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In vitro regenerative potentials of the medicinal plant *Abutilon indicum* (L.) Sweet

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The present study was undertaken to evaluate the most suitable concentration of growth regulators for callus induction and subsequent organogenesis in *Abutilon indium* (L.) Sweet (Malvaceae) leaf, stem and seed explants. Since the plant has a lot of medicinal importance, it was subjected to culture *in vitro*. Kinetin for seeds, 2,4-dichlorophenoxyacetic acid (2,4-D), benzyl amino purine (BAP), naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) for leaf as well as BAP and kinetin for stem were the growth regulators used at different concentrations individually. Results showed that growth regulators at different combinations on subculture resulted in the regeneration of shoots. The excised shoots were transferred to liquid medium for *in vitro* rhizogenesis and plantlets were acclimatized in the laboratory conditions before they were transferred to garden soil. Histologically, callus sections were with active centres, embryoids and multiple shoot buds. Cytological variations such as multinucleate, chromosome bridges, polyploidy and tracheary elements were noticed in callus cells. The regenerated plants were morphologically and cytologically similar to the *in vivo* plants.

Key words: Embryoids, multiple shoot buds, multinucleate, polyploidy, Malvaceae.

INTRODUCTION

Abutilon indicum (Malvaceae) is a hairy medicinal undershurb with golden yellow flowers, abundant throughout the hotter parts of India. In Karnataka it is called Srimudre. The bark finds use as febrifuge lessens (perspiration), allays thirst and vomiting. The root cures uterine haemorrhagic discharges. The leaves are cooked and eaten in the case of bleeding piles. Its decoction is used in bronchitis, jaundice, gonorrhoea and inflammation of the bladder. It is also used in mouth wash in the case of tooth ache (Kirtikar and Basu, 1918). Since this is an important medicinal plant, there is an increased demand of *A. indicum* biomass to meet the requirements of medicine and has generated need to undertake large scale cultivation by the donor plant. Propagating this plant through tissue culture may be a solution to the above problem. Regeneration of plants from callus may help to induce variability in this plant for future improvement. This may result in the generation of useful somaclonal variants not available by conventional methods. Over the last years, a number of micropropagative

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Table 1. Callus response of A. indicum explants on various types of media.

Medium	Growth regulator (mg/l)	Seed	Stem	Leaf
MS	2,4-D 1.0	-	+	++
	2,4-D 2.0	-	++	+++
	Kinetin 1.0	+++	++	+
	Kinetin 2.0	++	+	-
B-5	2,4-D 1.0	-	+	-
	2,4-D 2.0	-	-	+
	Kinetin 1.0	+	+	-
	Kinetin 2.0	+	-	-
White's	2,4-D 1.0	-	+	+
	2,4-D 2.0	-	-	-
	Kinetin 1.0	+	-	-
	Kinetin 2.0	+	-	-

+ Low yield, ++ moderate yield, +++ high yield.

protocols have been developed using a variety of explants like shoot tip, axillary buds, stem cuttings and leaf segments.

Callus mediated plant regeneration is an easy way to obtain somaclonal variants as has been emphasized in other several plants (Dhar and Joshi, 2005; Agrawal and Sardar, 2006). Moreover, *in vitro* morphogenesis without a callus phase is regarded as the most suitable strategy to obtain plants with high speed as well as genetical fidelity. In the present study, whole plant regeneration from various explants of *A. indicum* was reported.

MATERIALS AND METHODS

Explant preparation

Different explants like dry seeds, stem (both node and internode) and leaf segments were excised from the healthy plants maintained in the botanical garden, University of Mysore, Manasagangotri, Mysore. Seeds, stem and leaf segments were washed under running tap water for ten minutes followed by surface sterilisation with 0.1% $HgCl_2$ for 10, 7 and 5 min, respectively. This was followed by a dip in absolute alcohol and washed with sterile distilled water for 5-6 times. The excess water from explants was removed using sterile blotter discs. Stem and leaf explants were cut in appropriate sizes before inoculation.

Media preparation

Three types of media: White (1943), Murashige and Skoog (1962) and Gamborg et al. (1968) were prepared, supplemented with various concentrations and combinations of growth regulators; 2-3% sucrose was added to the medium as a carbohydrate source and 0.8-0.9% bacteriological grade agar was added as solidifying agent. pH of the medium was adjusted to 5.8-6 prior to autoclaving at 121°C for 15 min. For callus induction, 2,4-D and kinetin (1 and 2 mg/l) were used for all the explants used (Table 1). Callus was subcultured on MS basal medium as well as growth regulator fortified MS medium (Table 2).

