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EMBRYOLOGICAL OBSERVATIONS ON UNPOLLINATED OVARY CULTURE OF MULBERRY (*MORUS ALBA* L.)

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A three-step procedure was adopted for the induction of gynogenesis in two cultivars of mulberry (*Morus alba* L.). This includes in vitro flowering, inflorescence segment culture and isolated ovary culture. In the third step, that is, isolated ovary culture, the cultured ovaries burst and an embryo emerged from the ovary. The present paper investigates the ontogeny of the developing gynogenic embryo. The study confirmed that the gynogenic embryo emerged from the egg cell. Before the onset of division of the egg, all other cells of the embryo sac degenerated in the majority of ovules, but in exceptional cases the polar nuclei will be retained along with the dividing egg cell. The presence of the gynogenic embryo along with free-nuclear autonomous endosperm is the most important feature of the present investigation. Autonomous endosperm is formed from either the polar nuclei or secondary nucleus. In both cultivars used for the experiment, the percentage of ovaries showing proembryo induction during inflorescence segment culture is much higher than that of ovaries producing gynogenic plants during isolated ovary culture. This suggests the degeneration of some gynogenic embryos during the initial stages of induction.

Key words: *Morus alba*, mulberry, autonomous endosperm, embryo sac, gynogenesis, unpollinated ovary culture.

INTRODUCTION

Mulberry (*Morus alba* L.) is an important plant in sericulture, since its leaves are the sole source of feed for silkworm larvae (*Bombyx mori*). Qualitative and quantitative improvement of mulberry varieties plays a vital role in the progress of the sericulture industry. Being a highly heterozygous perennial plant, mulberry is not readily amenable to conventional methods of plant improvement. Production of homozygous lines of mulberry by inbreeding is not possible because of its dioecious nature and because the male and female lines are genetically diverse. Hence, production of haploid plants is the fastest and the only easy method for producing homozygous lines in mulberry.

Despite progress in haploid production from cultured anther, this approach is faced with many problems impeding its widespread application. In mulberry there are some attempts to produce haploid plants through anther culture (Shoukang et al., 1987; Sethi et al., 1992; Katagiri and Modala, 1993; Tewari et al., 1994; Jain et al., 1996). Except for Shoukang et al. (1987), however, the reports are of unsatisfactory results, with very poor response. Hence an alternative method of haploid production through gynogenesis can be employed. Many of the problems associated with androgenesis could be overcome by culturing unfertilized ovule/ovary/flower buds and inducing a haploid cell of the female gamete (egg) to develop into a haploid sporophyte.

In vitro culture of unpollinated ovaries and ovules has been successfully applied to many plant species that are not amenable to androgenesis. In such plants there is no other alternative method of haploid production. Gynogenic plants of mulberry

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TABLE 1. Gynogenic embryo induction and plant production in ovary cultures of mulberry cvs. K-2 and S-1. The cultures were raised in June-August and September-November seasons. Basal medium: MS

