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Ferula gummosa Boiss. Embryogenic Culture and Karyological Changes

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Abstract: *Ferula gummosa* Boiss. a highly valuable medicinal plant which naturally propagates in very limited areas of the Middle East with specific environmental conditions. The production of *Ferula gummosa* somatic embryos and the karyological analysis of somatic seedlings were the purpose of this study. High frequency indirect embryogenesis was induced in callus derived from zygotic embryonic axes. Embryogenesis was obtained when callus tissues were placed onto an agar induction Murashige and Skoog medium with 1-naphthalene acetic acid and after the transfer of the cultures in a thermoperiod regime of 16 h, 19°C/8 h, 7°C under photoperiod of 16 h light/8h dark. Embryogenic callus tissues were maintained by subculture on induction medium. Globular proliferation was achieved with suspension culture in the Murashige and Skoog medium added with 1-naphthalene acetic acid or 2,4-dichlorophenoxyacetic acid for two weeks. Maturation of embryos and development of plantlets arose on the induction agar medium, but was better after transfer into the hormone free Murashige and Skoog medium. However, the level of abnormal embryos was high. Direct embryogenesis was obtained from somatic seedlings. The best results were obtained from hypocotyl explants. Embryo induction was achieved by two week culture of the explants in 2,4-dichlorophenoxyacetic acid liquid medium; somatic embryo growth and maturation was recovered on the hormone free medium. High level of abnormalities was recorded in the culture. Karyological analysis showed a high incidence level of cytochimerism in somatic seedlings with chromosome stickiness, polypoidy and aneuploidy in metaphase cells of the same root tip. The frequency of these karyological changes varied with the type of somatic embryos with regard to morphological abnormalities. Normal and abnormal rooted somatic seedlings were able to grow until production of the first leaf and then entered dormancy in the same manner as zygotic plantlets.

Key words: Somatic embryos, cytochimerism, Galbanum

INTRODUCTION

Geographical distribution of Galbanum (*Ferula gummosa* Boiss., Apiaceae) is limited to some countries of the Middle East (Afghanistan, Iran, Pakistan, Turkmenistan, Turkey) and this species is considered to be the quintessential Iranian medicinal plant. It grows in high altitude mountain regions (from 1800-6000 m) with very specific environmental conditions so that the natural plant propagation of this species is limited. Root tubers are exploited for the high value gum. Galbanum is used in the production of cosmetics, rubber and explosives. However, the gum production is not sufficient to satisfy the trade needs. Because of the overexploitation, this species is going to be endangered.

Plant biotechnology offers new ways to propagate plants at a high rate of production. Among them, *in vitro* embryogenesis may provide an alternative to plant micropropagation. However, in a number of species because of morphological abnormalities or immaturity of embryos, plant recovery is limited (Ammirato, 1987; Janick, 1993).

To date, no published studies concern embryo somatic culture in Galbanum. This may be due to the difficulties of culture; in *ex situ*, this species has very specific growth requirements. In the present study, we report results concerning the production and development of Galbanum somatic embryos and cytogenetic variations observed in abnormal regenerated somatic seedlings.

MATERIALS AND METHODS

Source of explants: Seeds of *Ferula gummosa* (Boiss.) were collected from the region of Firouz kuh (Tangevashi) in the north of Iran. Seeds were surface sterilized by successive immersions into 70% (v/v) ethanol for 1 min, 10% (v/v) hydrogen peroxide for 10 min and 20% (v/v)

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sodium hypochlorite with 0.1% (v/v) 20 for 20 min. Embryonic axes from zygotic embryos axes were used as explants. Zygotic embryonic axes were excised from the seeds, rinsed in sterile ddH_2O and treated with 70% (v/v) ethanol for 30 sec and rinsed again in sterile ddH_2O before placement on culture medium.

Initiation and maintenance of embryonic callus: MS medium (Murashige and Skoog, 1962) with Gamborg vitamins (Gamborg *et al*., 1968) and 200 mg L^{-1} casein hydrolysate and 3% (w/v) sucrose, pH 5.8 was used as basal medium in this study.