Inoculation and incubation

Aseptically, the explants (4-5) were inoculated into the Erlenmeyer flasks on to the solid medium. After labelling, cultured flasks were transferred to incubation chamber maintaining the humidity of 75-80% at 21±1°C under artificial fluorescent white, cool light at 70 Em^2s^{-1} for 16 h. For each treatment, 20 flasks were replicated. Experiment to know the percentage of callusing of explants on different types of media was conducted and presented in Table 1. For multiple shoot induction and multiplication, callus was subcultured to the MS medium supplemented with different combinations and concentrations of growth regulators (Table 3). After regeneration of plantlets, the shoots were transferred to liquid medium containing auxins alone at different concentrations and in combinations. All the experiments were repeated at least thrice and were statistically analysed by ONE WAY ANOVA (SPSS).

For cytological preparations, the callus was pretreated with 8-hydroxyquinoline (0.2%) for 3.30 h, washed with water and fixed in Carnoy's fixative-1 for 24 h and stored in 70% alcohol. The callus was treated with 2% mordant for 10 min and washed in 45% propionic acid. Subsequently stained in 2% Heidenhain's haematoxylin and squashed in 10% propionic acid before the slides were made permanent.

For histological studies, fixed material was dehydrated in ethanol-xylene series and embedded in paraffin, sections of 9-11 µm in thickness were stained with Heidenhain's haematoxylin and made permanent.

RESULTS

Seeds germinated *in vitro* a week after inoculation and callused from the excised hypocotyl regions of the seedlings within three days. The proliferated callus exhibited yellow and friable nature. The callus on subculture to MS medium supplemented with a combination of auxins and cytokinins exhibited various morphological characters and presented in Table 2. The callus proliferation was observed in all the treatments and the callus on subculture at 3 mg/l benzyl amino purine (BAP) and 1 mg/l naphthalene acetic acid (NAA) and at 2 mg/l BAP and 1 mg/l NAA induced shoot buds (Figure 2) from hard green compact callus.

Stem explants particularly, internodes after callus induction were subcultured on the same medium supplemented with various growth regulators both individually and in combinations at different concentrations. The same is presented in Table 2. The response of stem callus on subculture showed little or no morphological changes except in the combination of BAP and NAA where multiple shoot buds were differentiated from the proliferated callus (Figure 3). The leaf explants callused from the ventral surface better than the dorsal surface. Morphologically, the callus of ventral surface was soft and coherent while that of dorsal surface was hard and nodular.

The callus that proliferated in the primary culture was subcultured on hormone-free medium as well as on the medium supplemented with growth regulators alone and

Table 2. Effect of subculture of calli from different explants on MS medium supplemented with various growth regulators.

Growth regulators	Seed callus	Stem callus	Leaf callus
Basal	-	Proliferation	-
2,4-D	Proliferation	--	Callus proliferation (Figure 1)
BAP	Friable	Proliferation	Shoot bud at 2 mg/l
Kn	Luxuriant callus	-	Root initiation
NAA	-	Root initiation	Cohesive callus
BAP+IAA	Root initiation	-	-
BAP+NAA	Shoot buds at 2mg/l + 1mg/l	Multiple shoots at 3mg/l + 1mg/l	Shoot buds at 3 mg/l + 1 mg/l
BAP+Kn	Green callus	--	--
BAP+CH*	--	--	Shoot buds

*CH- Casein hydrolysate.

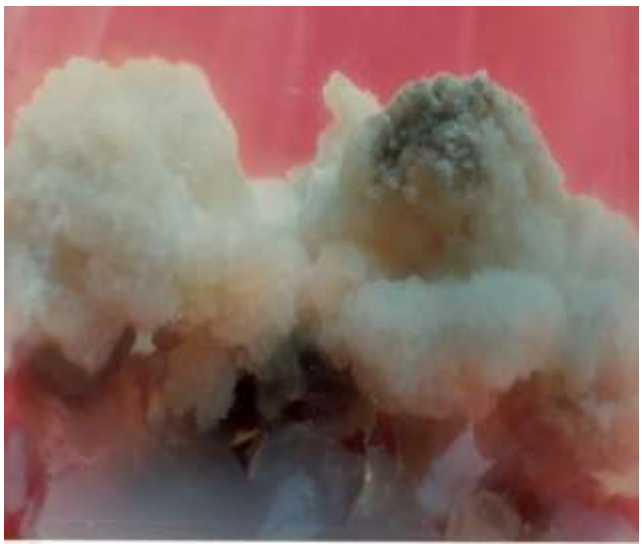

Figure 1. Leaf callus proliferated on MS + 2,4-D.

