J. Plant Biotechnology (2006) Vol. 8 (1). pp. 15~19

Journal of Plant Biotechnology

An Improved Method of Organogenesis from Cotyledon Callus of *Acacia sinuata* (Lour.) Merr. using Thidiazuron

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Abstract

An efficient protocol for *in vitro* multiple shoot bud induction and plant regeneration from mature green cotyledon derived callus tissues of *Acacia sinuata* has been developed. Callus formation occurs at all the concentrations of thidiazuron (TDZ) in Murashige and Skoog's (MS) medium, but 0.6 µM proved to be the best with maximum callus formation frequency. Supplementation of TDZ in combination with indole-acetic acid (IAA) in MS media accelerates shoot bud organogenesis in differentiating callus tissues with 60-70% conversion of shoot buds into shoot. Most efficient shoot organogenesis was recorded when TDZ induced calli were subcultured at different concentrations of 6-benzyladenine (BA). Optimum shoot bud induction and plant regeneration from callus was achieved when 0.6 µM (TDZ) induced calli were subcultured at 3.0 µM (BA) where 16.6 ± 0.74 shoots/unit callus were obtained. Rooting in *in vitro* differentiated shoots was achieved when transferred to medium containing different concentration of indole-3-butyric acid (IBA) in full & half strength MS medium. The well rooted plantlets were hardened and transferred to net house with 90% survival rate.

Key words: *Acacia sinuata*, cotyledon, thidiazuron, callus culture.

Introduction

The ability to culture plant cells and tissue provides the opportunity to micropropagate specific and often elite, plant clones by stimulation of axillary buds and laterally, through the production of somatic embryos and manipulation of culture conditions has facilitated the adventitious plant regeneration from whole organs, explants, callus and isolated protoplast (Davey et al. 1994). Furthermore, genetic transformation methods allow for the introduction of genetic material not normally accessible by conventional means, in contrast to traditional breeding methods which are limited by sexual incompatibility. Recombinant DNA technologies have tremendous potential in crop improvement. However, before biotechnological method can be utilized, a reliable regeneration protocol adaptable to transformation system is needed.

Acacia sinuata is an important succulent shrub occurring in tropical jungles throughout India, especially in Deccan and also in South Asian countries. The pods are extensively used as detergent and removes dandruff. Ointment prepared from the ground pod is good for skin diseases. The bark contains saponins which show spermicidal activity against human semen (Anonymous 2003).

Being an economic important tree, biotechnological approaches have not been utilized for its improvement. There have been a few reports on plant tissue culture work on *Acacia sinuata* (Vengadesan et al. 2000, 2002, 2003 a, b), but the existing protocols are lengthy and time consuming with the involvement of extensive manipulation of culture conditions and various combinations of PGRs. The present investigation was carried out with the objective to develop an efficient protocol for *in vitro* differentiation and plant regeneration from mature green cotyledon callus tissues using thidiazuron that could be useful for several *in vitro* manipulation studies.

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Received Jul. 24, 2005; Accepted Mar. 9, 2006

Material and Methods

Initiation of aseptic cultures

15 year old tree of *Acacia sinuata* maintained at Botanical garden, Aligarh Muslim University, was used as experimental material. Tissue cultures were initiated using single cotyledons as explant. Mature green pods of *A. sinuata* were collected from the mature plant and washed under running tap water for half an hour. Surface sterilization was carried out with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 10 min., and thoroughly rinsed five times with sterile deionise water before explant preparation.

Isolation of cotyledons

After sterilization, the pods were dissected out in a laminar flow cabinet using sterilized forceps and scalpel; cotyledons were harvested and transferred to different callus and shoot bud induction media.

Culture medium and culture conditions

The basal medium (BM) comprised the mineral salts and organic nutrients of MS medium with 3% sucrose (Qualigens Fine Chemicals, Mumbai). Depending on the experiment, BM was variously supplemented with various plant growth regulators such as TDZ, BA, IAA, and IBA in the different concentrations as indicated in the tables. In the first set of experiment, different concentrations of TDZ were used for callus induction. In the second set of investigation, combination of TDZ and IAA were used for the conversion of shoot buds into shoots on the callus obtained from different concentrations of TDZ. Third experiment was carried out to optimize the level of BA on TDZ induced calli for shoot bud organogenesis. All the constituents of medium were added to the molten agar and the pH of the medium was adjusted

Table 1. Effect of different concentrations of TDZ on callus induction from cotyledon explant of *Acacia sinuata* after four weeks of culture.

