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Stimulation of Embryogenesis and Haploid Production in *Brassica campestris* Anther Cultures by Elevated Temperature Treatments

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Summary. Culture of *Brassica campestris* anthers at 35°C for one or three days prior to culture at 25°C significantly stimulated the yield of microspore-derived embryos. More than 100 plants were regenerated from cultured embryos and haploids were identified amongst them. The haploid frequency was greater than 70% if all small-flowered sterile plants were considered to be haploid. The yield of microspore-derived plants in *B. campestris* is approaching the level where anther culture may be utilized as a practical breeding tool.

Key words: *Brassica campestris* – Embryogenesis – Haploid – Microspore – Temperature

Introduction

Previous reports on the occurrence of haploids in *B. campestris* have been limited. A single spontaneous haploid of *B. campestris* (Toria) was identified and described by Ramanujam (1941). Prakash (1974) reported the production of haploids in *B. tournefortii*, a 20-chromosome species closely related to *B. campestris*, following hybridization with *B. nigra*. Workers in the People's Republic of China have reported the regeneration of a haploid plant from callus in *B. campestris* anther cultures (Anon. 1975). Embryogenesis has been induced in cultured anthers of *B. campestris* cvs. 'Torch' and 'Yellow Sarson' and although haploids were not identified in the regenerated plants, it was demonstrated that they originated from microspores which may have undergone spontaneous doubling and/or polyploidization during anther culture (Keller et al. 1975).

In the closely related amphidiploid, *B. napus*, the occurrence of haploids has been more commonly reported. Stringam and Downey (1973) reported the spontaneous occurrence of haploids in several *B. napus* cultivars and suggested that there was a genetic basis to the haploid induction phenomenon. *B. napus* haploids have also been obtained through induction of embryogenesis in cultured anthers (Thomas and Wenzel 1975; Wenzel et al. 1977; Keller and Armstrong 1978). Keller and Armstrong (1978) reported that the frequency of embryogenesis was dramatically increased by initially culturing *B. napus* anthers at elevated temperatures followed by culture at 25°C and they also identified haploids which were present at a frequency of 22% among the anther-derived plants. The present study was initiated to determine if elevated culture temperature treatments stimulated embryogenesis in *B. campestris* and if haploids would subsequently be present among plants regenerated from anther-derived embryos.

Materials and Methods

The material utilized in this study was the *B. campestris* F_1 hybrid produced by crossing the summer turnip rape cultivars 'Torch' and 'R500'. The procedures for growth and maintenance of donor plants, bud selection, surface sterilization, anther plating and culture medium preparation have been previously described (Keller et al. 1975). The basic anther culture medium was modified by the addition of 100 mg/l L-serine which was shown to be beneficial for the culture of isolated tobacco microspores (Nitsch 1974). Culture temperature treatments included continuous culture at 25°C, culture at 30°C for 3, 7 or 14 days prior to transfer to 25°C, and culture at 35°C for 1, 3, 5 or 7 days prior to transfer to 25°C.

The cultures were maintained in darkness and were examined at 7-10 day intervals from the second to the eighth week of culture. Counts were made of the number of anthers producing embryos (embryogenic anthers) and of the number of embryos produced by each embryogenic anther. An efficiency value expressed as the expected yield of embryos from 1000 cultured anthers was calculated for each treatment.

The embryos were removed from the embryogenic anthers within a week of their detection and cultured at 25°C in continuous fluorescent light on hormone-free B_5 medium (Gamborg et al. 1968) containing 2% sucrose. Plantlets developing directly

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Table 1. Effect of culture temperature on embryogenesis in anthers of *Brassica campestris*

Culture temperature (°C)	Duration of treatment (days)	No. of cultured anthers	No. of embryogenic anthers	Frequency of embryogenic anthers (%)	No. of embryos	No. of embryos per embryogenic anther	Efficiency (expected no of embryos from 1000 anthers)
25	continuous	1326	6	0.5	12	2	9
30	3	432	3	0.7	6	2	14
30	7	726	2	0.3	2	1	3
30	14	1548	7	0.5	9	1.3	6
35	1	2460	215	8.7	376	1.7	153
35	3	2442	176	7.2	379	2.2	155
35	5	960	33	3.4	52	1.6	54
35	7	1236	24	1.9	32	1.3	26

from embryos on the embryo culture medium were transferred to Jiffy-7 peat pellets and maintained in mist chambers for 2-3 weeks prior to potting and transfer to greenhouse conditions. In cases where embryos developed abnormally, hypocotyl explants were cultured to induce shoot organogenesis followed by root induction and transfer to Jiffy-7 peat pellets as previously described (Keller and Armstrong 1977).

Anther-derived plants were maintained and grown to maturity under greenhouse conditions. Cytological analyses of microspore mother cells of anther-derived regenerates were carried out as described elsewhere (Keller and Armstrong 1977).

Results

Culture temperature treatments significantly influenced embryogenesis in *B. campestris* anthers (Table 1). Culture at 25°C resulted in embryogenesis in less than 1% of the anthers with an expected embryo yield of less than 10 from 1,000 anthers. Treatment at 30°C for 3, 7 or 14 days did not significantly stimulate embryogenesis and in the case of the 7 and 14 day treatments, the embryo yield had actually decreased. However, culture temperature treatments of 35°C stimulated the frequency of embryogenesis with up to 9% of the anthers producing embryos. Occasionally 30 or more embryos were produced by an embryogenic anther (Fig. 1), although in the majority of cases, 1-5 embryos were produced. The highest embryo yields were obtained when anthers were cultured at 35°C for 1 or 3 days prior to transfer to 25°C.