Treatment (μM)		No of ovaries analyzed histologically		Ovaries showing embryo induction in inflorescence segment culture (% ± SE)		Ovaries forming gynogenic plants in isolated ovary culture (% ± SE)	
Inflorescence segment culture	*Individual ovary culture	K-2	S-1	K-2	S-1	K-2	S-1
June-August							
BAP (4.5) + 2,4-D (4.5)	2,4-D (4.5) + glutamine	65	34	13 ± 1.3	14 ± 1.0	0.8 ± 0.02	0
Kn (9)	2,4-D (4.5) + IAA (5.5) + glycine	52	47	13 ± 1.6	9 ± 1.3	0.4 ± 0.05	0
BAP (2.5)	BAP (10)	48	55	10 ± 2.0	7 ± 1.4	0	0.5 ± 0.04
2,4-D (4.5)	2,4-D (4.5) + proline	73	45	8 ± 1.8	11 ± 1.6	0	0.4 ± 0.03
2,4-D (4.5)	2,4-D (4.5) + glycine +proline	68	44	10 ± 1.1	7 ± 1.1	0	0.6 ± 0.01
September-November							
BAP (10)	IAA (5.5) + Kn (2.3)	77	67	23 ± 2.1	16 ± 2.0	3.1 ± 0.3	0
BAP (8.5) + 2,4-D (4.5)	2,4-D (4.5) + glycine + proline	85	68	39 ± 2.3	15 ± 2.1	16.0 ± 3.6	0
BAP (8.5) + 2,4-D (4.5)	2,4-D (4.5) + IAA (5.5)	96	47	21 ± 2.2	23 ± 2.2	4.2 ± 2.8	0
BAP (4.5) + 2,4-D (4.5)	2,4-D (4.5) + glycine	84	57	15 ± 1.8	28 ± 2.3	0	1.4 ± 0.2
Kinetin (2)	BAP (4.4) + 2,4-D (4.5)	98	74	11 ± 1.1	19 ± 2.2	0	1.04 ± 0.2

*glutamine, proline and glycine were added at 500 mg/l, 200 mg/l and 500 mg/l, respectively.

were reported by Lakshmi Sita and Ravindran (1991) and Thomas et al. (1999). Lakshmi Sita and Ravindran (1991) cultured 'individual ovaries before or after fusion to form sorosis under field conditions' without taking any measure to prevent chance pollination. In our earlier paper (Thomas et al., 1999) we used inflorescences developed in vitro for gynogenesis, which were protected from chance pollination. An additional advantage of using inflorescences formed in vitro is that they do not require surface sterilization. The origin of the mulberry gynogenic embryo has not yet been investigated.

The present study aims to reveal the ontogeny of developing gynogenic plants from cultured unpollinated ovaries in mulberry (*M. alba* L.).

MATERIALS AND METHODS

INITIATION OF ASEPTIC CULTURES

Single node cuttings were collected from female mulberry (*M. alba* L.) plants (cvs. K-2 and S-1) in two seasons: September-November and June-August. After removal of the leaves, the cuttings were thoroughly rinsed in 1% solution of Savlon (Johnson and Johnson U.S.A.; contains cetrimide, a detergent and antiseptic) followed by a rinse in 70% ethanol for 10–15 s. The cuttings were then surface-sterilized in 0.1% mercuric chloride solution for 7 min. After washing three times in sterile distilled-water, the stem pieces were allowed to surface-dry in sterilized Petri dishes lined with blotting paper inside a laminar airflow cabinet. Such surface-sterilized single node cuttings were slightly trimmed at the cut ends to expose fresh tissue and planted on hormone-free MS (Murashige and Skoog, 1962) medium or supplemented with various growth regulators.

The media were solidified with 8 g/l agar and the pH was adjusted to 5.8 before autoclaving at 1.5 kg cm^{-2} and 121°C for 15 min. All cultures were maintained at 25 ± 2°C under a 16 h photoperiod supplied by two Philips TL 40 W fluorescent tubes. At least 24 cultures were raised for each treatment and all experiments were repeated three times.

OVARY CULTURE

Aseptic inflorescences from 4-week-old cultures of single node cuttings were excised, cut into pieces each bearing 4 or 5 florets, and planted on MS medium variously supplemented with BAP (6-benzyl amino purine), Kn (kinetin), 2,4-D (2 ,4-dichlorophenoxyacetic acid) and IAA (Indole-3-acetic acid), individually or in combinations (Tab. 1). Various amino acids such as glutamine (500 mg l^{-1}), glycine (500 mg l^{-1}) and proline (200 mg l^{-1}) were also added to the isolated ovary culture medium. After 3 weeks, individual ovaries were excised from the

