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Somatic embryogenesis and regeneration of triploid plants in endosperm cultures of *Acacia nilotica*

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Abstract. Immature endosperm of *Acacia nilotica* formed a nodular callus on MS medium supplemented with 2,4-D, BAP and CH. In the third passage on this medium, in the dark, the callus differentiated somatic embryos. The embryos germinated on MS only after 15 d pre-treatment on modified MS medium in which major salts were replaced by those of major salts of B_5 medium and supplemented with glutamine, CH and CW. Triploid nature of the somatic embryos was confirmed by Feulgen cytophotometry.

Key words: *Acacia nilotica* - Somatic embryogenesis - Endosperm culture - Triploid production - Leguminous tree.

Abbreviations: ABA = abscisic acid; AC = activated charcoal; BAP = 6-benzylaminopurine; B_5 = Gamborg et al. (1968) medium; CH = casein hydrolysate; CW = coconut water; d = days; MS = Murashige and Skoog (1962) medium; PEG 4000 = polyethylene glycol (MW = 3500-4000); 2,4-D = 2,4-dichlorophenoxyacetic acid.

Introduction

Triploids are seed sterile and, consequently, undesirable for plants where seeds are of commercial importance. However, for profusely seeding tree species which are grown for biomass and/or soil conservation, such as *Acacia nilotica*, triploidy could be desirable as it is likely to promote vegetative growth by conserving the photosynthates normally consumed in seed and fruit production. As compared to their diploids, the triploids of *Populus tremuloides* have better pulp qualities (Bhojwani and Razdan 1983). Some other examples where triploids are being commercially used are apple, banana, tea and watermelon.

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The common approach to producing triploids is to produce tetraploids and cross them with diploids. However, such crosses are not always successful. Endosperm is a natural triploid tissue and regeneration of plants from this tissue offers a direct method to produce triploids. Since the first demonstration of totipotency of endosperm cells (Johri and Bhojwani 1965), triploid production through endosperm culture has been reported for several species, including apple, citrus, kiwifruit and sandalwood (see Bhojwani and Bhatnagar 1992, p 210). This paper describes somatic embryogenesis and triploid plant regeneration from immature endosperm cultures of *Acacia nilotica*, an important leguminous tree species suitable for afforestation of arid and marginal lands.

Material and Methods

Endosperm culture. Immature pods of *Acacia nilotica* (Linn.) Willd. ex Delile ssp. *indica*, were collected, in January-April, from trees planted by the Forest Department of Delhi Administration. The fruits were rinsed in 1% Alpilon solution for 30 min and, after washing in tap water for about 30 min, surface sterilized with 0.1% solution of mercuric chloride for 10 min. The pods were washed with sterile distilled water and seeds were excised under aseptic conditions. Endosperm and endosperm with intact embryo were removed with the aid of a stereoscopic microscope and explanted on culture medium. In *Acacia*, endosperm is of the nuclear type and is consumed by the time the seed attains full development. Therefore, immature cellular endosperm from seeds containing 0.5-3 mm long dicotyledonous embryo (mature embryos are 6-10 mm long) was cultured. Five explants were placed in a disposable 55x10 mm petri plate containing 10 ml of medium. Endosperm callus was subcultured in 25 x 150 mm culture tubes capped with cotton plugs wrapped in cheese cloth. For each treatment, 50 explants were cultured.

MS (Murashige and Skoog 1962) and a modified MS medium, were used as the basal media. In the modified MS medium the major salts were replaced by those of B_5 medium (Gamborg et al. 1968) and supplemented with 500 mg/l inositol, 10% coconut water (CW), 500 mg/l glutamine and 200 mg/l casein hydrolysate (CH). Modified MS medium was gelled with 0.2% phytagel. The standard MS medium was variously supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine

(BAP), CH and CW. The MS media were gelled with 0.8% agar. Media pH was adjusted to 5.8 before autoclaving at 1.06 kg/cm^2 for 15 min.

Cultures were maintained at 25°C ± 2°C in light (35.5 μ mol m^{-2}s^{-1}) supplied by two Philips TL 40 W/54 fluorescent tubes or darkness.