Callus tissues were obtained from embryonic axes cultures treated by 1 mg L^{-1} BAP (6-benzylaminopurine) added to the agar (0.8% (w/v) agar) basal medium. The cultures were incubated at 25°C and 16 h light/8 h dark photoperiod regime. Maintenance of callus was performed by regular subculture onto the same medium for several months. After 3 months of culture, indirect embryogenesis was induced by 1 mg L^{-1} NAA (1-naphthalene acetic acid) and a particular thermoperiod (18°C day/8°C night) regime. Visible embryos in the globular, heart-shape, torpedo and cotyledonary phases were observed using a stereomicroscope (model SZH-ILLB, Olympus Optical Co. LTD), at a magnification of 10 X.

Suspension cultures were used to produce somatic embryos. For this purpose, 1 g of embryogenic callus of *Ferula gummosa* was inoculated in the liquid basal culture medium with two different hormonal treatments, 1 mg L^{-1} NAA or 0.5 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid). After two weeks of culture at 25°C with 16 h light/8 h dark photoperiod and agitation on an orbital shaker at 120 rpm, cultures were recuperated by filtration on filter paper and washed by sterile ddH_2O three times. Tissues were then distributed in 4 culture jars on the agar maturation medium (basal medium) without phytohormones. Three series were done for each treatment. Somatic embryos were examined with a stereomicroscope and the number of regenerated somatic seedlings was registered after 40 and 80 days of culture on maturation medium.

Secondary direct embryogenesis: Somatic seedlings, 40 days old, obtained as above were used for this purpose. Different hormonal treatments were applied on total seedlings or various explants by several ways.

For the first treatment, samples were placed in suspension basal initiation medium with 1 mg L^{-1} NAA or 0.5 mg L^{-1} 2,4-D for 15 days and then were transferred onto maturation medium without hormones. Three series of samples were treated: (1) total somatic seedlings in initiation medium were transferred into the maturation medium; (2) total somatic seedlings in initiation medium were cut in different explants then cultivated on the maturation medium; (3) the different explants were first isolated from seedlings followed by inoculated into the initiation liquid medium and then transferred to the maturation medium after 15 days.

In a second treatment, samples were directly cultivated on agar basal medium with different hormonal combinations of 2.4-D (0. 2. 5 mg L^{-1}), Kinetin (0, 0.5 mg L^{-1}), NAA(0, 5 mg L^{-1}), BAP (0, 0.5 mg L^{-1}). After two months, they were transferred to the basal medium.

All suspension cultures were maintained at 25°C with 16 h light/8 h dark photoperiod and agitation on an orbital shaker at 120 rpm for 15 days. Cultures on solid medium were maintained under a thermoperiod (18°C day/ 8°C night) and photoperiod (16 h light/8 h dark) regime.

Cytological analysis: Chromosome number was determined from somatic cells at metaphase of the root tip meristems of randomly selected somatic and zygotic seedlings. To determine chromosome number, the root tips were pretreated with 0.002 M hydroxyquinolein for 2 or 3 h. Roots were rinsed with tap water and were dipped into the fixative solution, glacial acetic acid/ ethanol 96% (1/3), for 24 h. Roots were then rinsed with ddH_2O and blotted dry with filter paper. The material was then conserved in 70% (v/v) ethanol at 4°C. After a suitable time, root tips were rinsed again and treated with 1 N HCl at 60°C for 5-6 min. Root tips were rinsed again in water and maintained in water for 1 min. Roots were sectioned transversely (1 mm or less). Sections were stained with 2% (w/v) acetoorcein for 8-10 min and squashed.

Statistical analysis: Data were compared by using T test and Chi square analysis done by the mean of SPSS, version 9.

RESULTS AND DISCUSSION

Callus induction and indirect somatic embryogenesis: Direct and indirect somatic embryogenesis has been described in literature for many other species (Thorpe, 1995). In the present case initially it concerns indirect embryogenesis as embryonic axes first have developed callus tissues on MS basal medium with 1 mg L^{-1} BAP at 25°C (Fig. 1A) and the embryonic competence of these tissues has been induced only by 1 mg L^{-1} NAA treatment combined with a reduction of temperature conditions of growth with a particular thermoperiod regime (18°C day/8°C obscurity). After 3 months of culture in these conditions globular structures with a

