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EMBRYOGENESIS IN MICROSPORE CULTURE OF SUNFLOWER (*Helianthus annuus L.*)

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SUMMARY

In the experiments presented here initial steps for the development of successful microspore culture of sunflower have been carried out. Both division of uninucleate microspores and embryogenesis were achieved - although in low rates - with two of the four genotypes tested. Further studies with other genotypes, at varying flower bud stages, with different media compositions and culture conditions will be necessary in order to develop an efficient microspore culture technique applicable to sunflower breeding.

INTRODUCTION

Haploid plants are considered to be useful in plant breeding programmes as intermediates leading to doublead haploid, i.e., homozygous diploid lines after chromosome doubling. In order to produce haploid individuals on a large scale, efficient techniques are required (e.g., Chuong and Beversdorf, 1985). Androgenesis has been reported to be an applicable procedure in many species now (Dunwell, 1986) and in some species - like rapeseed, potato and barley - androgenetic doubled haploids have already been used in practical breeding. The main aims of anther and microspore culture are to increase the frequency of embryoid induction and to achieve plant regeneration from the embryoids, respectively. In sunflower (*Helianthus annuus*), androgenetic (Alissa et al., 1985; Bohorova et al., 1985; Mix, 1985; Mezzarobba and yonard, 1986) and gynogenetic haploid plants (Gelebart and San, 1987) have already been produced by using anther culture, nonfertilized ovaries and ovule culture, respectively. However, the results of anther culture were rather unsatisfactory for application in sunflower breeding up to now. Like in other species, anther response of sunflower is stroungly affected by physical, nutritional, physiological and genetic factors. By testing a number of different culture procedures, i.e., donor plant stages, culture media and conditions, an appropriate anther culture protocol could be worked out for the successful regeneration of shoots (Gürel et al., 1991). In this case, shoot development was achieved from the sunflower hybrid 'Do 131', two inbred lines ('B11 A3', 'MH 1-2') and an interspecific hybrid (*H. annuus* cv. Baso x *H. mollis*). By further adjustment of donor plant cultivation, anther stage, as well

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as media for initiation and regeneration it should be feasible to further improve the technique for its successful application in practical sunflower breeding.

In the present study, we tried to develop an efficient microspore culture for sunflower as already described for other species, e.g., in the genus *Brassica.*

MATERIALS AND METHODS

Sunflower plants were grown in the greenhouse under semi-controlled conditions, i.e., under 16 h photoperiod, at a light intensity of 22,000 lux, with temperatures of 12-20°C (day) and 8-10°C (night). Three hybrids, 'Do 131', 'Frankasol' and 'Sunbred 262' and an inbred line ('B11 A3') were used in the experiments.

Flower buds were surface sterilized by immersion in 70% ethanol and 2.5% NaOCl for 2 min each. Then the buds were rinsed in sterile distilled water twice. Flower buds mainly contained microspores in the uninucleate stage, but occasionally buds with microspores in earlier and later stages were used, too. For culture induction the modified NLN-medium (Gland et al., 1988) was used, whereas for regeneration, the MS-medium (Murashige and Skoog, 1962) was applied. The components for the NLN-medium were filter sterilized, whereas the regeneration medium was sterilized by autoclaving.

Two methods of isolation and incubation of flower buds - differing in the number of buds - and two pH values of the medium (6.0, 6.2) were used. Each treatment was carried out in 4 replications. In each case the buds were put into a centrifuge tube (10 ml) with 1 ml of NLN medium added. In order to release the microspores, buds were crushed with a glas stick. The suspension obtained was filtered by using a steel filter (44 um). Tube and filter were rinsed with 7 ml NLN-medium and this suspension was then poured into a new centrifuge tube (10 ml). After washing once by centrifugation (350 rpm, 55 g, 10 min) the microspore pellet of 30 buds was dissolved in 3 ml of fresh NLN-medium (method 1). The suspension was subdivided into two petri dishes (35 mm diameter, each containing 1.5 ml of it). Alternatively, the microspores of 20 buds were cultured in one petri dish of the same size containing 2 ml of medium (method 2). Microspore densities were adjusted to 40,000 per ml. Small petri dishes were set into a large one (9 cm) sealed with parafilm. The microspores were incubated at 30°C under continuous light (white fluorescent lamps, 1,000 lux). After one day in culture the medium was changed by an additional centrifugation as described above.

RESULTS

Within two days of culture, an increase in volume was observed first followed by the subsequent bursting of microspore walls in all genotypes tested, although at low frequencies. Cell division occurred in microspores of 'B11 A3' and 'Sunbred 262', only. Microspore divisions were always followed by bursting of the cell walls. Most of the uninucleate microspores and all microspores at the other stages died after 3-4 days. However, embryoids were obtained from genotypes 'B11 A3' and 'Sunbred 262' within fifteen days of culture (Table 1, Figure 1). Unfortunately, the embryoids either stopped further development in the initiation medium or turned brownish and died when transferred to the MS-medium.