Feulgen cytophotometry. Since the chromosomes of *A. nilotica* are very small and their number very high (2n=52), ploidy of the plantlets regenerated from endosperm callus was determined by Feulgen cytophotometry (Raina and Rees 1983). Root tips from seedlings of the parent plant served as the diploid control.

Root tips were fixed in 4% formaldehyde, at pH 7, for 2 h. After washing under running tap water for 24 h the root tips were refixed in 1:3 acetic-ethanol for 24 h. The fixed roots were thoroughly washed in distilled water, hydrolysed in 5N HCl at room temperature (*ca* 25°C) for 60 min. The roots were given four washes in distilled water and treated with Schiff's reagent for 1 h, in the dark. This was followed by washing the roots in 0.5% solution of sodium metabisulphite for 10 min. The root tips were again washed in distilled water four times and squashed in a drop of 50% glycerol on a glass slide and covered by a no. O cover glass. The intensity of stain was measured using Leitz MPV compact cytophotometer at a wavelength of 565 nm. Root tips from three plantlets of endosperm origin and three seedlings were used for DNA measurement, and for each root 28-37 nuclei at the late telophase stage were scanned. The intensity of stain is expressed in arbitrary units.

Results and Discussion

Initiation of callus

On MS basal medium the excised endosperm turned brown and eventually died without showing any callusing. Even in the presence of the embryo, the endosperm did not proliferate on the basal medium, although the embryo germinated normally.

When the MS medium was supplemented with 10 μM 2,4-D, the endosperm formed a brown nodular and friable callus irrespective of the presence or absence of embryo. However, the degree of callusing was higher when the endosperm was cultured without the embryo. In the cultures of endosperm + embryo, the embryo did not show any sign of callusing.

Callusing of endosperm was promoted by the combined presence of 2,4-D, BAP and CH. The MS medium was supplemented with these substances at two levels: (1) 2.5 μM 2,4-D; 5 μM BAP and 500 mg/l CH, and (2) 10 μM 2,4-D; 25 μM BAP and 1000 mg/l CH. Of these, the latter combination induced better callus proliferation. On this medium, even the embryo callused when cultured along with endosperm (Fig. 1A). However, the embryo callus was carefully discarded at the initial stages to ensure the purity of endosperm callus. The endosperm callus formed in the presence of 2,4-D, BAP and CH was nodular, friable and light brown with green loci (Fig. 1C). Irrespective of the explant and the medium, endosperm callusing always started at the chalazal end (Fig. 1A,B).

Initiation of embryogenic cultures

In the first and second subcultures on MS + 2,4-D + BAP + CH, the endosperm calli maintained in light grew well but remained unorganized. However, in the third passage, the calli incubated in the dark exhibited somatic embryogenesis in 7% of the cultures (Fig. 1D). Embryogenesis was asynchronous. In subsequent subcultures of the embryogenic calli, in the dark, the proliferation of somatic embryos increased, and the embryogenic cultures could be maintained by 8-week subcultures. The embryos occurred in large clusters, and the adjacent embryos appeared interconnected.

Several authors have highlighted a role of embryo in the proliferation of mature endosperm (Bhojwani 1984). Immature endosperm generally does not require the association of an embryo to form callus. In *Acacia* the presence of embryo was not only unnecessary but deleterious for the initial proliferation of endosperm callus.

Germination of somatic embryos

Clusters of somatic embryos from the embryo differentiation medium were transferred to the modified MS medium, in light, for germination. None of the embryos showed normal germination or plumular leaf development. However, some of the embryos, which appeared disconnected from the rest of the clusters, developed

Table 1. Pretreatment effects on germination of somatic embryos on the modified MS medium, in light, at 25°C. The pretreatments were given in the dark at 25°C or as specified. 1/2-MS salts was used during pretreatment.