TDZ (μM)	% response	Mean no. of green spots	Nature of callus
0.0	0.00±0.00^{e}	0.00±.0.00^{f}	-
0.1	40.0±2.88^{d}	3.20±0.37^{e}	Yellowish, compact
0.2	58.3±4.40^{c}	10.4±0.67^{d}	Yellowish green, compact
0.4	92.6±3.71^{a}	19.2±0.73^{b}	Yellowish green, compact
0.6	98.3±1.67^{a}	27.0±0.89^{a}	Greenish slightly compact
0.8	92.6±3.92^{a}	15.6±0.81^{c}	Greenish friable
1.0	80.0±2.88^{b}	9.40±0.87^{d}	Greenish friable

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the DMRT at 0.05% probability level.

Table 2. Effect of different concentrations of TDZ in combination with IAA (0.5 μM) on callus for shoot bud induction and conversion into shoots, after six weeks of culture.

TDZ (μM)	% response	Mean no. of shoot buds	Mean conversion of buds into shoots	Mean shoot length (cm)
0.0	0.00±0.00^{d}	0.00±0.00^{f}	0.0±0.00^{d}	0.0±0.00^{d}
0.1	64.3±4.70^{c}	2.20±0.48^{e}	0.0±0.00^{d}	0.0±0.00^{d}
0.2	65.0±2.88^{c}	5.60±0.50^{d}	0.0±0.00^{d}	0.0±0.00^{d}
0.4	87.3±3.71^{b}	8.60±0.50^{c}	2.8±0.37^{c}	1.7±0.32^{c}
0.6	94.3±3.48^{ab}	14.0±0.50^{b}	3.2±0.58^{c}	2.2±0.43^{bc}
0.8	98.3±1.66^{a}	18.6±0.60^{a}	9.2±0.66^{a}	3.6±0.40^{a}
1.0	95.0±2.88^{ab}	17.0±0.94^{a}	5.0±0.31^{b}	3.0±0.35^{ab}

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the DMRT at 0.05% probability level.

to 5.8 using 1N NaOH before autoclaving at 1.06 Kg cm^{-2} (121℃ for 15 min in culture tubes (150 x 25 mm, Borosil, India). The cultures were maintained in a growth room at 25 ± 2℃ under 16 hr photoperiod provided by cool white fluorescent tubes (Phillips, India) with the light intensity of 50 μ mol $m^{-2}s^{-1}$ and 55 ± 5% relative humidity.

Rooting in microshoots

Regenerated microshoots of appropriate size (3-4 cm) were transferred to tubes containing 20 ml of MS, ½ MS medium supplemented with various concentrations (1, 2, 5 μM) of IBA for root induction. Culture conditions for root initiation were same as described for shoot differentiation.

Establishment in soil

Seven-eight week old regenerated plantlets with well developed root system were washed with tap water to remove the agar medium. These were then dipped in 0.2% Bavistin (a systemic fungicide) for 2-5 minutes and subsequently transplanted in plastic pots containing soilrite. The plantlets were covered with clear polyethylene bags to ensure high humidity around them and were acclimatized at 25 ± 2℃ under 16-hr photoperiod, watered regularly with ¼ MS medium lacking vitamins for one week and then with tap water. After 3-4 weeks, polythene bags were removed gradually and established plantlets were transplanted to earthen pots containing garden soil in a green house.

Statistical analysis

Each treatment was replicated 20 times and all experiments were repeated thrice. The regeneration frequency, number of shoot buds per unit callus, conversion of shoot buds into shoot, shoot length, number of root and root length were recorded periodically. Data obtained were analyzed using one way analysis of variance (ANOVA) and significant differences between means were assessed by LSD at $P < 0.05$ and the means were compared using DMRT with statistical software, SPSS version 10 (SPSS INC, Chicago; USA).

Results and Discussion

The objective of first experiment was to identify the most effective concentration of TDZ for callus induction, six concentrations of TDZ (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 μM) were tested and the successful induction of callus tissue was observed from the cut ends of cotyledon after 7 days