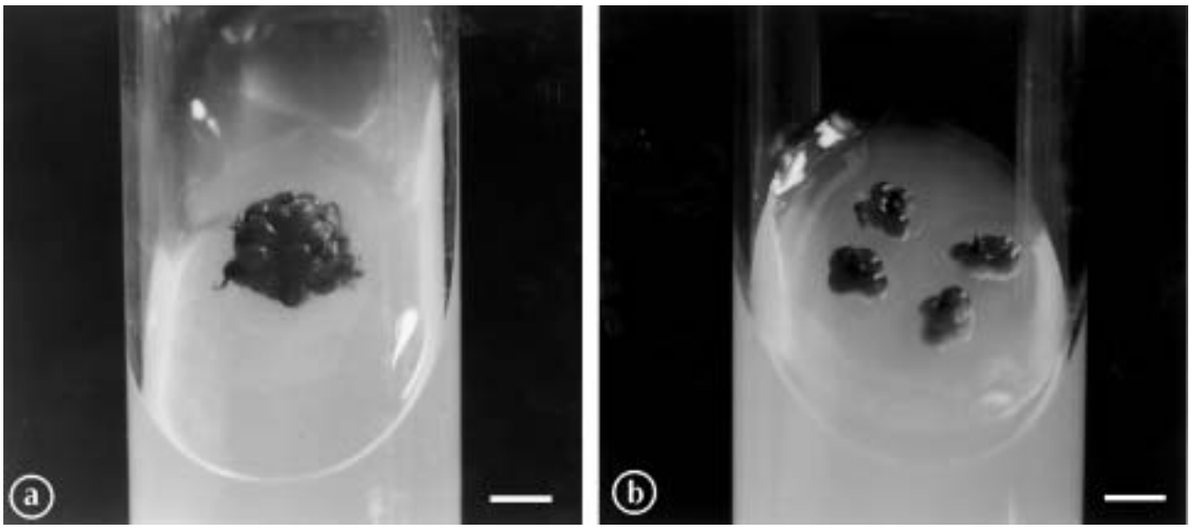

Fig. 1. Effect of in vitro culture. (**a**) Three-week inflorescence segment culture on MS + 4.5 µM BAP + 4.5 µM 2,4-D; ovaries have enlarged, (**b**) Excised ovary culture on MS + 4.5 µM 2,4-D + 500 mg l^{-1} glycine + 200 mg l^{-1} proline. Here the enlarged ovaries from inflorescence segment culture were excised and individual ovaries cultured. Bar = 10 mm.

inflorescence segments and transferred to fresh medium supplemented with growth regulators (Tab. 1).

EMBRYOLOGICAL STUDY

For investigating the ontogeny of the gynogenic embryos, the ovaries were fixed for 24 h in FAA (Formalin:glacial acetic acid:70% ethanol in a 1:1:18 ratio by volume). Following dehydration in tertiary butanol, the material was infiltrated and embedded in paraffin wax (melting point 60˚C) (Johansen, 1940). Sections 8 µm thick were cut on a rotary microtome and stained with safranin-Astrablau (Thomas, 1999).

RESULTS

IN VITRO FLOWERING AND INFLORESCENCE SEGMENT CULTURE

Two cultivars of mulberry (*M. alba* L.), K-2 and S-1, were used to produce gynogenic plants by unpollinated ovary culture. Irrespective of the cultivar and the medium, almost all the cultures showed shoot development and produced flowers 4 weeks after single node cuttings. The inflorescences developed in the axil of the leaves and were visible as soon as bud-break occurred. The inflorescences were generally restricted to the lower 3–4 nodes of the shoots. Supplementation with 5 µM BAP was optimum for inflorescence development, but the number of flowers per inflorescence was at maximum with 7 µM BAP (data not shown).

In ab initio isolated ovary culture (i.e., individual ovaries cultured from the beginning), growth of ovaries was slow and poor. In none of the several hormonal media used for culture did a gynogenic plant develop. Therefore, flowers attached to the rachis were cultured on MS medium supplemented with BAP, Kn, 2,4-D and IAA, individually or in different combinations (Fig. 1a; Tab. 1). After three weeks, individual ovaries were excised from the inflorescence segments and transferred to MS medium with a different hormonal combination (Fig. 1b; Tab. 1).

