont enracinées (tableau II) sur milieu MS à la dilution 1/2 contenant de l'AIB (3 $mg.l^{-1}$*). L'ANA s'est avéré moins efficace que l'AIB pour l'enracinement. L'adjonction de concentrations d'auxine plus élevées (> 3* $mg.l^{-1}$*) favorise la formation de cals à la base des pousses. Des plantules développées* in vitro *ont avec succès été élevées pendant 2 mois en conditions non stériles. Le système de régénération ainsi obtenu peut être utile pour la multiplication en masse d'*A tortilis.

Acacia tortilis / *Mimosoïdées / forêt / micropropagation / plant* in vitro

INTRODUCTION

Acacia tortilis subsp *raddiana* (Forsk) Hayne is a medium sized (4–15 m tall), multipurpose nitrogen-fixing tree and the second most extensively planted taxon after *Prosopis* species in the Thar desert of northwestern India. It occurs naturally in Africa, the middle East, Sahel, Sudan and India. The plant can withstand arid conditions better than other species of *Acacia* and *Prosopis cineraria* (Anon, 1980). It is economically important for fuel, fodder, timber and for sand-dune stabilization and afforestation.

Tissue culture has been used for cloning superior genotypes and in breeding programmes of woody perennials (Karnoski, 1981; Boulay, 1987). Because of the immense importance of nitrogen-fixing trees and shrubs in afforestation and soil erosion control programmes, attempts have been made to regenerate important leguminous tree species through tissue culture, *eg, Albizzia falcatoria* (Sinha and Mallick, 1993), *A richardiana* (Tomar and Gupta, 1988), *Dalbergia sissoo* (Mukhopadhyay and Mohan Ram, 1981), *D latifolia* (Rai and Chandra, 1989), *Tamarindus indica* (Jaiwal and Gulati, 1991), *P tamarugo* (Nandwani and Ramawat, 1992), *P cineraria* (Nandwani and Ramawat, 1993), and *Sesbania sesban* (Shankar and Mohan Ram, 1990). Although tissue culture of *Acacia* has received little attention (Skolmen, 1986), it was in fact one of the first plants grown in culture (Bonner, 1942). A few reports are available on cell suspension cultures or plantlet regeneration from embryonic explants *viz A albida* (Duhoux and Davies, 1985). *A auriculiformis* (Mittal *et al,* 1989), *A koa* (Skolmen, 1978, 1986), *A senegal* (Dave *et al,* 1980), *A mangium* (Galiana *et al,* 1991) and *A nilotica* (Dewan *et al,* 1992). None of them have described the large-scale multiplication of *Acacia* species.

The present communication reports *in vitro* shoot multiplication and plantlet regeneration in *A tortilis*, which is a part of our long-term goal in development of technology for regeneration of forest trees.

MATERIALS AND METHODS

Plant material

Seeds of *A tortilis* subsp *raddiana* were obtained from the selected genotypes through the silviculture division, Central Arid Zone Research Institute, Jodhpur, India. Seeds were pretreated with H_2SO_4 (30%) for 15 min and washed under a runnning tap water for 2–3 h. Seeds were surface disinfected by ethanol (90%) for 2 min followed by mercuric chloride (0.1%) for 5 min and then washed with sterilized distilled water. Finally, seeds were transferred into half-strength Murashige and Skoog (1962) medium (MS) containing 0.6% agar and 2% sucrose with no growth regulators. Various explants, *eg*, hypocotyls, cotyledons, and cotyledonary node (cotyledons +

axillary meristem) were excised from the 7–10-day-old seedlings.

Culture medium

MS medium was used throughout the investigations. Various growth regulators, *eg*, 1-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), 6-benzyl adenine (BA) and kinetin, were used for regeneration studies at a range of concentrations. The pH of the medium was adjusted to 5.8 with 0.01 N KOH or 0.01 N HCl before autoclaving at 121°C for 20 min.