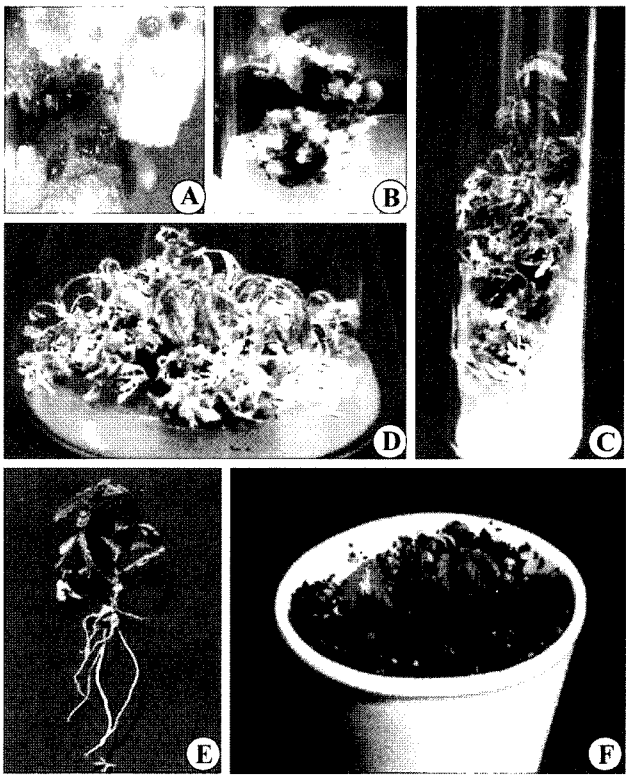

Figure 1. Organogenesis in cotyledon derived callus of *Acacia sinuata* (Lour.) Merr. (A) Callus showing green smooth spots on MS + TDZ (0.6 μM); (B) Adventitious shoot buds from callus on TDZ (0.8 μM) + IAA (0.5 μM); (C) Adventitious elongated shoots on MS + BA (3.0 μM); (D) Shoot multiplication on MS + BA (3.0 μM); (E) *In vitro* produced shootlet with adventitious roots 1/2 MS + IBA (5.0 μM); (F) An acclimatized plantlets.

of inoculation in all the media containing various concentrations of thidiazuron and the percent response of explant ranges between 40-98%. MS medium lacking TDZ did not show any response. Use of low level of TDZ (0.4-0.6 μM) supported better dedifferentiation and callus growth with numerous green smooth spots (Figure 1A). Calli obtained in this experiment were relatively compact and slightly green in colour. A stimulatory effect of TDZ on callus induction at lower concentration has been documented in tissue culture studies of various woody tree species (Huettman & Preece 1993).

Second experiment was carried out with an aim of induction of shoot bud organogenesis and subsequent plant regeneration involving transfer of callus tissue to shoot bud induction and proliferation medium containing different concentrations of TDZ with the combination of 0.5 μM (IAA). The transferred calli produce more greenish callus and were found highly competent for shoot bud initiation particularly in media containing 0.8 μM (TDZ) + 0.5 μM (IAA) with 98.3 % shoot bud induction frequency and about 70% conversion

Table 3. Effect of various concentrations of BA on shoot morphogenesis from calli obtained from different concentrations of TDZ after six weeks of culture.

TDZ (μM) Induced calli	BA (μM)					
	1.0		3.0		5.0	
	Mean No. shoots	Mean shoot length (cm)	Mean No. shoots	Mean shoot length (cm)	Mean No. shoots	Mean shoot length (cm)
0.1	1.8±0.37^{c}	1.7±0.25^{b}	2.6±0.24^{e}	1.8±0.25^{b}	2.0±0.54^{c}	1.9±0.40^{b}
0.2	2.4±0.40^{bc}	2.1±0.43^{ab}	4.2±0.58^{de}	2.0±0.31^{b}	2.6±0.67^{c}	2.2±0.33^{b}
0.4	3.2±0.48^{ab}	2.5±0.41^{ab}	8.2±0.73^{b}	2.2±.040^{b}	6.0±0.44^{a}	2.7±0.60^{ab}
0.6	4.2±0.37^{a}	3.0±0.44^{a}	16.6±0.74^{a}	3.3±0.37^{a}	7.4±0.50^{a}	3.8±0.43^{a}
0.8	2.0±0.54^{bc}	2.5±0.31^{ab}	7.2±0.48^{bc}	2.7±0.46^{ab}	4.4±0.50^{b}	3.2±0.56^{ab}
1.0	1.6±0.40^{c}	1.9±0.36^{ab}	5.8±0.66^{cd}	1.9±0.33^{b}	3.6±0.67^{bc}	2.5±0.57^{a}

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the DMRT at 0.05% probability level.

Table 4. Effect of IBA on rooting in *in vitro* raised microshoots of *Acacia sinuata* cultured on MS and 1/2MS medium after 4 weeks of incubation.

