COMPARATIVE INVESTIGATION OF IMMATURE EMBRYOS GROWING OF INTERSPECIFIC SUNFLOWER HYBRIDS

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ABSTRACT

Comparative investigation of immature embryos growing of interspecific sunflower hybrids was carried out in vitro conditions. Four hybrid combinations, in which wild annual and perennial sunflower species participated, were used. These were the hybrid combinations 807A x E-131 (*Helianthus argophyllus*), 3A x E-130 (*H. argophyllus*), 807A x M-129 (*H.divaricatus*) and 3A x M-146 (*H.tuberosus*). In in vivo conditions the embryos necrotized and died because of incompatibility between species. In in vitro conditions, the method of embryo rescue was applied. On the base of this method, different number of hybrid embryos was isolated. The tissue culture of Azpiroz et al. (1987) was applied, when some modifications of the tissue were used. The results of the investigation showed that using embryo rescue the hybrid plants could be grown. The seed set of hybrids, originated from perennials was too low than that of hybrids, originated from annual species. The obtained hybrid plants were cultivated in greenhouse conditions and sufficient quantity of seeds was obtained. Next generations were grown in field conditions. They were used as initial material for developing sunflower lines with valuable agricultural characters, resistant to biotic and abiotic stress factors.

INTRODUCTION

For the breeding of most crops was necessary to accelerate or shorten the process repeatedly. That was due to the need of fast developing of hybrids and uniformed lines, fast transfer of new genetic material, valuable for sunflower breeding programs. Shortened vegetation period, improving the quality of seed production, overcoming of new pathogens races and overcoming of incompatibility of cultivated and wild forms were the subjects of the main research work. The method of embryo culture, used in this connection, allowed faster creation of lines. This method led to reducing the duration of lines obtaining. Six generations could be produced in one year as contrasted with classical methods (Alissa et al., 1986; Aspiroz et al., 1987). Applying the embryo rescue method was a precondition for developing the effective method for decreasing the sterility and obtaining of hybrid forms in *Helianthus* genus (Chandler & Bear, 1983; Bohorova *et al*., 1985; Krauter & Fried, 1991 and etc.). Paul & Barthou (1994) suggested method for cultivation of embryos from commercial hybrids in unsterile conditions. Special equipment, preliminary treatment of embryos and different chemical substances were necessary for their cultivation in "in vitro" conditions. In this study we presented the data of successful obtained crosses between cultivated sunflower and annual and perennial species of genus *Helianthus* using embryo rescue method and classical breeding methods.

MATERIAL AND METHODS

Plant material

The investigation was carried out in Dobrudzha agricultural institute (DAI), General Toshevo. The embryos were isolated from preliminary crosses, obtained using classical methods in sunflower breeding – isolation, pollen collecting, pollination. Cultivated sunflower lines 807A and 3A, developed in DAI were used as maternal parent. Some accessions of wild sunflower species were used as paternal component. The annual species *Helianthus argophyllus*, (accession *E-131*)

and perennial species *H. divaricatus* (accession *M-129*) and *H.tuberosus* (accession *M-146*) were used as pollinators.

Explants and sterilization

The immature embryos are isolated from the inflorescence from the 3rd to the 14th day after crossing. The day of performing embryo isolation is different for each cross. Depending on the used genotype, the embryo size is from 2 to 7 mm. The formed young embryos are removed from the inflorescence and placed in a lint bag. Sterilization is done with commercial bleaching solution (without diluting it) for 20 min, then the bags are transferred to a laminar box and washed with sterile distilled water. Using scalpel and pincers, the husk is removed from the not well formed seed, and the embryo is separated from the endosperm and immediately placed on a nutrition medium.

Nutrition media, sterilization and cultivation

For the separated 2-3 mm large embryos the nutrition media suggested by Chander and Beard (1983) are used. The initial medium on which the embryos are cultivated is B5 with added amino acids as follows: L-alanine – 100 mg/l, L-glutamine – 800 mg/l, L-serine – 160 mg/l, L-tryptophane – 5- mg/l, L-cysteine – 10 mg/l and NOK – 0.05 mg/l. Sucrose is 120 g/l, and the agar is 7g/l; pH of the medium is 5.7. The nutrition medium is distributed in 10 cm Petri dishes after autoclaving, and 10 embryos are plated on each dish. Five to seven days after chlorophyllization of the cotyledons, the embryos are transferred to solid agar nutrition medium. The nutrition medium contains only B5 – salts and 10 g/l sucrose.

For cultivation of embryos bigger than 3-4 mm, the methodology of Azpiroz et al (1987) is applied, which is simpler and with a shorter cycle. The medium for cultivation of the isolated embryos is MS, the macrosalts being reduced in half, the vitamins are of medium B5, the sucrose is 20 g/l, with 100 mg/l of inositol, pH being 5.7.This medium is distributed in 5 Petri dishes each with diameter 10 cm. Ten embryos are placed in each dish.

The cultures are placed in a phytosanitary room at temperature 24±2° C and illumination 2500 – 3000 lux, with photoperiod 18/8 h. The plants are grown in the cultivation constructions till beginning of budding stage at temperature 18-20° C, and the next stages occur at temperature 22-26° C. When roots reach length 2-3-4 cm, the plants are transferred to a soil under non-sterile conditions, covering them with glass cover for about 4-6 days to ensure successful rooting and acclimatization.