Table 3. Effect of different concentrations and combinations of BAP and NAA on multiple shoot formation from various explants in *A. indicum*

Growth hormone (mg/l)	Leaf		Stem		Seed	
	Response (%)	Average no of shoots/explant	Response (%)	Average no of shoots/explant	Response (%)	Average no of shoots/explant
BAP						
0.5	10	4±0.5	4	1.5±0.6	-	-
1.0			11			1.8±0.5
2.0	18	6.5±0.8	15	5.1±0.9	8	3.1±0.4
3.0	25	8.3±0.6	21	7.2±1.4	10	4.3±0.3
5.0	15	5±0.2	12	4.4±0.5	6	2.8±0.1
BAP+NAA						
1+0.5	40	10±0.2	15	5±1.0	7	3.0±0.8
2+1	55	18.0±0.5	22	7.5±1.1	11	4.5±0.3
3+1	75	20.2±2.2	30	8.6±0.9	13	4.7±0.5
4+1	45	10.6±0.3	14	4.9±0.8	10	4.4±0.6

*Each treatment consists of 25 replicates and each treatment was repeated at least thrice.

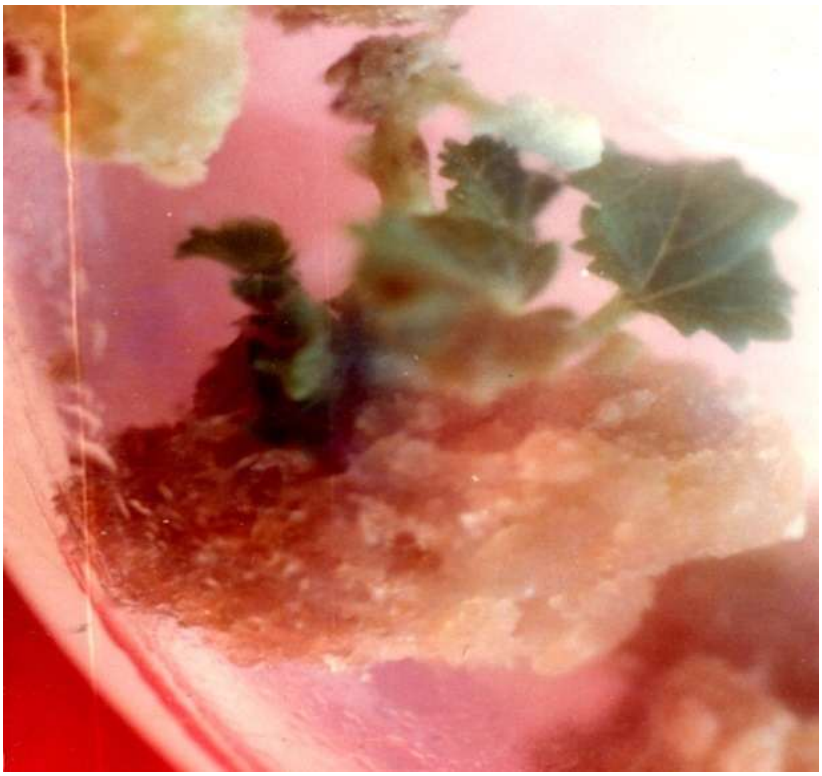

Figure 2. Differentiation of shoot buds from seed callus on MS+2 mg/l BAP + 1 mg/l NAA.

Figure 3. Differentiation of shoot buds from stem callus on MS+3 mg/l BAP + 1 mg/l NAA.

in combinations and their responses are presented in Table 2. On BAP (2 mg/l) alone as well as on BAP and NAA combinations, morphological nature of the callus was observed wherein several shoots were differentiated (Figure 4). The excised shoots after rooting and acclimatisation were transferred to pots (Figure 5).

Shoot proliferation from all these explants differed according to concentrations of cytokinin alone or in combinations of cytokinin and auxin used (Table 3). Of the three explants, the best and rapid regeneration was

Figure 4. Regeneration of shoots from leaf callus on MS +3 mg/l BAP + 1 mg/l NAA.