Culture conditions

Hypocotyls (0.5–0.7 cm) and cotyledons (0.8–1.0 cm) were placed upright and adaxial, respectively, 2 in each culture tube (125 x 25 mm, Borosil) or Erlenmayer flask containing 20 and 40 ml culture medium, respectively. Cotyledonary node explants (1.0–1.2 cm) were cultured singly in culture tubes or in pairs in Erlenmayer flasks. Culture vessels were incubated under fluorescent and incandescent lights with 16 h illumination (2 500 lux) at 26 ± 1°C temperature and 55% (± 5%) relative humidity. Each treatment consisted of 24–30 replicates, and cultures for multiplication and elongation were grown for at least 3 passages of 4 weeks duration using conditions similar to those described above except that cotyledons were removed from the multiple shoot buds prior to subculture in the second passage.

Rooting and transplantation

Well-elongated leafy shoots (3.0–4.0 cm) of 8–12 weeks age were transferred into half-strength MS medium supplemented with 3-indole butyric acid (IBA) or NAA at various concentrations (1.0–5.0 mg/l). *In vitro*-developed plantlets were removed carefully from the culture vessels and adhered agar was removed to avoid contamination. Plantlets were washed with distilled water and transferred to plastic pots containing garden soil and vermiculite (1:1, v/v). Pots were kept in a plant growth chamber for 2–4 weeks before transferring to the glass-house and finally to the field.

Scanning electron microscopy (SEM)

Cotyledonary node explants were removed at 5 d intervals from the bud induction medium and fixed in formaline acetic acid and dehydrated in a graded acetone/amyl acetate series to absolute amyl acetate. They were then critical-point dried with liquid CO_2, mounted and sputter coated with 100–200 Å gold film. Explants were viewed at 10 kV in a Jeol 35 CF SEM and photographed (Rhonda and Dodd, 1990).

RESULTS AND DISCUSSION

Of all the explants used, cotyledonary node segments were found to be best for the multiple shoot induction. Hypocotyl and cotyledon explants produced only callus and no regeneration of shoot buds was achieved from such explants. Shoot bud formation from cotyledonary node explants was observed on almost all of the treatments with growth regulators incorporated in MS medium, singly or in combination (table I). On MS medium containing NAA (0.1 mg/l) and BA (5.0 mg/l) 13–15 shoot buds were differentiated from the explants (fig 1). The original cotyledonary node explants with multiple shoot buds were repeatedly transferred to fresh culture media for shoot multiplication and enlongation. Formation of new shoot buds (adventitious) and development of older shoots were observed simultaneously. Well-developed shoots (fig 2) could be harvested after up to 5 passages at an interval of 4 weeks. The same technique has been used for the micropropagation of *P tamarugo* (Nandwani and Ramawat, 1992), *P cineraria* (Nandwani and Ramawat, 1993) and *A nilotica* (Dewan *et al*, 1992). It was observed that production of shoot buds was not affected by the size of explants used, but if the cotyledons were removed in the first passage of 4 weeks, the number of shoot buds formed was decreased (data not shown) as reported earlier (Nandwani and Ramawat, 1992, 1993). Of the cytokinins

Table I. The morphogenetic response of cotyledonary node explants of *A tortilis* to various growth regulators incorporated in the MS medium. [a]

Growth regulator (mg/l)		*No of shoot buds/ explants (± SD)*	*Shoot length (cm) (± SD)*	*Explants regenerated (%)*	*Callusing* [b]
Control		–	–	–	–
BAP					
0.1		0.7 ± 0.48	1.77 ± 0.74	20	–
0.5		1.7 ± 0.53	1.85 ± 0.64	35	–
1.0		1.9 ± 0.99	2.03 ± 0.79	40	–
2.5		4.55 ± 1.13	4.05 ± 1.71	65	+
5.0		8.6 ± 2.22	2.67 ± 2.24	80	+
Kinetin					
0.1		1.0 ± 0.47	1.15 ± 0.65	15	–
0.5		1.6 ± 0.966	1.33 ± 0.47	25	–
1.0		2.8 ± 0.788	1.67 ± 0.46	40	–
2.5		6.5 ± 2.01	2.2 ± 0.758	70	+
5.0		8.2 ± 1.75	2.94 ± 1.2	90	+
Kinetin	IAA				
0.5	0.25	2.7 ± 0.82	1.65 ± 0.46	20	+
2.0	0.25	4.7 ± 1.159	2.46 ± 0.61	30	+
4.0	0.25	7.9 ± 1.37	2.8 ± 0.76	70	++
5.0	0.25	9.2 ± 1.549	3.5 ± 1.1	65	++
BAP	NAA				
0.5	0.1	1.9 ± 0.737	1.71 ± 0.52	15	+
2.0	0.1	4.1 ± 0.87	2.61 ± 0.598	55	+
4.0	0.1	9.5 ± 1.08	2.98 ± 0.82	80	+
5.0	0.1	11.66 ± 2.12	3.54 ± 1.06	95	++

