Acta Universitatis Latviensis, Biology, 2004, Vol. 676, pp. 153–158

Embryogenesis, callogenesis and plant regeneration from anther cultures of spring rape (*Brassica napus* L.)

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Abstract

Anther culture is a very important and useful tool in plant breeding for haploid production. The investigation was carried out with three spring rape cultivars, 'Trend', 'Landmark' and 'Auksiai'. Rape anthers were cultivated in modified Nitch and Nitch induction medium supplemented with 13 % sucrose and 0.4 % agarose. Embryo regeneration took place in a modified B_5 Gamborg nutrient medium supplemented with 0.1 mg l^{-1} GA_3 and 0.8 % agar. Temperature pretreatment of flower buds was at 35 °C for 72 h. After 72 h thermal shock pretreatment, the callus formation frequency differed for the tested genotypes. Thermal shock pretreatment on the callogenesis process appeared to be genotype dependent, since this treatment failed to significantly increase for 'Auksiai' and significantly decrease callus formation for the 'Landmark' and 'Trend' cultivars. Further embryo development was noted only in genotype 'Trend'. The frequency of embryo formation from anthers treated with high temperature was higher than the control. It was observed that in haploids the stomata length was reduced by 1.4 and their number in the vision field increased by approximately 1.1 in comparison with the diploid parent plants. The height of the haploid plants was 1.4 times less than parent plants. Plant regenerants were 100 % haploids.

Key words: anther culture; *Brassica napus* L; callogenesis; embryogenesis; nutrient media; regeneration; temperature pretreatment.

Introduction

The oilseed rapes (*Brassica napus*, *B. rapa* and *B. juncea*) are now the third most important source of edible vegetable oil in the world. Over the last decade, researchers have made great efforts into developing biotechnological methods to facilitate rape breeding (Kott 1998). Anther culture is an important technique for immediate fixation of homozygosity and shortening the breeding cycle in varietal improvement. The selection efficiency with the doubled haploid lines is higher, especially when dominance variation is significant. Through anther culture, considerable progress and success have been achieved for a large number of economically important crop species, such as barley, wheat, rapeseed, rice, and maize (Lelu et al. 1990). Application of heat stress pretreatment has been an essential factor to increase the efficiency of androgenesis in different species (Kumar et al. 2003). Heat pretreatment has been used to induce embryogenesis from isolated anthers, as it disrupts the cytoskeleton in microspores in the initial phase (Ferrie et al. 1995). Optimal temperature and duration of the pretreatment varies from species to species (Ferrie et al.

1995).

High temperature pretreatment disrupts the normal integrated development of somatic anther tissue and subsequently may synchronise the physiological states of the two tissues, thereby stimulating the induction process (Dunwell et al. 1983).

There are many factors that influence the response frequencies from *in vitro* androgenesis, and these factors may also interact. Some of major factors are genotype, donor plant growth conditions, anther pretreatment, cultivation media composition and environment conditions.

The aim of this research was to investigate the morphogenesis *in vitro* in anther culture of spring rape.

Materials and methods

The investigation was carried out with three spring rape cultivars, 'Trend', 'Landmark' and 'Auksiai'. The donor plants were grown in the growth room with a light intensity of 5000 lx and a 16-h photoperiod. Buds (3 - 4 mm in length) were collected when the microspores were at the mid uninucleate stage. Temperature pretreatment of flower buds was performed at 35 °C for 72 h. Buds were surface sterilized in 70 % ethanol for 2 min and rinsed three times with sterile distilled water. Rape anthers were cultivated in modified Nitsch and Nitsch (Fletcher et al. 1998) induction medium supplemented with 13 % sucrose and solidified 0.4 % agarose. The macro, micro salts and agarose were autoclaved at 115 °C for 30 min whereas the other components of the medium were 0.2 mm filter sterilized. The ready medium was poured into 90 mm diamter sterile plastic Petri dishes (15 - 20 ml medium per dish). Anthers from each bud were removed under a dissecting and inoculated onto the induction medium (20 anthers per Petri dish). The dishes were wrapped with double layers of parafilm and cultivated in the dark. For each treatment, 100 anthers were cultured and the experiment was repeated three times. The maturity morphologic embryos in 28 days after anther isolation were transferred to B_5 Gamborg (Fletcher et al. 1998) regeneration medium supplemented with 0.1 mg l^{-1} GA_3 and 0.8 % agar and cultivated at 25 °C temperature, a 16-h photoperiod, and 5000 lx light intensity. After 30 days, the plantlets with roots, stems and at least three true leaves were transferred to soil. The ploidy level of regenerated plants was determined according to the stomata length at the seventh leaf unfolded growth stage. At flowering, the regenerated plants were evaluated by inspection of the morphological characteristics of flower buds, anthers and pistils, and production of mature pollen grains (Chen et al. 1994).