Figure 5. Acclimatised plant.

observed in leaf explants on MS medium supplemented with 3 mg/l BAP and 1 mg/l NAA. This treatment yielded highest number (75) of regenerated shoots than stem explant (30) followed by seed explants showing least frequency (13%). Regenerated plants from these explants subcultured on the same medium yielded more number of shoots and remained constant after 4th subculture.

Shoots were harvested every 25-35 days and 70% of shootlets produced roots on the rooting medium containing NAA (1.0 mg/l) after 15 days and showed highest rooting efficiency (Table 4). Indole-3-acetic acid (IAA) and IBA also induced more number of root initials.

The well-developed plantlets were transferred to the cups containing vermiculite and peat in 1:1 ratio followed by acclimatisation in moist chamber having 85-90% humidity and finally transferred to garden soil.

Histological studies of the callus from all the explants have been studied. Seed callus in section possess growth or active centres with compact cells having dense cytoplasm and conspicuous nuclei (Figure 6). In the embryogenic calli, embryoids and shoot buds were observed. The stem callus in section showed homogenous mass of large and vacuolated parenchyma cells in the centre while peripherally the cells are densely cytoplasmic and compact from which multiple shoot buds were differentiated. The leaf callus in section showed well organised shoot buds interspersed with embryoids of various shapes (Figure 7). Each shoot bud is with a prominent shoot apex flanked by two primordial leaves (Figure 8) and each embryoid possesses an outer jacket layer and inner mass of cells connected by a vascular strand at the base of the embryoid.

The calli of all the explants from primary cultures showed normal mitotic behaviour. After subsequent subcultures, the callus of 2-month-old was found to possess cytological observations like multinucleate cells, chromosomal bridges (Figure 9) at anaphase, ploypoid cells (Figure 10) and tracheary elements.

Morphology of regenerated plants

Well established regenerated plants from the seed and stem cultures resemble the *in vivo* plants in all respects. Regenerated plants from the leaf cultures though cytologically similar to *in vivo* plants, the growth of the plant is robust and measured two meters in height. The flowers from these tall plants showed gorgeous flowers with good seed setting.

DISCUSSION

In the present investigation, it was found that MS medium was an effective nutrient medium for callus maintenance and plant regeneration in all the explants studied. Plant regeneration achieved in this species depend on the concentration of cytokinin along with auxin in a proper

Table 4. Effect of auxins on root induction in *in vitro* raised shoots of *A. indicum.*

Auxin concentration (mg/l)	Shoots rooted (%)	Number of roots/shoot
NAA (0.5)	45	11.7±0.2
NAA (1.0)	70	18.1±0.4
NAA (2.0)	30	9.9±0.1
IAA (0.5)	35	8.1±0.9
IAA (1.0)	60	15.3±0.5
IAA (2.0)	40	10.6±0.7
IBA (0.5)	40	10.4±0.6
IBA (1.0)	55	14.7±0.3
IBA (2.0)	25	8.4±0.2

*Each treatment consists of 25 replicates and each experiment was repeated at least thrice.