[a] Each experiment was repeated 3 times and data were scored after 4 weeks. [b] Relative value of callus formation: – zero; +, poor; ++, moderate.

used, BA was found to be better than kinetin for induction and multiplication of shoots. Addition of kinetin instead of BA in the medium resulted in the production of a lower number of shoots. Incorporation of auxin (either IAA or NAA) enhanced shoot induction but promoted callusing in the explants. SEM observations (fig 3) supported the fact that shoot bud induction was started rapidly in 5 d after inoculation. One large shoot from the apical meristem (main shoot) developed quickly in 4–5 d followed by multiple shoot bud induction (5–6 d) at the cotyledonary nodes. The number of shoot buds was increased (12–13 d) as the explants were grown in successive subcultures.

Rooting of shoots

Induction of roots in regenerated shoots was observed on MS medium containing IBA or NAA (1.0–5.0 mg/l) (table II). Half-strength MS medium supplemented with IBA

Table II. Effect of auxin on root formation of *in vitro* regenerated shoots of *A tortilis* grown on MS medium (1/2 strength) under low light intensity (1 000–1 500 lux).

Auxin (mg/l)	*Number of roots/ shoot (± SD)*	*Rooted shoots (%)*	*Callusing* [a]
Control	–	–	–
IBA			
1.0	3.0 ± 0.89	25	–
3.0	7.5 ± 1.048	65	–
5.0	3.66 ± 1.36	55	+
NAA			
1.0	2.16 ± 1.16	20	–
3.0	4.0 ± 1.41	45	+
5.0	4.5 ± 1.04	40	++

[a] Relative value of callus formation: – zero; +, poor; ++, moderate.

(3.0 mg/l) was found suitable for rooting as compared to NAA (fig 4). Five to 7, thin, white-creamish, roots developed directly at the base of shoots without callus formation. Up to 65% rooting was achieved in differentiated shoots of *A tortilis.* Higher concentrations of auxins (5.0 mg/l) favoured callusing in the shoots. *In vitro*-developed plantlets were transferred to pots and grown for up to 2 months in non-sterile conditions (fig 5).

Multiple shoot formation and plantlet regeneration from cotyledonary nodes has been achieved in *A tortilis.* Several species of *Acacia* have been grown *in vitro* (Skolmen, 1986) and shoot formation from juvenile tissues has been reported in *A albida* (Duhoux and Davies, 1985). *A auriculiformis* (Mittal *et al,* 1989; Rao and Prasad, 1991), *A nilotica* (Dewan *et al,* 1992). In all cases, high concentrations of cytokinin along with low amounts of an auxin, or sometimes without an auxin, produced a few shoots from cotyledonary nodes or hypocotyl explants. In our case, shoot buds were initiated very rapidly after the explant was transferred to culture medium, as supported by SEM studies. However, multiplication and elongation of regenerated shoot buds was slow in *A tortilis* as compared to *P cineraria* (Nandwani and Ramawat, 1993). The system developed in *A tortilis* requires further experimentation to increase the growth rate and multiplication. It is suggested that the technique described can be utilized for afforestation and conservation of germplasm of *A tortilis.*