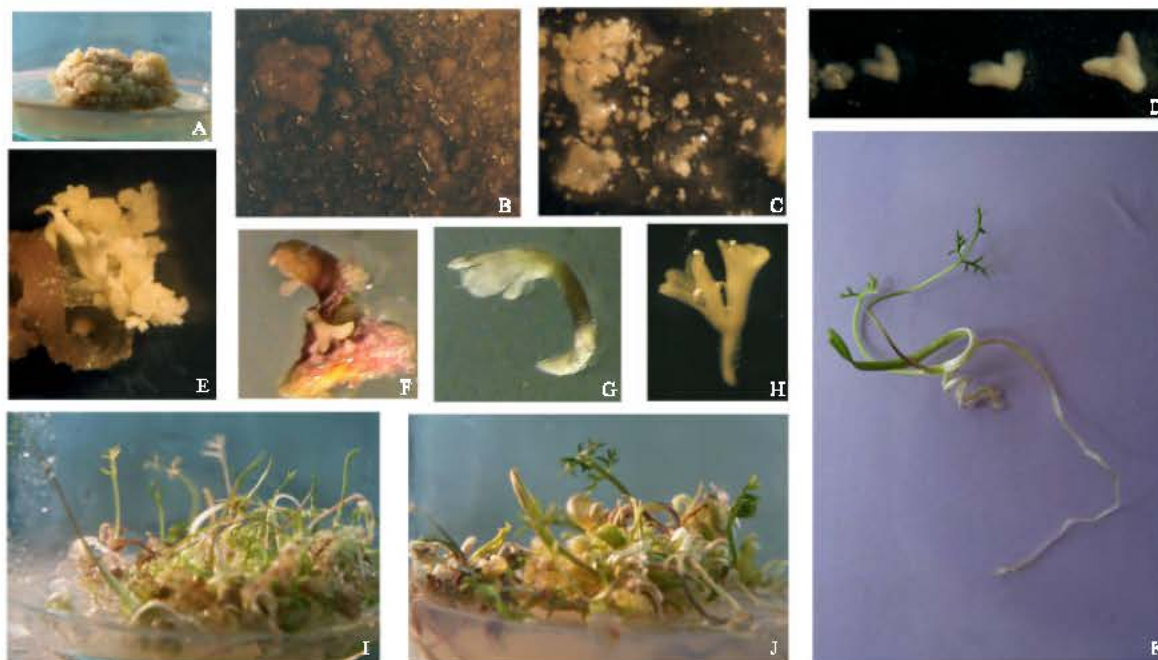

Fig. 1: Different developmental stages of somatic embryogenesis of Ferula gummosa Boiss, (A) Callus derived from embryonic axis of zygotic embryos growing on MS medium supplemented with BAP 1.0 mg LG^{-1}, (B) Globular stage of somatic embryo in culture with NAA 1.0 mg LG^{-1}, (C) Embryos maturation in MS medium supplemented with NAA 1.0 mg LG^{-1}, (D) different stages of somatic embryo development, (E) Clusters of secondary somatic embryos derived from hypocotyl tissues of the primary somatic seedling, (F, G and H) Different shapes of abnormal somatic embryos, (I) Somatic seedlings germination on MS medium supplemented with NAA 1.0 mg LG^{-1}, (J) Somatic seedlings germination on free hormone MS medium and (K) somatic plantlet

pronounced bright white color differed from the rest of the tissues of the culture and proliferated on the medium. Globular proliferation was improved with suspension culture in the basal medium added with 1 mg L^{-1} NAA or 0.5 mg L^{-1} (Fig. 1B). Auxin potential in the induction of somatic embryos has been well documented in many plants but environmental factors may also induced somatic embryogenesis by mimic auxin effect (Thorpe, 1995). Cold treatment can have stimulated the formation of proembryonic masses in *Ferula gummosa* callus culture as it has been recorded with carrot cell culture (Krul, 1993). One may consider that stress situation may trigger defense mechanisms of the cells that respond by activating the embryogenic potential and so the self reproduction of the system to withstand the stress. Exogen auxin treatment may perhaps be counted as a stress situation by the tissues. And in that hypothesis one may think that embryonic aptitude of tissues may reflect their tolerance capacity with regard to stress.