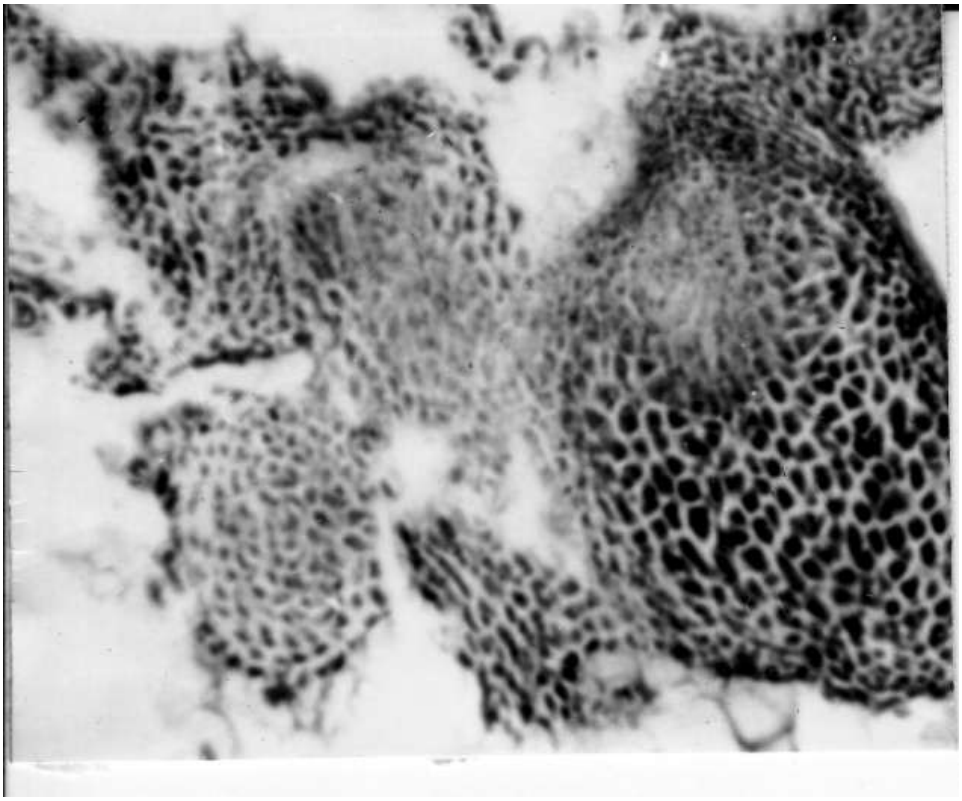

Figure 6. Active centres in callus section.

initial ratio unlike in *Cucumis* (Trulson and Shahin, 1986), where differentiation depend on the presence of lower or higher concentrations of auxins and are independent of cytokinins. During subcultures, the calli produced plantlets in the presence of auxin and cytokinin combinations similar to the work on *Hoslundia opposita* (Prakash and Van Standen, 2007) and *Withania somnifera* (Parashuram et al., 2009). In all the explants of the present study, root formation was observed only at very low concentration of auxins and was inhibited at higher concentrations. Contrary to this, Bhansali and Arya (1978) reported that root formation was completely inhibited at lower levels of auxin in *Citrus* stem and leaf cultures. The excised shoots failed to induce roots in the combinations of auxins contrary to the work of Sri Rama Murthy (2010) who demonstrated that the combinations of different auxins IBA and NAA had cumulative effect and played a great role in the induction of roots. The best root development was recorded at 1 mg/l NAA. Anuradha and Pullaiah (1992) reported that NAA was a more effective rooting agent for *Morus alba*.

Histological evidences revealed that all these explants

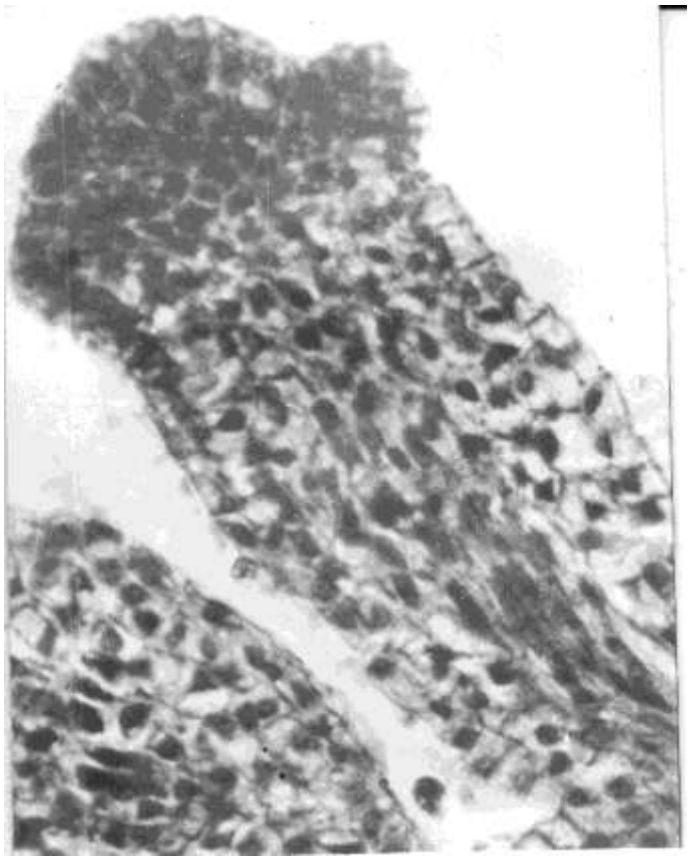

Figure 7. An embryoid in embryogenic callus.