Histological analyses of the ovaries at different stages of ovary culture were made. The ovules at the time of inflorescence segment culture showed a mature embryo sac. Longitudinal sections through ovules at this stage showed most of the embryo sac cells, and egg cells were especially clear (Fig. 2a). Most of the ovaries showed degenerating synergids and antipodal cells.

All other cells of the embryo sac except the egg cell degenerated just before egg division. Hence it is confirmed that the gynogenic embryo originates by division of the egg cell. The first division of the egg cell was periclinal (Fig. 2b), and repeated division of the egg cell formed a proembryo. Usually the first division of the egg took place 5 or 6 days after initiation of inflorescence segment culture. The proembryo underwent anticlinal and periclinal divi-

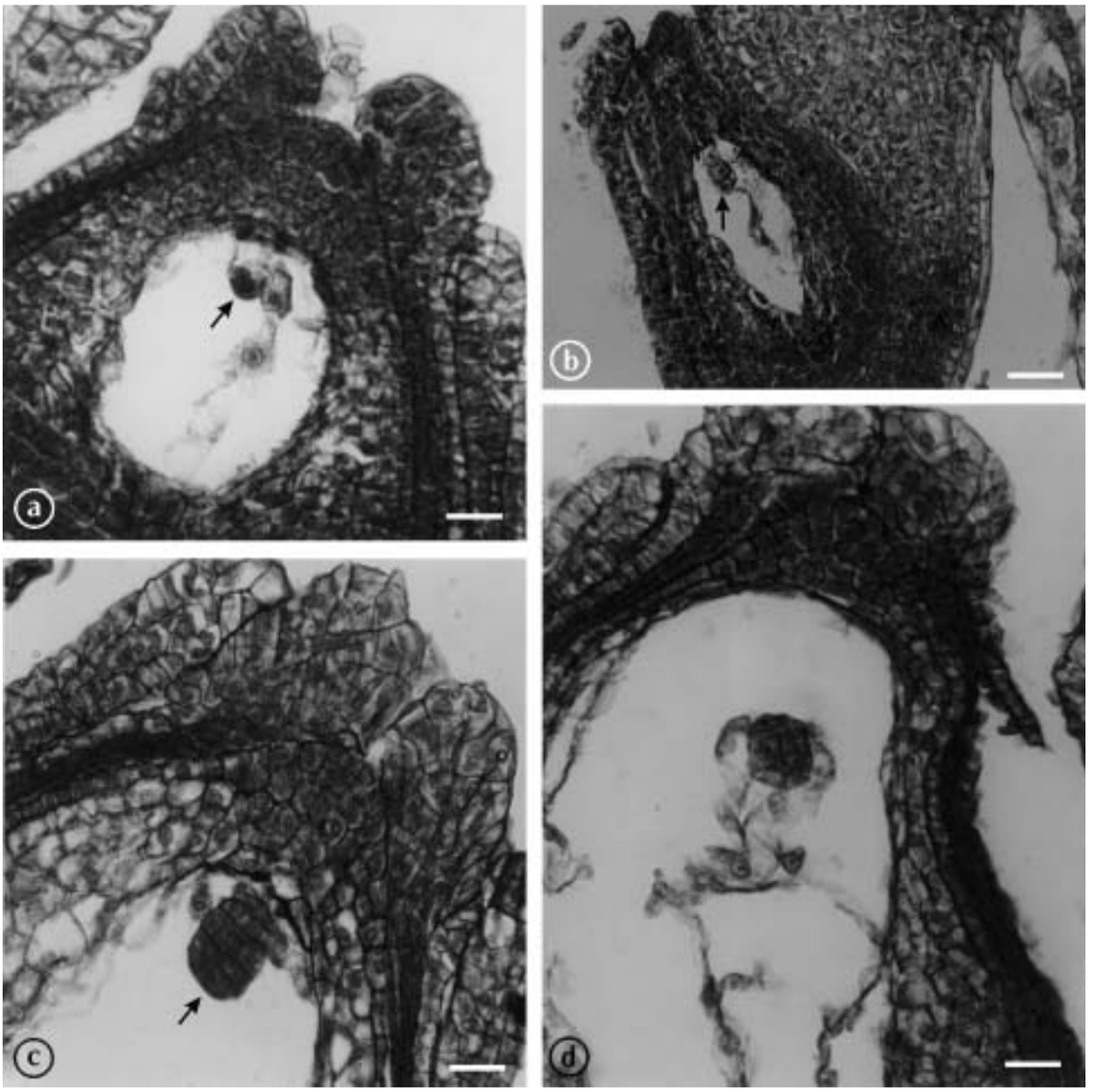

Fig. 2. Longitudinal section of ovules cultured in vitro. (**a**) Inflorescence segment culture. Note the prominent egg cell (arrow) with one synergid and polar nucleus. All other cells of the embryo sac degenerated, (**b**) Parthenogenetic 3-celled proembryo (arrow). Note the first periclinal division of the egg, resulting in an upper cell and a lower cell. The lower cell has divided into daughter cells, (**c**) An ovule at the time of excised ovary culture. A 26–32-celled globular proembryo (arrow) has developed at the micropylar end, (**d**) An ovule containing the gynogenic proembryo and autonomous endosperm formed by division of the polar nucleus or secondary nucleus. Sections were taken 3 days after isolated ovary culture. Bars = 30 µm in (a), 80 µm in (b) and (c), 60 µm in (d).

sions to form a multicellular globular embryo after three weeks of inflorescence segment culture.

The percentages of ovaries showing proembryo induction during inflorescence segment culture and the percentages of ovaries forming gynogenic plants during isolated ovary culture are given in Table 1. The percentage of ovaries showing proembryo induction during inflorescence segment culture was much higher than that of ovaries yielding gynogenic plants during isolated ovary culture in both cultivars, K-2 and S-1, possibly indicating degeneration of some gynogenic embryos during the initial stages of induction.