Embryo production was usually completed by the fourth week of culture. A range of embryo developmental stages varying from small globular types (< 1 mm) to large structures (up to 10 mm) with well developed cotyledons was detected. The embryos do not develop further if maintained on the anther culture medium and eventually blacken and die. Transfer of embryos to the embryo culture medium resulted in survival frequencies of 40-50%. Approximately 20-30% of the surviving embryos developed directly into plantlets. The remainder did not develop shoot apical meristems and usually underwent abnormal hypocotyl elongation or cotyledon enlargement. Plantlets could be regenerated from hypotcotyl explants in approximately 30% of these abnormal embryos. One hundred and thirty plants were regenerated directly from embryos or indirectly from hypocotyl explants.

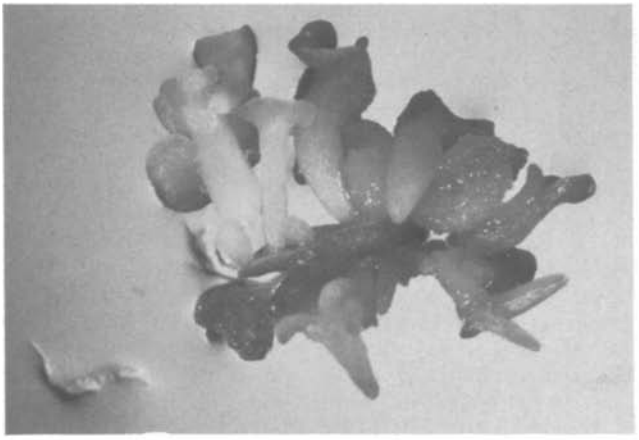

Fig. 1. Production of a large number of embryos from a *B. campestris* anther cultured at 35°C for 3 days prior to transfer to 25°C

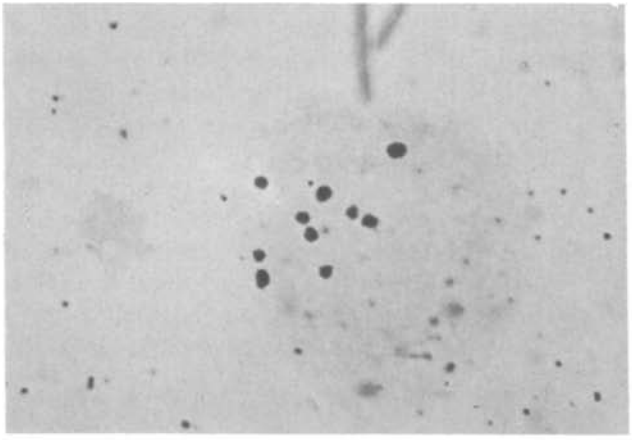

Fig. 2. Metaphase I of haploid *B. campestris* showing 10 univalents

Observations on anther-derived flowering plants revealed that a large number had small, sterile flowers. Cytological evaluation of 29 small-flowered, sterile plants revealed that 27 were haploid with a chromosome number 2n = x = 10 (Fig. 2). It was observed that diploid microspore mother cells were present at frequencies approaching 50% in some anthers of five of these plants. All cytologically identified haploids were derived from anthers which had received a 35°C treatment. In a population of 85 anther-derived regenerates, 63 had small sterile flowers and failed to set seed upon bud pollination. If all small flowered, self-sterile plants are considered to be haploids, then the haploid frequency in the anther-derived population would be 74%. The remaining 26% were fertile and set seed and were considered to be diploid although some tetraploids may have been present.

Discussion

As in *B. napus* (Keller and Armstrong 1978) culture temperature influenced the frequency of embryogenesis in *B. campestris* anthers although the optimal temperature treatment differed for the two species. In *B. campestris* maximum embryo yields were obtained when anthers were cultured at 35°C for 1 or 3 days. Similar embryo yields were obtained from *B. napus* anthers receiving this treatment (Keller and Armstrong 1978) but these yields were only 25% of the maximum yield which could be obtained when the anthers were cultured at 30°C for 14 days. In *B. campestris* embryogenesis was not stimulated by this latter treatment.

The haploid frequency in anther-derived *B. campestris* plants was estimated to be 74% which is higher than the 22% haploid frequency in anther-derived *B. napus* regenerates (Keller and Armstrong 1978). The remaining plants which were fertile and considered to be diploid presumably originated as a result of fusion and/or endoreduplication in the microspores during anther culture (Keller et al. 1975). It is possible that the haploid frequency could be slightly over estimated since one of the parent cultivars ('Torch') is self-incompatible and consequently some self-sterile diploid microspore-derived regenerates may inadvertently have been considered to be haploids. This likely was the case for the two diploids found among the 29 self-sterile plants that were cytologically analyzed.

This study indicates that culture temperature plays a role in determining haploid frequency in *Brassica*. Haploids were not detected among 54 *B. campestris* plants derived from anthers cultured at 25°C (Keller et al. 1975). However, in the present study, high frequencies of haploids were regenerated from anthers receiving elevated culture temperature treatments. Similar observations were made in studies with *B. napus* where haploids were detected when the anthers had received elevated temperature treatments (Keller and Armstrong 1978) but were not present when anthers were cultured at 25°C (Keller and Armstrong 1977).

The yield of microspore-derived *B. campestris* plants obtained under optimal culture conditions is approaching the level where anther culture can be evaluated as a practical plant breeding tool. Furthermore, the production of haploids from this diploid species provides a good source of monoploid material for the isolation of cells or protoplasts or the initiation of cell cultures for use in mutagenesis studies.

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