Data were analyzed using analysis of variance and mean comparisons were made by protected least significant difference at 1 % level of probability.

Results

The whole androgenetic process of anther culture in this study was similar as described previously (Kupriene et al. 2004). Microscopic calli were visible on the surface of the anther following 12 to 14 days culture in induction medium. When thermal shock was not applied the callusgenesis process in induction medium was most intensive for the genotypes 'Trend' and 'Landmark' in comparison with 'Auksiai' (Fig. 1). After 72-h thermal shock pretreatment the callus formation frequency differed for the tested genotypes.

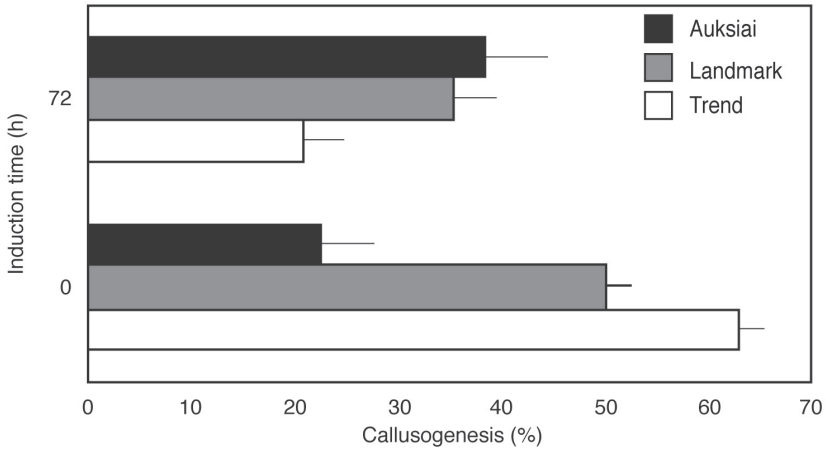

Fig. 1. Callogenesis in spring rape anther culture after 28 days of cultivation

Thermal shock significantly improved the percentage of anthers producing calli for 'Auksiai' from 22.5 % to 38.3 %. However, this same treatment decreased the overall efficiency of callusogenesis for 'Trend' and 'Landmark', in comparison with control, with decreases from 62.9 % to 21.0 % and from 50 % to 35.4 % respectively. After 28 days of cultivation of rape anthers, the callus was yellow white and had watery structure (Fig. 2A). It was transferred to regeneration medium where it did not develop further and did not induce shoot formation.

Direct embryogenesis of anther culture was obtained on induction medium. Embryo development for the genotypes 'Auksiai' and 'Landmark' often stopped after several divisions or during the globular embryo to the heart-shaped embryo stage, followed by embryo death. Further embryo development was noted only in anthers of the genotype Trend when they were cultivated after 72 h of thermal shock. The developmental stages (globular, torpedo and cotyledonary embryos) were observed in embryos developed from generative cells by direct embryogenesis (Fig. 2B, C, D). The frequency of embryo formation from anthers treated with high temperature was higher than for the control (data not show). After 19 days the first morphologically mature embryos were noted. In regeneration medium, embryos first developed a strong primary root with masses of root hairs and in the following week the shoot apex begun to produce young leaves. However not all the embryos were physiologically ready for morphogenesis after 28 days of cultivation. When transferred onto regeneration medium, the morphologically mature embryos formed 62.5 % plants with roots and 37.5 % without roots. Regenerants with roots, stems and at least three true leaves were transferred to soil. Plant survival following transfer to soil was 100 % (Fig. 2F). At the seventh leaf unfolded growth stage, the haploids plants were evaluated for leaf morphometric parameters and stomata length. It was observed that for the haploids, the stomata length was reduced by 1.4 and their number in the vision field increased by approximately by 1.1 in comparison with the diploid parent plants (data not shown). At flowering, the haploids had smaller flowers and did not produce pollens (Fig. 3). The height of the haploids was 1.4 times less than that of parent plants. The plant regenerants were 100 % haploids.