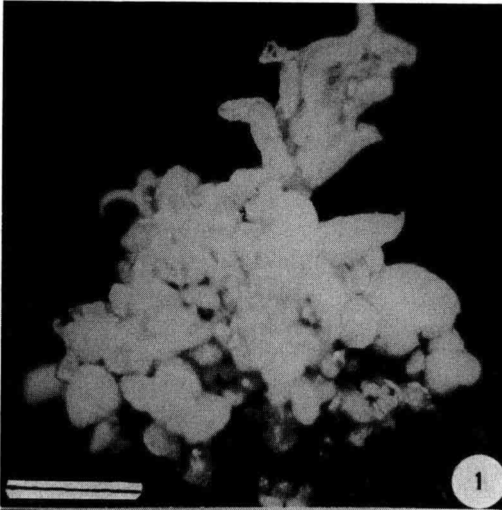
ACKNOWLEDGMENT

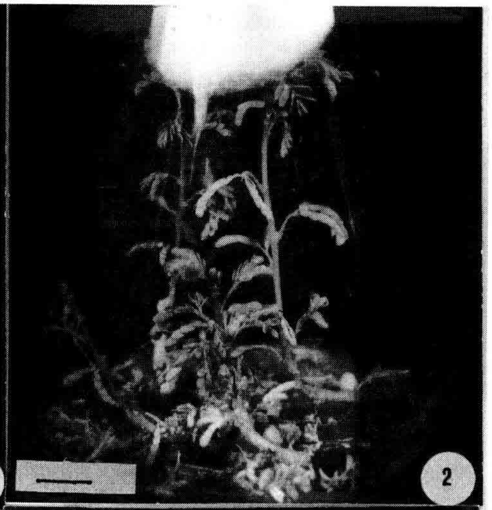
The author thanks the head of the RSIC of NE Hill University, Shillong for scanning electron microscopy and KG Ramawat for valuable suggestions. Financial support from CSIR, New Delhi is gratefully acknowledged.

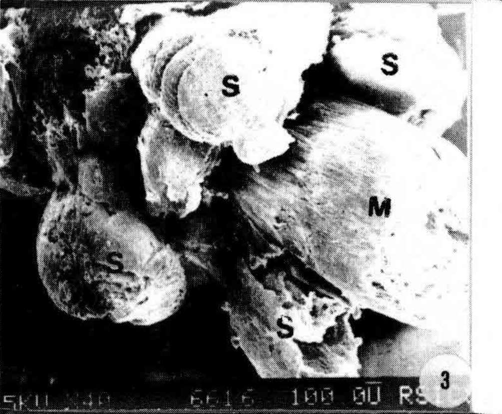
REFERENCES

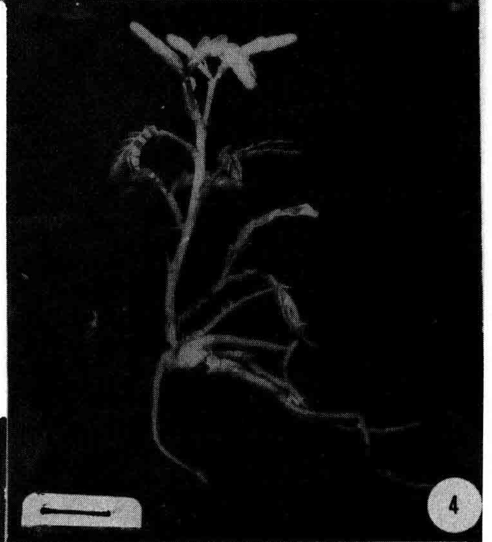
Anon (1980) Tropical legumes: Resources for the future. Nat Acad Sci, Washington, DC, USA, 231

Bonner J (1942) Culture of isolated roots of *Acacia melanoxylon. Bull Torr Bot Club* 69, 130-133


1


2


S
S
M
S
S
3


4

5

Boulay M (1987) *In vitro* propagation of tree species. *In: Plant Tissue and Cell culture* (CE Green, DA Somers, WP Hachett, DD Biespoer, AR Liss, eds) Acad Press Inc, New York, USA, 367-382

Dave VS, Goyal Y, Vaishnava GR, Surana NM, Arya HC (1980) Clonal propagation of desert plants through tissue culture. III. Plantlet formation in *Acacia senegal* stem culture. *J Ind Bot Soc* 59, 57