2,4-D induced embryogenesis in carrot but it has been shown that NAA may also trigger embryonic competence of the carrot tissues such as we found with Galbanum and even NAA were significantly more efficient for the production of *Ferula gummosa* somatic embryos and seedlings (Table 1). Maturation of *Ferula gummosa* embryos occurred without any subculture and embryogenic culture contain somatic embryos in various stages in development that range from globular to torpedo-shaped embryos (Fig. 1C and D) and somatic seedlings that have developed the first leaf (Fig. 1I and J). Complete development of plantlets was achieved in 3 months (Fig. 1K). Removal of auxin from the culture medium is recommended for the successful completion of embryos development (Zimmerman, 1993). Nevertheless even without NAA in the maturation medium a high proportion of abnormal embryos were registered in the *Ferula gummosa* cultures.

Secondary direct embryogenesis and somatic seedlings recovery: In spite of our numerous attempts we did not success to obtain direct embryogenesis from zygotic seedlings but secondary direct embryogenesis was observed on regenerated somatic seedlings (Table 2).

Table 1: Effect of NAA and 2,4-D in initiation medium on somatic embryos maturation

Hormonal treatment in initiation liquid medium	Average no. of somatic seedlings per jar after 40 days on maturation medium±SD	Average number of somatic seedlings per jar after 80 days on maturation medium±SD
NAA (1 mg L^{-1})	39.75±3.07	69.25±6.44
2,4-D (0.5 mg L^{-1})	17.75±4.43	52.25±2.75

Each numerical value represents the mean and standard deviation from 12 replications

Table 2: Secondary direct embryogenesis in *Ferula gummosa*

Samples	Excision time with regard hormonal treatment in liquid medium	1st hormonal treatment (mg L^{-1}) in liquid basal medium, 15 days	Hormonal treatment (mg L^{-1}) in agar basal medium 2 months	Growth and development on agar medium after 1 month	Embryo production per explant
Somatic seedling (SS)		2,4-D 0.5	--	Embryogenesis	8-10
		NAA 1	--	Embryogenesis	
			2,4-D 5 + Kinetin 0.5	--	
			2,4-D 5 + BAP 0.5	--	
			2,4-D 2 + BAP 1	--	
			2,4-D 5	--	
			NAA 5 + BAP 0.5	--	
			NAA 5 + Kinetin 0.5	--	
			NAA 5	--	
SS cotyledon	Before	2,4-D 0.5	--	Embryogenesis	1-2
	Before	NAA 1	--	Embryogenesis	1-2
	After	2,4-D 0.5	--	Embryogenesis	3-5
	After	NAA 1	--	Embryogenesis	1-2
			2,4-D 5 + Kinetin 0.5	--	
			2,4-D 5 + BAP 0.5	--	
			2,4-D 2 + BAP 1	--	
			2,4-D 5	--	
			NAA 5 + BAP 0.5	--	
			NAA 5 + Kinetin 0.5	--	
			NAA 5	--	
SS hypocotyl	Before	2,4-D 0.5	--	Embryogenesis	3-5
	Before	NAA 1	--	Embryogenesis	1-2
	After	2,4-D 0.5	--	Embryogenesis	10-8
	After	NAA 1	--	Embryogenesis	5-7
			2,4-D 5 + Kinetin 0.5	--	
			2,4-D 5 + BAP 0.5	--	
			2,4-D 2 + BAP 1	--	
			2,4-D 5	--	
			NAA 5 + BAP 0.5	Callogenesis	
			NAA 5 + Kinetin 0.5	--	
			NAA 5	--	
SS root apex	Before	2,4-D 0.5	--	--	
	Before	NAA 1	--	--	
	After	2,4-D 0.5	--	--	
	After	NAA 1	--	--	
			2,4-D 5 + Kinetin 0.5	--	
			2,4-D 5 + BAP 0.5	--	
			2,4-D 2 + BAP 1	--	
			2,4-D 5	--	
			NAA 5 + BAP 0.5	Callogenesis	
			NAA 5 + Kinetin 0.5	Callogenesis	
			NAA 5	--	
SS root without apex		2,4-D 5 + Kinetin 0.5	--		
			2,4-D 5 + BAP 0.5	--	
			2,4-D 2 + BAP 1	--	
			2,4-D 5	--	
			NAA 5 + BAP 0.5	--	
			NAA 5 + Kinetin 0.5	Calls	
			NAA 5	Calls	
SS leave	Before	2,4-D 0.5	--	--	
	Before	NAA 1	--	--	
	After	2,4-D 0.5	--	--	
	After	NAA 1	--	--	
SS petiole	Before	2,4-D 0.5	--	--	
	Before	NAA 1	--	--	
	After	2,4-D 0.5	--	--	
	After	NAA 1	--	--	
SS root apex	Before	2,4-D 0.5	--	--	
	Before	NAA 1	--	--	
	After	2,4-D 0.5	--	--	
	After	NAA 1	--	--	