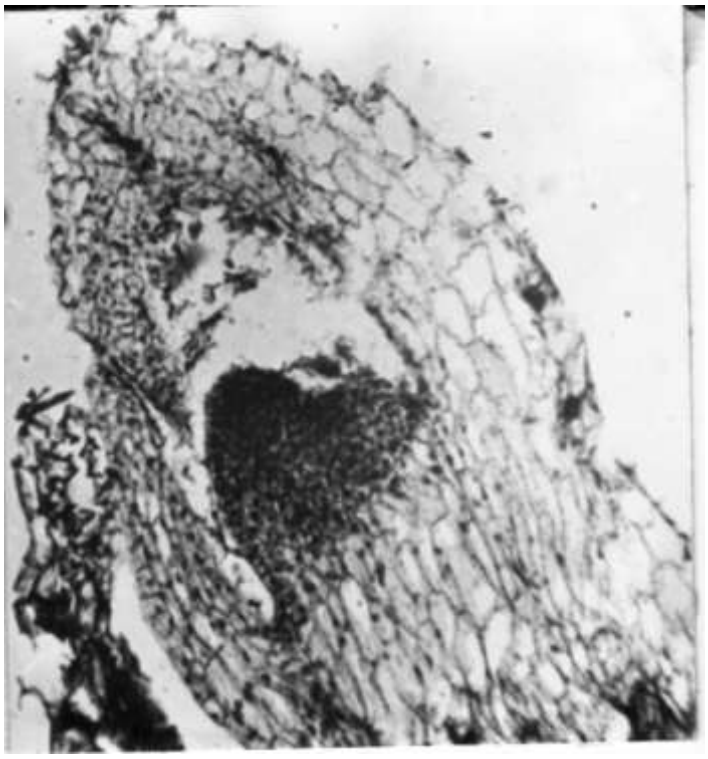

Figure 8. A differentiated shoot bud in section with a pair of primordial leaves.

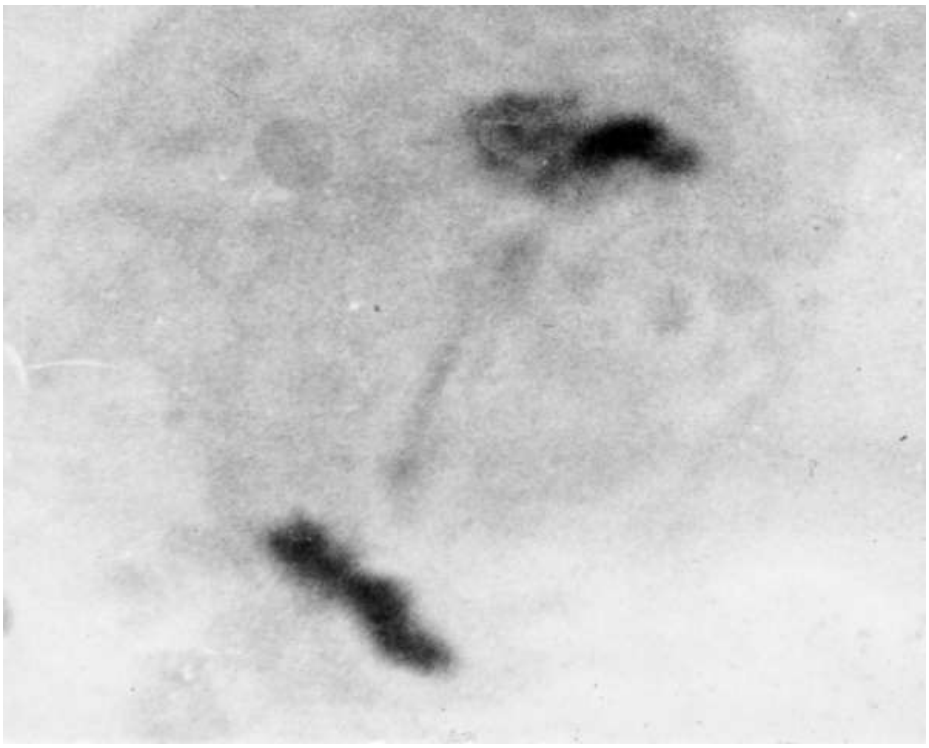

Figure 9. Chromosome bridge at anaphase.