Dewan A, Nanda K, Gupta SC (1992) *In vitro* micropropagation of *Acacia nilotica* subsp *indica* Brenan *via* cotyledonary nodes. *Plant Cell Rep* 12, 18-21

Duhoux E, Davies D (1985) Shoot production from cotyledonary buds of *Acacia albida* (synonym *Faidherbia albida*) and influence of sucrose on rhizogenesis. *J Plant Physiol* 121, 175-180

Galiana A, Tibok A, Duhoux E (1991) *In vitro* propagation of nitrogen-fixing tree legume *Acacia mangium* Willd. *Plant Soil* 135, 151-159

Jaiwal PK, Gulati A (1991) *In vitro* high frequency plant regeneration of a tree legume *Tamarindus indica* (L). *Plant Cell Rep* 10, 569-573

Karnoski DF (1981) Potential for forest tree improvement *via* tissue culture. *Bioscience* 31, 114-120

Mittal A, Aggrawal R, Gupta SC (1989) *In vitro* development of plantlets from axillary buds of *Acacia auriculiformis* – a leguminous tree. *Plant Cell Tissue Organ Cult* 19, 65-70

Mukhopadhyay A, Mohan Ram HY (1981) Regeneration of plantlets from excised roots of *Dalbergia sissoo*. *Ind J Exp Biol* 19, 1113-1115

Murashige T, Skoog F (1962) A revised medium of rapid growth and bioassays wih tobacco tissue culture. *Physiol Plant* 15, 473-496

Nandwani D, Ramawat KG (1992) High frequency plantlets regeneration from seedling explants of *Prosopis tamarugo*. *Plant Cell Tissue Organ Cult* 29, 173-178

Nandwani D, Ramawat KG (1993) *In vitro* plantlets formation through juvenile and mature explants in *Prosopis cineraria*. *Ind J Exp Biol* 31, 156-160

Rai RV, Chandra KSJ (1989) Micropropagation of Indian rosewood by tissue culture. *Ann Bot* 64, 43-46

Rao GVR, Prasad MNV (1991) Plantlet regeneration from the hypocotyl callus of *Acacia auriculiformis* – multipurpose tree legume. *J Plant Physiol* 137, 625-627

Rhonda LG, Dodd WA (1990) An *in vitro* technique for the production *de novo* of multiple shoots in cotyledon explants of cucumber (*Cucumis sativus* L). *Plant Cell Tissue Organ Cult* 20, 177-183

Shankar S, Mohan Ram HY (1990) Plantlets regeneration from tissue cultures of *Sesbania grandiflora*. *Curr Sci* 59, 39-43

Sinha RK, Mallick R (1993) Regeneration and multiplication of shoot in *Albizia falcataria*. *Plant Cell Tissue Organ Cult* 32, 259-261

Skolmen RG (1978) Vegetative propagation of *Acacia koa* Gray. *In: Proc Sec Conf Nat Sci* (CW Smith, ed), Hawaii, USA, 260-273

Skolmen RG (1986) Acacia (*Acacia koa* Gray). *In: Biotechnology in Agriculture and Forestry* (YPS Bajaj, ed), vol 1, trees I, Springer Verlag, Berlin, Germany, 375-384

Tomar UK, Gupta SC (1988) Somatic embryogenesis and organogenesis in callus cultures of a tree legume *Albizia richardiana* King. *Plant Cell Rep* 7, 70-73

Fig 1. Multiple shoot bud formation from the cotyledonary node on MS medium containing NAA (0.1 mg/l) and BA (5.0 mg/l) in the first passage.

Fig 2. Well-developed leafy shoot on MS medium supplemented with BA (2.0 mg/l) after 8 weeks of culture.

Fig 3. SEM showing induction of shoot buds from the cotyledonary node explants of *A tortilis* after 5 d of incubation (M – main shoot, S – shoot buds).

Fig 4. Root formation in regenerated shoots on half-strength MS medium containing IBA (3.0 mg/l).

Fig 5. Two-month-old *in vitro* raised plantlet of *A tortilis* (bar represents 1.0 cm).