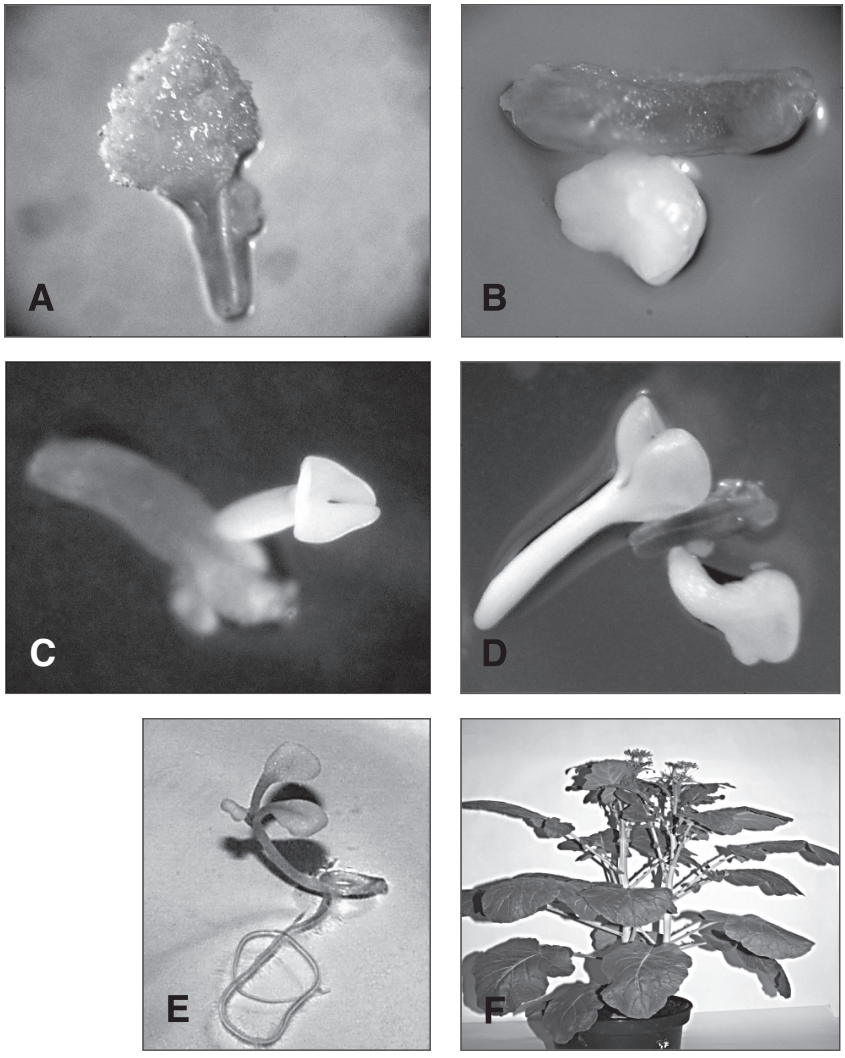

Fig. 2. Embryogenesis, callogenesis and plant regeneration in cultured anthers of rapeseed. A, callus emerging from anthers after 14 days of anther isolation; B, embryos after 15 days of anther isolation; C, direct embryo formation from cultured anthers after 19 days; D, microspore-derived embryo after 28 days of anther isolation; E, regenerated plantlet on B_5 medium; F, haploid plant.

Discussion

High temperature stress is one of the most important but least studied abiotic stresses affecting plant development in tissue culture. The reproductive stage is the most susceptible stage for temperature stress in most crops in which temperature response has been studied (Paulsen 1994; Angadi et al. 2000). Our previous results showed that 35 °C temperature pretreatment for 72 h had a positive effect on morphogenesis process of

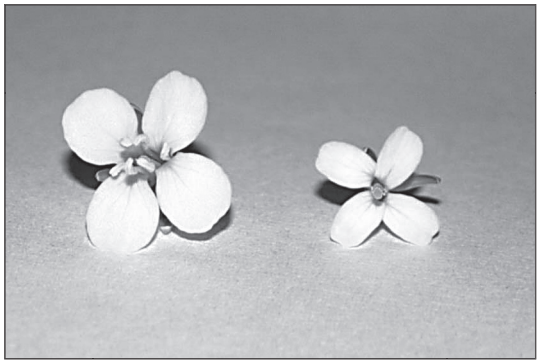

Fig. 3. Flowers of rape regenerants (left diploids, right haploids).

spring rape in isolated anther culture (Kuprienė et al. 2004). However, the effect of anther pretreatment on callogenesis appeared to be genotype dependent since this treatment failed to significantly increase for 'Auksiai' and significantly decreased callus formation for the 'Landmark' and 'Trend' cultivars. We found that the high temperature pretreatment was beneficial for embryogenesis only for the Trend genotype. Morphological variability of embryos among individual cultures is a result of the specific embryogenic competence of the original culture (Burbulis et al. 2000). Although incubation temperature and other cultivation conditions influenced embryo quality, the genetic factor appeared to play an important role in normal development (Chuong et al. 1987; Kott, Beversdorf 1990): the frequencies of normal embryos characterized by a root-shoot axis and cotyledons depended on the genotypes. Frequencies of normal embryos were generally constant in Trend cultures after 35 °C temperature pretreatment (data not shown) while embryos were not found in cultures of 'Landmark' and 'Auksiai'. Genotypic differences in anther culture responses have been previously reported in flax (Nichterlein et al. 1991) and rape (Dunwell et al. 1983).

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