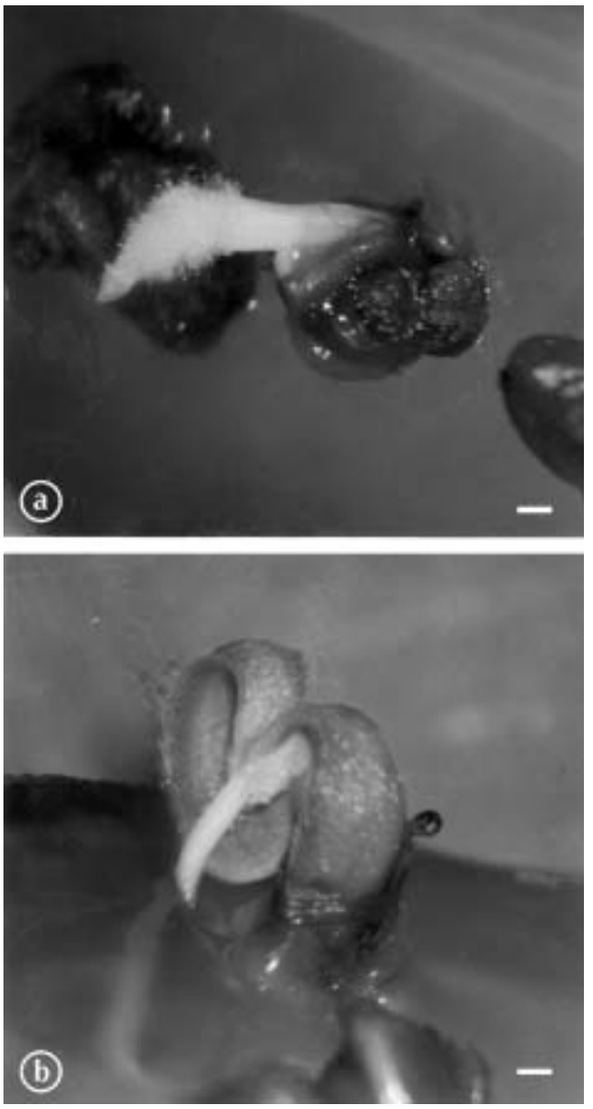

Fig. 3. (**a**) Ovary three weeks after isolated ovary culture. Note the emergence of hypocotyl and radicle regions from the ovary, (**b**) Gynogenic embryo 4 weeks after isolated ovary culture. The cotyledons and radicle are very clear. Also note the shrivelled ovary wall attached to the gynogenic plant. Bars = 10 mm.

In all combinations tested, the ovaries enlarged and turned red after 3 weeks of culture. Serial section of ovaries at this stage showed a 12–16-celled globular proembryo. On MS basal medium the growth of ovaries was very poor (data not shown). Addition of BAP, Kn, 2,4-D and IAA alone or in combination substantially improved ovary growth. However, the maximum number of ovaries attained size ≥ 6 mm × 4 mm on MS medium supplemented with 4.5 μM each of BAP and 2,4-D. In terms of ovary growth, cv. K-2 was more responsive than S-1 (data not shown). In responding ovaries the gynogenic embryo developed at the micropylar region and remained there until the emergence of gynogenic plants.

ISOLATED OVARY CULTURE AND PLANT REGENERATION

In inflorescence segment culture the ovaries showed good growth but did not produce gynogenic plants. However, histological studies revealed that gynogenesis was induced in inflorescence segment culture but the embryos failed to develop beyond the globular stage (Fig. 2c). In prolonged cultures of inflorescence segments the embryos had degenerated. It was therefore decided to detach the ovaries from the rachis after 3 weeks of culture of inflorescence segments and to culture the isolated ovaries in order to promote embryo development (Fig. 1b).

On some media the unfertilized ovaries developed gynogenic plants after 4 weeks of individual ovary culture (Tab. 1). The percentage of cv. K-2 ovaries forming gynogenic plants reached maximum (16%) when ovaries from 3-week-old inflorescence segments cultured on MS + BAP (8.5 μM) + 2,4-D (4.5 μM) were transferred to MS + 2,4-D (4.5 μM) + glycine (500 mg/l) + proline (200 mg/l) (Tab. 1).

In mulberry, about 4% of the responding ovaries retained the polar nuclei or secondary nucleus and divided along with the egg cell to form free-nuclear endosperm. The number of endosperm nuclei ranged from 10 to 40; they occupied the embryo sac along with the gynogenic embryo (Fig. 2d). There was no cellular endosperm development. Invariably only one embryo was formed in an ovary, which germinated in situ. The radicle region of the embryo was oriented towards the micropyle and the cotyledons towards the chalazal end. A globular 26–32-celled gynogenic embryo was formed one week after isolated ovary culture. The radicle emerged from the responding ovaries first, and was followed by the cotyledons (Fig. 3a,b) at 4 weeks. The dried ovary wall was found attached to the developing gynogenic embryo (Fig. 3b). The emerged gynogenic embryos were healthy, with green cotyledons, and no callus phase of growth was found.

The ovaries were cultured in two seasons: June-August and September-November. The cultures initiated during the June-August season responded very poorly (Tab. 1). Of the two cultivars used for gynogenic experiments, K-2 showed better response

than S-1 in terms of the percentage of ovaries showing proembryos and gynogenic plant regeneration.

DISCUSSION

Ovary culture technique is easier than ovule culture technique, because ovaries can be dissected more efficiently than ovules can. Moreover, the chance of injuring the ovule during dissection is greater; that danger can be avoided in ovary culture.