Pretreatments		No. of cultures	% Cultures showing		
Supplements	Duration (days)		Only root formation	Only shoot formation	Bipolar germination
1. Control (no treatment)		21	14	-	-
2. 10 μM ABA	10	12	50	-	-
3. 10 μM ABA	20	12	25	-	-
4. 10 μM ABA	30	16	38	-	-
5. 10% sucrose	10	15	27	-	-
6. 10% sucrose	20	18	50	-	-
7. 10% sucrose	30	24	29	-	-
8. 10 μM ABA + 7.5% PEG 4000	15	14	21	-	-
9. 7.5% PEG 4000	15	23	26	4	-
10. 4% mannitol	15	41	42	2	-
11. 16 gm/l agar	15	19	21	5	-
12. 7.5% PEG 4000 + 0.25% charcoal	15	32	19	3	-
13. As in 12 but 5 d at 4°C and 10 d at 25°C	5+10	21	5	-	-
14. As in 12 but 5 d at 35°C and 10 d at 25°C	5+10	25	12	28	4

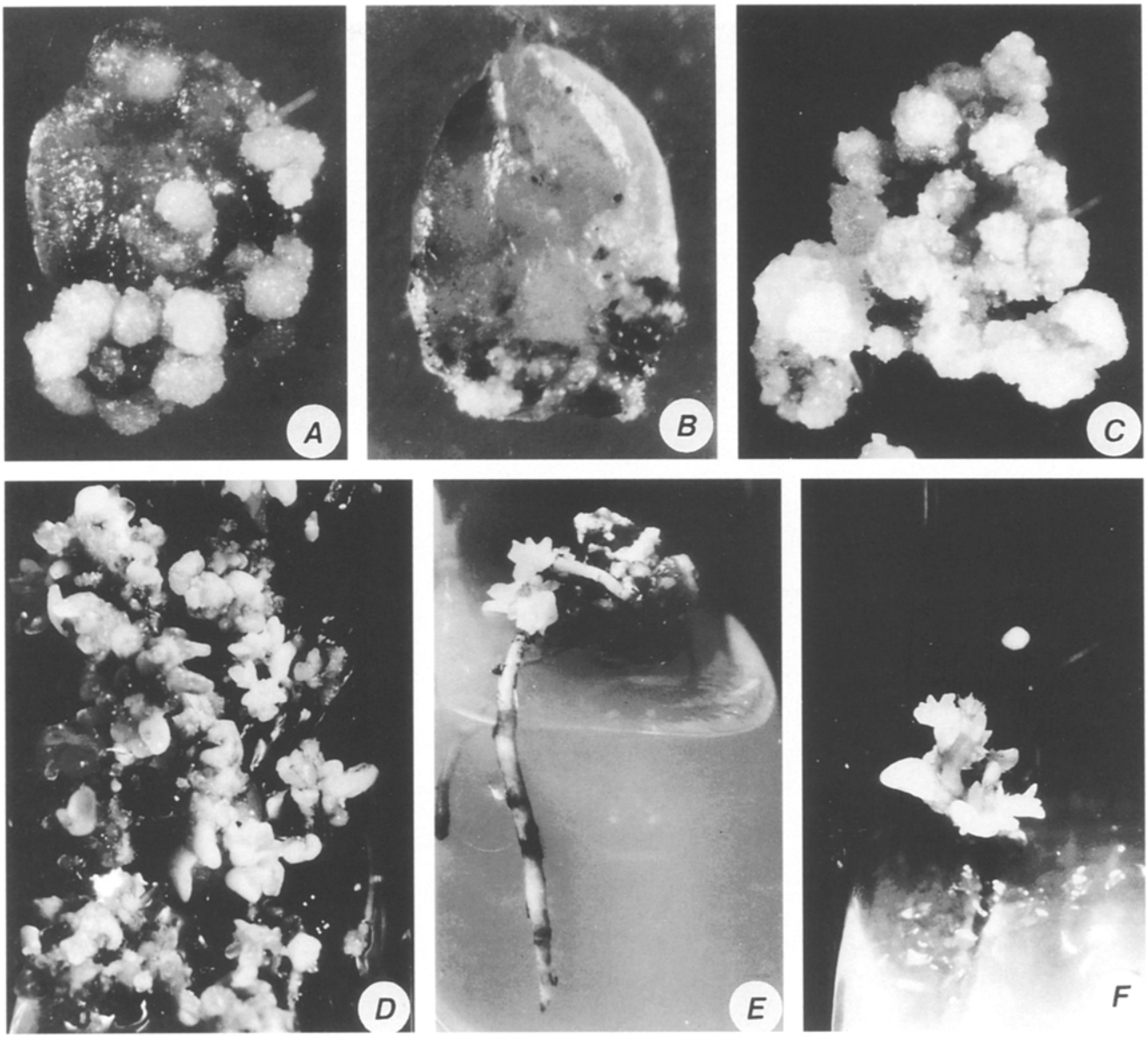

Figure 1. (A, B) Five-week-old culture of only endosperm (B) and endosperm with intact embryo (A) on MS + 10 μM 2,4-D + 25 μM BAP + 1000 mg/l CH. Note that callusing of endosperm started at the chalazal end of the endosperm; in (A) the embryo has also callused. (C) Endosperm callus 33 d after subculture. (D) Eleven-week-old subculture of endosperm callus, in the dark, bearing numerous somatic embryos at different stages of development. (E) A cluster of somatic embryos 7 weeks after transfer to the modified MS medium; some of the embryos have developed thick, brown roots. (F) A rooted embryo from (E) 30 d after transfer to the medium as in (E), showing the development of plumular leaves.

a primary root (Fig. 1E) after 5 weeks of culture. When such rooted embryos were individually transferred to fresh medium of the same composition, they developed plumular leaves (Fig. 1F).

Since the embryos isolated from the cluster showed shoot development, in the next experiment individual embryos or small embryo clusters (3-4 embryos) were transferred to the embryo germination medium directly or after a pretreatment (Table 1). The primary root developed in all the treatments, but plumular leaves were formed only when the embryos were exposed to a medium of high osmolarity. The best pretreatment for plumular leaf development was PEG 4000 and activated charcoal. In this treatment, one of the embryos exhibited bipolar germination, which was not observed in any other treatment.