Several factors acted in this process as the nature of initiation medium (liquid or agar medium), the growth regulators in the initiation and maturation medium, the nature of explants from regenerants, the timing of explants excision. As shown in Table 2 secondary direct embryogenesis occurred if the samples were cultivated into liquid initiation medium and only on cotyledons or hypocotyls (Fig. 1E), isolated or *in situ* on the regenerated seedlings. Hypocotyl excision from regenerated seedlings after the step of induction in liquid medium improved the production of embryos and 2,4-D increased the performance of the culture slightly. Nevertheless a high percent of abnormal embryos (about 74%) with trumpet form, cup form or fasciated embryos (Fig. 1F, G and H) was noted whatever the hormonal treatment given at the initiation stage. With respect to the developmental pattern of these somatic embryos, somatic seedlings presented different morphological aspects and were clustered in several categories: A, somatic seedlings with fused cotyledons and non elongated roots; B, somatic seedlings with elongated, narrow, fused cotyledons and tall roots, C, somatic seedlings with a twisted and wasted cotyledon and a tall root; D, somatic seedlings with branched cotyledons and a tall root; E: other forms (Fig. 2).

Rooted seedlings despite their morphological abnormalities of cotyledons were able to continue their growth and development. This situation as been well described with rooted embryos of peanut where the recovery of plantlets was optimized by special culture conditions (Chengalrayan *et al*., 1997). An unsuitable hormonal treatment may have triggered malformation of *Ferula gummosa* embryos and seedlings. To obtain an efficient system of regeneration with vigorous embryos probably it requires special hormonal treatment we have to definite in the future.

Cytological approach: But embryo abnormalities may relieve also from genetic changes in cells because plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin and Scowcroft, 1981). Cytological abnormalities are one of the manifestations of somaclonal variation. The primary cytological changes observed among regenerated plants include chromosome rearrangements and changes in chromosome number (Kaeppler *et al*., 2000). In this investigation karyological analysis was done on somatic seedlings of categories A and B as described before, proportionally more abundant in the culture. A previous investigation reported chromosome number of 2n = 22 for *Ferula gummosa* (Retina and Pimenov, 1977). We found the same chromosome number but detected a high incidence of cytochimerism in somatic seedlings (Fig. 3). Chromosome stickiness, polyploidy and aneuploidy were recorded in the root cells (Fig. 3 C-E) and in a different and very significant ($p<0.005$) proportion between the two groups (Fig. 4). In abnormal seedling of group A, different polyploidy levels i.e., 2n = 35,36 … were found in the same root tip. In group B a high proportion of cell (64.1%) showed chromosome reduction with 2n = 14, 16, 18, 19, 20.