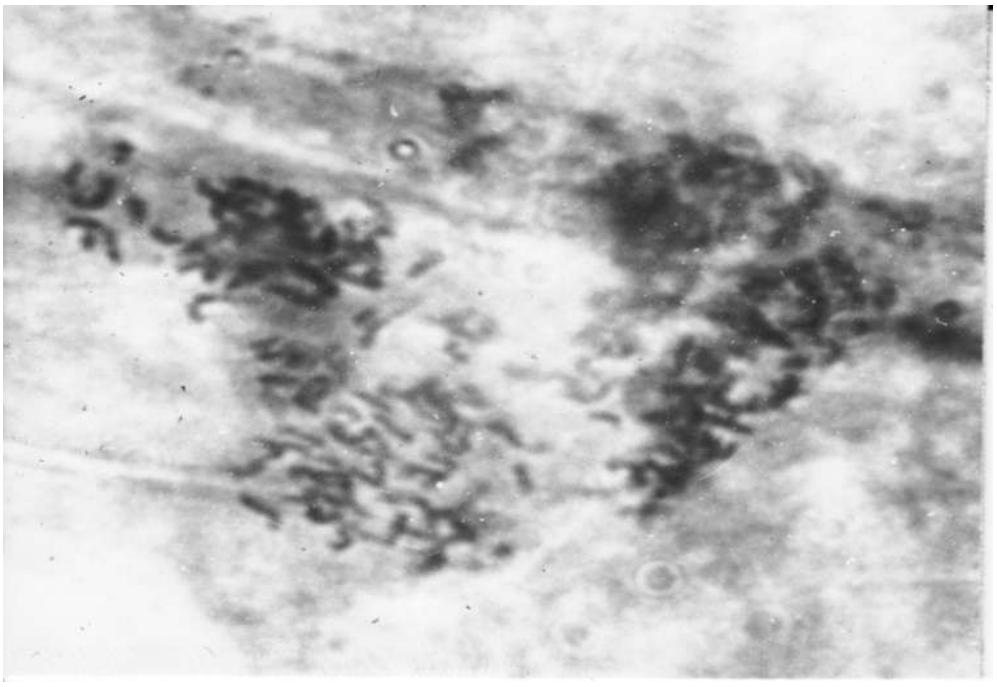

Figure 10. A polyploid cell at early anaphase.

exhibited differentiation in the form of shoot buds and also embryoids. After four weeks, from the embryogenic calli, protuberances of somatic embryo initials were observed. In most cases, regeneration of shoots from all these explants was obtained through organogenesis, while some plants were regenerated through embryogenesis. Somatic embryogenesis in *A. indicum* is frequent, in contrast to the work of Pillon et al. (1996) on

Arabidopsis cultures, but at par with the work of Fras et al. (2002), wherein they were able to achieve highest frequency of advanced somatic embryos. The callus showed ability for normal organogenesis upto 5th and 6th subcultures which decline further in its regeneration potential.

Mitotic abnormalities in the primary cultures were not seen as there was induction of differentiation in cultures and there was a stable chromosomal state in the callus. Similar observations were reported in the callus cultures of *Beta vulgaris*, where a large number of diploid regenerants were obtained (Jacq et al., 1992). In the later subcultures, chromosomal abnormalities such as chromosomal bridges, multinucleate, multinucleolate, asynchrony and polyploidy cells were evident. The occurrence of polyploidy phenomenon may be due to endoreduplication, a case found in cultures of Singh (1984). *In vivo* cytological studies showed no polyploid cells but *in vitro*, the callus cells exhibited polyploid cells (3%) as compared to the cultures of tomato (van den Bulk et al., 1990) where there is a polyploidy both *in vivo* and in vitro. Moreover, occurrence of this polyploid behaviour may be due to the culture conditions they are subjected to as well as genotype of explant.

The callus that proliferated on the medium supplemented with cytokinin was subcultured on the hormone-free medium to find tracheary elements suggesting that the initial application of an exogenous cytokinin is sufficient to induce xylogenesis in the preceding cultures without the addition of growth hormones and subsequently inducing shoot and root. Similar type of behaviour has been observed in tissues of *Allium sativum* (Novak, 1981). Xylem differentiation has been studied in tissue culture for a number of years but still what controls, where and when in the differentiation pathway is still a conundrum.

The plantlets with well-developed shoot-roots were transferred to pots containing soilrite and the acclimatized plants were finally transferred to soil with 80% survival rate. It is inferred that the technique described here provides a promising method for rapid propagation of this medicinal plant species.

Conflict of interests

The author(s) did not declare any conflict of interest.

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