The stage at which the explants are cultured is critical for the gynogenic response. Generally, explants cultured at the mature or nearly mature embryo sac stage have given the best results (Beaville, 1980; Davojan, 1985; Yang et al., 1986; Yang and Zhou, 1990; Mukhambetzhanov, 1992; Castillo and Cistue, 1993). In mulberry, ovules had a mature embryo sac (8-nucleate) at the time of inflorescence segment culture.

In mulberry, gynogenic plants emerged from the egg cell. Origination of gynogenic plants from the egg cell (parthenogenesis) has been reported in *Beta vulgaris* (Ferrant and Bouharmont, 1994), *Hordeum vulgare* (Huang et al., 1982; San and Gelebart, 1986) and *Nicotiana tabacum* (Pavlova and Hvedynich, 1984). The mode of development of gynogenesis in mulberry is via direct embryogenesis. A similar mode of regeneration is reported in *Allium cepa* (Keller and Korzun, 1996) and *Melandrium album* (Mól, 1992). In contrast to these observations there are reports of gynogenic callus and embryo development from synergid and antipodal cells. In rice, synergid apogamy has been the main source of gynogenic plants (Zhou et al., 1986; He and Yang, 1988). Occasionally, antipodal cells may divide and form a multicellular mass, as observed in barley (San, 1976; Huang et al., 1982), *Crepis tectorum* (Pavlova, 1983) and sunflower (Yang et al., 1986), but their further development into plants has not been established so far.

Gynogenic embryos were seen along with endosperm nuclei formed by the division of the polar nuclei or secondary nucleus in some ovules, but it is not clear whether the endosperm nourishes the gynogenic embryo in any way. Autonomous endosperm formation is a common phenomenon during ovary/ovule culture experiments in a number of systems such as *Beta vulgaris* (Ferrant and Bouharmont, 1994), *Brassica napus* (Rojek et al., 2002), *Melandrium album* (Mól, 1992), *Oryza sativa* (Zhou and Yang, 1981) and *Viola odorata* (Wijowska, 1999 a,b). Musial et al. (2001) divided cultured ovaries of onion into three categories: ovule containing endosperm only, ovule containing the egg apparatus, and ovule with both endosperm and embryo.

The ovaries were cultured in two seasons, June-August and September-November. The September-November season gave the optimum response. Such a seasonal effect on gynogenesis has been observed in other plants. Lux et al. (1990) reported that the gynogenic response of greenhouse-grown plants of *Beta vulgaris* varied significantly with the time of the year. Maximum response was observed in summer (May-September). Doctrinal et al. (1989) further demonstrated that June was the most favorable of the summer months for gynogenesis in cultivars of *B. vulgaris*. The best results with *Cucumis melo* were obtained in summer, with an average of 3 embryos per 100 ovules (Sauton, 1988). In *Gerbera* the frequency of gynogenic callus induction was higher in the autumn than in spring, but the morphogenic capacity of the callus showed an inverse relation with the season (Cappadocia et al., 1988).

The gynogenic response differed markedly between the two studied cultivars of mulberry: cv. K-2 was most gynogenic, with a maximum response of 16%, whereas cv. S-1 exhibited very poor response. This genotype-dependent response is a serious limitation in the application of this technique, which may be compounded if the process of gynogenesis involves gametic selection. Genotypic differences in gynogenic response have been reported in *Hordeum vulgare* (San, 1979), *Allium cepa* (Campion and Azzimonti, 1988; Muren, 1989; Keller, 1990a; Keller, 1990b), *Helianthus annuus* (Gelebart and San , 1987; Badea et al., 1989) and *Beta vulgaris* (Van Geyt et al., 1987; Lux et al., 1990).

The present study confirms the origin of the gynogenic embryo from the egg cell. The percentage of ovaries showing proembryo induction was much higher than that of ovaries yielding gynogenic plants, suggesting the degeneration of some gynogenic embryos during the initial stages of induction. The presence of a gynogenic embryo along with autonomous endosperm is a rare event; these phenomena are independent of each other.

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