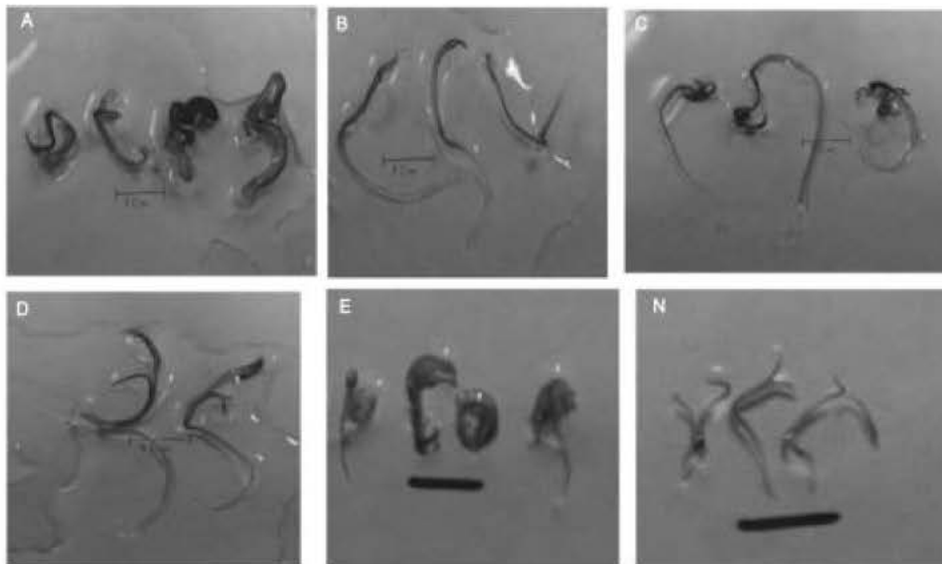

Fig. 2: Abnormal development of somatic seedlings of *Ferula gummosa*, (A) Somatic seedlings with fused cotyledons and non elongated roots, (B) Somatic seedlings with elongated, narrow, fused cotyledons and tall roots, (C) somatic seedlings with a twisted and wasted cotyledon and a tall root and (D) Somatic seedlings with branched cotyledons and a tall root and (E) Other forms; N: Normal seedlings

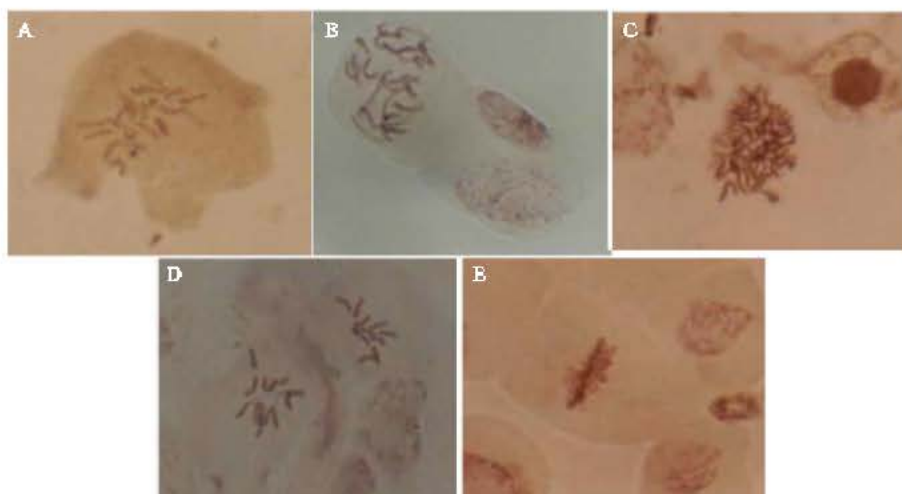

Fig. 3: Instability karyologic in somatic seedlings of *Ferula gummosa,* (A) Zygotic seedling metaphasic cell, 2n = 22, (B) Somatic seedling metaphasic cell, 2n = 22, (C) Somatic seedling polyploidy cell, (D) Somatic seedling aneuploid cell and (E) Chromosomes stickiness in somatic seedling cell

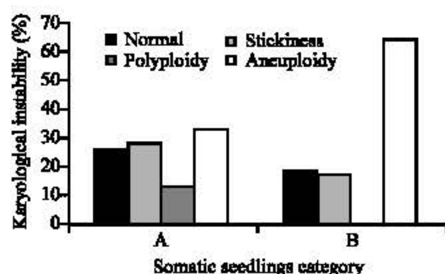

Fig. 4: Karyological instability in somatic seedlings. A, somatic seedlings with fused cotyledons and non elongated roots. B, somatic seedlings with elongated, narrow, fused cotyledons and tall roots

Karyological stability has been obtain in haplopappus culture with colcemid treatment (Fujishige *et al.*, 1996) and we can envisage such a possibility to improve *Ferula gummosa* embryonic system.

CONCLUSIONS

Ferula gummosa somatic embryos can be successfully obtained in culture. However, there is a probability of a high level of abnormalities in somatic embryos and seedlings. Normal and abnormal rooted seedlings developed the first leaf and then arrested their development to enter in dormancy according to zygotic seedlings development pattern. As somatic plantlets seem to be also conditioned by their natural biological rhythm, it was very difficult to search the optimum conditions for acclimation and transfer to soil. Therefore, we aim our research towards the production of artificial seeds that may permit the somatic embryo maturation and seedling growth in soil directly.

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