A major problem associated with somatic embryogenesis is the poor conversion into plants. The embryos differentiated from endosperm callus of *Citrus grandis* did not develop

to the dicotyledonous stage (Gmitter et al. 1990). The endosperm callus of *C. sinensis* produced dicotyledonous embryos, which developed shoots but not roots. Complete plants were established by micrografting of the shoots onto the diploid root-stock seedling. Contrary to this, the somatic embryos formed by the endosperm callus of *A. nilotica* formed roots more readily than shoots. Only two triploid plantlets have been obtained so far. Efforts are being made to improve the frequency of normal germination of these embryos.

DNA estimation

The Feulgen cytophotometric data (Table 2) revealed that the DNA content in the root tip cells of endosperm origin was 1.5 times that of cells of the seedling root tip of *A. nilotica*. It is, therefore, concluded that the embryos and root tips differentiated from endosperm callus were triploid.

Table 2. DNA content of root tip cells from zygotic seedlings and plantlets regenerated from endosperm callus. The values are expressed in arbitrary stain intensity units.

Source of root tip	Replicate	DNA content	Mean DNA content ± S.E.
1. Zygotic seedling	1	72.8	
	2	71.3	71.7 ± 0.54
	3	71.1	
2. Somatic embryos of endosperm origin	1	107.1	
	2	106.6	107.1 ± 0.26
	3	107.5	

Conclusions

Mature and immature endosperm of several species have been reported to yield continuously growing callus and differentiation of shoots, embryos or complete plants directly or after callusing (Bhojwani 1984). Some of the tree species where triploid regenerants have been obtained are *Citrus sinensis* (Gmitter et al. 1990), *Putranjiva roxburghii* (Srivastava 1973) and *Santalum album* (Lakshmi Sita et al. 1980). In *Citrus* and *Santalum* regeneration occurred via somatic embryogenesis, as in *Acacia* in the present study. However, in *Putranjiva* regeneration involved organogenesis. This is the first successful report of establishment of tissue cultures and regeneration of triploid plantlets from the endosperm of a leguminous species.

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