MS+IBA (μM)	1/2MS+IBA (μM)	% response	Mean No. roots/shoot	Mean root length (cm)
0.0		0.00±0.00^{f}	0.0±0.00^{d}	0.0±0.00^{e}
1.0		25.0±2.88^{e}	1.4±0.24^{bc}	0.9±0.18^{de}
2.0		34.3±3.48^{de}	1.8±0.37^{bc}	1.3±0.33^{cd}
5.0		55.0±2.88^{c}	2.4±0.24^{b}	1.7±0.43^{bcd}
10.0		36.6±4.40^{d}	1.6±0.40^{bc}	1.1±0.24^{cde}
	1.0	36.6±3.33^{d}	1.2±0.20^{c}	1.6±0.33^{bcd}
	2.0	51.6±4.40^{c}	2.0±0.31^{bc}	2.2±0.33^{abc}
	5.0	82.6±3.71^{a}	3.4±0.50^{a}	2.9±0.57^{a}
	10.0	65.3±2.90^{b}	2.0±1.44^{bc}	2.7±0.48^{ab}

Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the DMRT at 0.05% probability level.

rate of shoot buds into shoot (Figure 1B). The earliest sign of shoot bud formation was noticeable within three weeks of incubation. The effectiveness of combination of auxin IAA with cytokinin (TDZ) for shoot bud induction was also reported earlier in *Acacia sinuata* (Vengadesan et al. 2000).

Third experiment was carried out to optimize the level of BA for maximum shoot bud induction and elongation from the calli obtained in the first experiment. In this experiment calli obtained from the TDZ exposed cotyledons were transferred to MS medium composed of different concentration of BA (1.0, 3.0 and 5.0 μM). The amount of shoot bud organogenesis observed per unit callus (500 mg fresh weight) was 100% during 30 days of incubation and the optimum level of shoot bud formation was recorded on the medium containing 3.0 μM BA with (16.6 ± 0.74) shoots/unit callus (Table 3), Figure 1 C and D, which was initially obtained on 0.6 μM TDZ. However media containing 5.0 μM (BA) produced lower frequency of shoot bud induction and multiplication. Shoot buds initially arose as clusters within the callus as well as on abaxial surface of callus. Similar type of shoot bud differentiation was also reported in callus tissues of *Sesbania bispinosa*, a woody legume (Sinha and Mallick 1991). Almost all calli on medium containing BA showed friable growth and characterized by soft yellowish to greenish brown in appearance. Shoot were regenerated mostly from these friable calli. The effective nature of friable callus for shoot bud induction and plant regeneration was also documented in *Leucaena leucocephala* (Saafi and

Borthakur 2002) and *A. sinuata* (Vengadesan et al. 2000). The response to shoot regeneration was observed within 3-4 weeks after transference of calli from media comprises TDZ to the medium containing low concentration of BA (3.0 µM) in obtaining optimum results.

A continuous process of shoot bud multiplication was also achieved through repeated subculturing of a portion of the differentiating callus onto the same shoot induction medium. This was done by splitting the callus tissue into pieces and subcultured onto the medium containing 3.0 µM (BA). Induction of shoot buds on the surface of the competent callus tissue and pre-existing shoots were observed after three weeks of culture. Subsequent, shoot elongation to optimum length (>4 cm) was achieved on the same medium with low level of BA (3.0 µM). This continuous process of induction and multiplication of shoots from primary callus tissue could be successfully repeated five to six times (Data not shown). Adventitious or axillary shoots produced in clusters in medium containing cytokinin generally lacks roots. To obtain complete plantlets, regenerated shoots must be transferred to rooting medium which is generally carried out on an auxin containing medium. Therefore, the last experiment was carried out to optimize the IBA concentration in full and half strength MS medium. Individual microshoots of appropriate size (>4 cm) were excised from differentiating shoot clusters and transferred onto the MS and 1/2MS medium containing different concentrations of IBA (1, 2, 5 and 10 µM) separately for root induction. Of several media tested, about 80% of the regenerated microshoots produced roots with 1/2MS+IBA (5.0 µM) Figure 1 E. The requirement of half strength MS salts for *in vitro* rooting has been documented in various tissue culture studies of this woody legume (Vengadesan et al. 2002, 2003). Plantlets with well developed root system were hardened according to the procedure given in material and methods. Out of 150 plantlets transferred to soil, on an average 130 survived and grew well. The established plantlets were uniform and no morphological variations were observed (Figure 1 F) till the communication of this paper.

The noteworthy aspect of our experiment is the efficacy of thidiazuron in callus and multiple shoot induction and plant regeneration from cotyledon tissues. Thidiazuron was primarily developed to defoliate the plant before cotton ball harvest (Arndt et al. 1976) and show high cytokinin activity, similar to that of N^6- substituted adenine derivatives (Mok et al. 1982). TDZ expresses high degree of morphogenetic response in various tissue culture studies of woody plants species (Hutemann and Preece 1993, Faisal et al. 2005).

It may be concluded that for the first time an efficient method for successful regeneration and multiplication of *Acacia sinuata* by *in vitro* green mature cotyledon callus cultures using thidiazuron is described. The protocol presented here is simple, reproducible and efficient. Moreover, the system is rapid with initiation of tissue culture to transplanting of plantlets to green house was completed in 16 weeks and this could possibly be employed in genetic transformation procedures of this economic woody tree.

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