RESULTS AND DISCUSSION

The process of creating of sunflower lines, parental lines of a hybrid, was long, difficult, and needed at least 10-12 years. For acceleration of that process, some alternative ways in the field of plant biotechnology were searched. Different theoretical opportunities and in vitro technics existed for intensification of the breeding process, but not all of them could be applied, because of their low effectiveness in sunflower. One of the methods, which could be successfully used, was embryo cultivation. This method was used in sunflower for quick obtaining of lines, restorers of fertility, as well as sterile analogues (Plotnicov, 1983). In perfect conditions this method allowed vastly shortening the breeding process and obtaining 6 generation in one year, which was impossible to be done by classical methods (Alissa *et al*., 1986; Azpiroz *et al.,* 1987). This method was easy and cultivated sunflower embryos could be grown on simple synthetic tissue with small quantity of hormone supplements. The cultivation of interspecific embryo rescue was disparate. Our investigations showed that they had small size and aborted prematurely. In this case the tissues were more complicated and at least one preliminary investigation had to be done and determined when

exactly the embryos died. It depended on type of interspecific crosses – crosses with annuals and perennials (Drumeva, M. & Nenova, N., 2012; N. Nenova *et al.,*2014; Valkova D. *et al.,*2014) described in the previous chapter. описани в предишния раздел.

Based on our previous investigations, in this study we included interspecific hybrids obtained with participation of annuals and perennials. Hybrid combinations, seed set and number of obtained plants, grown in the soil were presented on table 1.

Table 1. Number of isolated embryos and obtained plants grown in the soil from F_1 interspecific sunflower hybrids.

№	Hybrid combination	Seed set, %	Number of embryos	Number of plants
1	*807 A x H.argophyllus E- 131*	53	62	57
2	*3A x H.argophyllus E -131*	71	117	66
3	*3A x H.tuberosus M-146*	0.2	5	1
4	*807 A x H.divaricatus M-129*	0.2	5	2

The results showed, that the highest seed set was determined for the hybrid combination *807 A x E-* 131 (*H. argophyllus*). The species *H.argophyllus* (acc. E- 131) was included in other cross too but the difference in seed set was 18%, which showed that the maternal component also had an effect for the successful crossability. In this combination there were 55 more isolated embryos, which was precondition for surviving of bigger number of plants after sowing in the soil. The embryos from both crosses with participation of *H.argophyllus* (E- 131) were with different size. The embryo size of combination *807 A x E-131* (*H. argophyllus*) was 4-7 mm, and for the other hybrid combination *807 A x E-131* (*H. argophyllus*) - 3-4 mm. It was suggested that larger embryos possessed more endosperm than the others. They contained more nutrients and their survival mechanisms were better.

The testing results of hybrids, obtained with participation of perennials were quite different. The seed set was too low 0.2%, and five embryos were isolated from both hybrids - *3A x M-146 (H. tuberosus)* and *807A x M-129* (*H. divaricatus*). Obviously, the incompatibility of wild annuals and cultivated sunflower was lower than that of perennial species.

These three species, *H. argophyllus*, *H. divaricatus* and *H. tuberosus*, included in the investigation possessed valuable economic characters and could be used as sources of new genetic material to be transferred to the genome of cultivated sunflower. The barriers of incompatibility between most of wild species and cultivated sunflower were hardly overcome using conventional methods. This enforced using of rescue embryo method. All F_1 plants were planted and grown in nursery conditions and after that in the field. Each plant was isolated separately. Selection on morphological characters, evaluation for resistance to downy mildew, phoma, phomopsis and broomrape were carried out. The obtained plants from the cross *cultivated sunflower x perennial species* died during their vegetation. The presented results concerned the plants from hybrid combination *cultivated sunflower x annual species*.

The results of evaluation for resistance to diseases and parasite broomrape were presented on table 2.

Table 2. Resistance of interspecific hybrids to diseases and parasite broomrape.

Resistance, %	Hybrid combination
Resistance 100% to *Pl.helianthi* Novot. and 76-99% to *Orobanche cumana* Wallr.	807 A X E-131
Resistance 76-99% to *Pl.helianthi* Novot. and *Orobanche cumana* Wallr.	3A X E-131
Type of reaction	Hybrid combination
Immune to *Phomopsis helianthi* and *Phoma macdonaldi*	807 A X E-131
Resistant to Phomopsis helianthi and *Phoma macdonaldi*	3 A X E-131

One of the main purposes of interspecific hybridization was directed to transfer of genetic material from wild *Helianthus* species into the genome of cultivated sunflower. The obtained materials with participation of wild annual species *H. argophyllus* (accession E-131) possessed resistance to *Pl. helianthi* from 76% до 100%. The resistance to broomrape varied from 76% до 99%. The type of reaction to the leaves pathogens (*Phomopsis helianthi* and *Phoma macdonaldii*) varied from immune to resistant. The present resistance was transferred from wild accessions because the cultivated sunflower was susceptible regarding the studied pathogens.

Seed oil content was an important character in developing new sunflower forms for including in breeding programs. Wild sunflower species were characterized with low seed oil content. Hybrid combinations were distinguished with higher seeds oil content. The highest seed oil content 41.7% was determined for the cross 3 A x E-131, followed by 39.5% for hybrid combination 807 A x E-131. The pointed values oil content in hybrid seeds were comparatively low, but after backcrossing and self-pollination it could be increased.

CONCLUSIONS

On the base of the pointed results in this investigation, some conclusions could be made:

- Wild annual species were characterized with lower incompatibility with cultivated sunflower than wild perennial species. The improved by us embryo rescue method was suitable for overcoming the incompatibility of wild annual *Helianthus* species and cultivated sunflower.

- The species *H.argophyllus,* accession E-131 was a source of Rf genes and genes for resistance to economically important diseases and parasite broomrape. Transfer of these genes was determined by evaluation of hybrid material.

- Seed oil content was low, but in next generations after backcrossing and self-pollination it could be increased.

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