Fig. 1. Microspore - derived embryoid of sunflower. (Gürel et al.)

Table 1. Reaction of sunflower microspores cultured *in vitro* on NLN media with different pH values

Genotype	pH	Increase of cell volume		Division of microspores		No. of embryoids obtained	
		method 1	method 2	method 1	method 2	method 1	method 2
B11 A3	6.0	+/–	+/–	–	+/–	–	1
	6.2	+/–	+/–	–	+/–	–	–
Do 131	6.0	+/–	+/–	–	–	–	–
	6.2	+/–	+/–	–	–	–	–
Frankasol	6.0	+/–	+/–	–	–	–	–
	6.2	+/–	+/–	–	–	–	–
Sunbred 262	6.0	+/–	+/–	–	+/–	–	–
	6.2	+/–	+/–	–	+/–	–	1

+/– only a few microspores showed these reactions.

DISCUSSION

As far as we know this is the first report on successful initiation of embryogenesis from culture of isolated microspores of sunflower (*Helianthus annuus*). Microspores reacted on a high sucrose medium with two pH values. High sucrose contents were also used for anther culture by Mezzarobba and Jonard (1986) and for culture of young zygotic embryos of sunflower by Chandler and Beard (1983) and Espinasse (1985).

We observed cell divisions and development of embryoids in only two of the four genotypes tested, i.e., 'B11 A3' and 'Sunbred 262'. According to Mezzarobba and Jonard (1986) embryogenic capacity of sunflower anthers is strongly affected by genotype. Such genotypic effects on microspore embryo yield were also described in other species like *Brassica napus* (e.g., Dunwell et al., 1985; Siebel and Pauls, 1989).

The frequency of microspore division and regeneration of embryoids reported here is still very low. Most of the microspores and all cells in the tetrad and binucleate stages died after 3-4 days. This effect has also been reported for microspore culture of *Datura innoxia* (Sangwan-Norrel, 1977) and *Brassica* species (Lichter, 1982; Sato et al., 1989). In our experiments only a few microspores showed cell division, but further development was usually not observed. Similar findings were reported by Lichter (1989) for *Brassica oleracea, B. nigra* and *Raphanus sativus*.

It is well documented by many experiments with different species that the appropriate microspore stage is very important for induction of embryogenesis (e.g., Pechan and Keller, 1988.). In our experiments the flower buds were selected by cytological examination of microspores from anthers from the same row on the flower head. However, microspores in unrespores may produce inhibitors that affect the reactive ones. In raspeseed for example, binucleate microspores showed particularly toxic effects on the development of embryogenic microspore cultures (Kott et al., 1988).

A more exact procedure for selection of anthers containing microspores in the appropriate stage, for exmaple the cytological examination of each bud or characterization of the microspore stage by closely correlated bud characteristics, may prevent extrusion of inhibitors into the culture medium. However, if later and earlier stages can not entirely be excluded from culture, one or two more changes of medium or lower densities of microspores may also provide an efficient way to prevent or reduce toxic compounds in the medium.

In accordance with Lichter (1982) it can be supposed that the physiological condition of donor plants additionally affects microspore reaction *in vitro*. Experiments designed to define the best conditions for an optimum development of donor plants may help to increase embryo yield. For example, donor plants of *B. napus* grown under lower temperature conditions (15°C) yielded more embryogenic microspores than plants grown at 20°C (Dunwell et al., 1985).

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EMBRYOGÉNÉSE DANS LES CULTURES DE MICROSPORES DE TOURNESOL (*Helianthus annuus* **L.**)

RÉSUMÉ

Au cours des expériences présentées dans cette publication, nous avons réalisés les étapes initiales du dévloppement de cultures de microspores. La division de microspores uninucléés ainsi que l'embryogénése ont été obtenues, bien qu'avec un faible taux de réussite pour les quatre génotypes, des stades de floraison variés, des milieux de cultures de compositions variables et diverses conditions de culture seront nécessaires pour mettre au point une technique de culture de microspores applicable à la sélection du tournesol.

EMBRIOGENESIS EN EL CULTIVO DE MICROSPORAS DE GIRASOL (*Helianthus annuus* **L.**)

RESUMEN

En los experimentos presentados aquí los pasos iniciales para el desarrollo del cultivo satisfactorio de microsporas han sido llevados a cabo. Ambas divisiones de microsporas uninucleadas y embriogensis fueron alcanzados aunque en baja proporción con dos de los cuatro genotipos testados. Estudios posteriores con otros genotipos, con diferentes estados de floración y diferentes composiciones de medios y condiciones de cultivo serán necesarios para desarrollar un cultivo de microsporas eficiente aplicable a la mejora de girasol.