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ИЗПОЛЗВАНЕ НА ЕМБРИОКУЛТУРА ЗА ПОЛУЧАВАНЕ НА МЕЖДУВИДОВИ ХИБРИДИ ЦАРЕВИЦА
APPLICATION OF AN EMBRYO RESCUE TECHNIQUE IN INTERSPECIFIC MAIZE HYBRIDSATION

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Резюме

Проучена е възможността за приложение на ембриокултурата като достъпен метод и реална алтернатива за получаване на разнообразни кръстоски на културната царевица (*Zea mays* L.) с нейни диви родствени форми (*Tripsacum dactyloides* L). Реализирането на такива междуродови хибриди представлява интерес за селекцията, но получаването им чрез масово използваните в практиката конвенционални методи рядко е възможно. Настоящото изследване има за цел да анализира и да проучи поведението на тестираните генотипове в условията на отглеждане *in vitro* и да изясни някои от критичните елементи за развитието им в изкуствено създадена среда. На базата на основна хранителна среда на Chu (N6) е проследено действието на различни комбинации от хормони върху развитието на зародишите. По този начин е потърсено балансиране на нейния състав и оптимизиране на условията за получаване на хибридно потомство от желаните кръстоски. Най-благоприятен ефект върху регенерационните процеси е постигнат при хранителна среда, съдържаща 0,00044 µmol 2,4-Д в комбинация с 0,00011 µmol kinetin.

Abstract

The possibilities for application of embryo culture as a practical method and an alternative for obtaining hybrids from different crosses of cultivated maize (*Zea mays* L.) with wild related forms (*Tripsacum dactyloides* L) were explored. Obtaining such hybrids is of a particular interest for breeding, but the wide-spread conventional techniques rarely succeed in producing them. The present study aims at analyzing the performance of the tested genotypes grown *in vitro* and at clarifying some of the key elements of their development when grown on artificial media. The effects of different combinations of plant hormones on young immature embryos were evaluated based on the main medium of Chu (N6). The results were used to optimize both the nutritive media content and the conditions for obtaining hybrid progeny from the desired crosses. The medium containing 0.00044 µmol 2,4-D in combination with 0.00011 µmol kinetin was found to exert the most favourable effect on the regeneration processes.

Ключови думи: *Zea mays, Tripsacum dactyloides*, ембрио спасяване, регенерация, междуродови хибриди.
Key words: *Zea mays, Tripsacum dactyloides,* embryo rescue, regeneration, interspecific hybridsation, hybrids.

INTRODUCTION

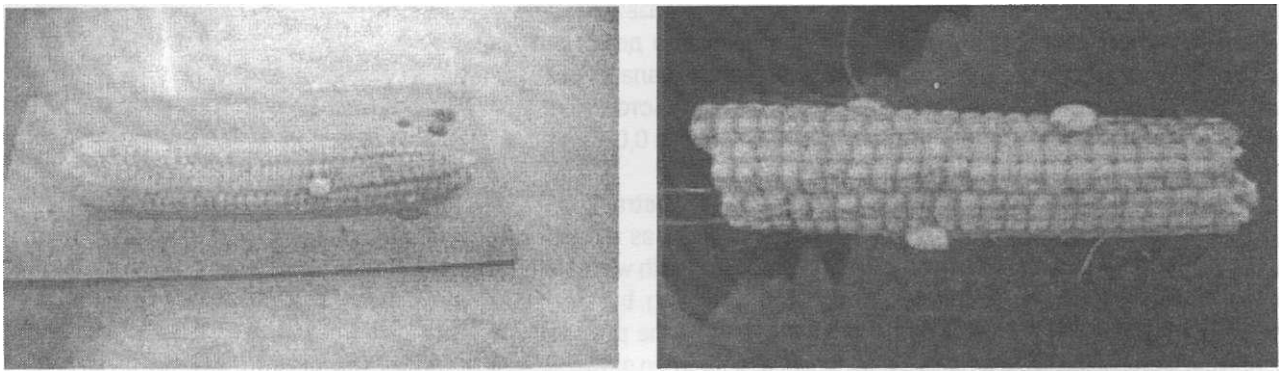
The importance of maize as feed, food and industrial crop makes the research on its improvement an everlasting effort with multitude of approaches. Recently emerged use of the crop as a major source of raw material for biofuel production re-invigorated the interest in developing new varieties with novel combinations of traits. In many crops sources of novel traits could be found in more or less related wild species and in the case of cereals the use of such wild relatives was a subject of plentitude of studies. *In vitro* cultivation of cells, tissues and organs is particularly advanced in the *Poaceae* (*Gramineae*) family. Methods for tissue culture and callus induction were successfully used for regenerating whole plants from immature embryos in a number of maize genotypes. In spite of the success in regenerating plants through somatic embryogenesis from immature embryos reported by many authors (Bronsema et al., 1998; Bronsema et al., 2001; Matthys-Rochon et al., 1998; Vasil et al., 1985) our search through the available literature did not yield any publications on regeneration of plants either through direct organogenesis or through somatic embryogenesis of interspecific crosses between maize and wild relatives. Embryo rescue was used in other crops to successfully produce hybrid progeny. This technique is of

particular interest when incompatibility arises between the hybrid embryo and the endosperm in the early stages of development (Bojinov, 2000). When the two species can produce hybrid embryo but it is aborted due to such incompatibility excising the immature embryo and cultivating it on artificial media proved to be effective in rescuing the progeny and obtaining crosses with prospective donors of important traits. Such hybrids increase the gene pool available for classical breeding and provide options for introduction of novel traits. With the recent rising of the concerns about potential food and feed shortage on a worldwide scale, introduction of novel traits that can increase the adaptability and the efficiency of use of the major crops becomes of particular importance.

In the case of maize embryo rescue can provide the means to obtain crosses between the cultivated corn (Zea *mais* L) and the wild relatives belonging to the genus *Tripsacum.* In the present paper we report the development of a protocol for obtaining hybrids between advanced breeding lines and hybrids of maize with the wild species *Tripsacum dactyloides* through the use of embryo rescue.

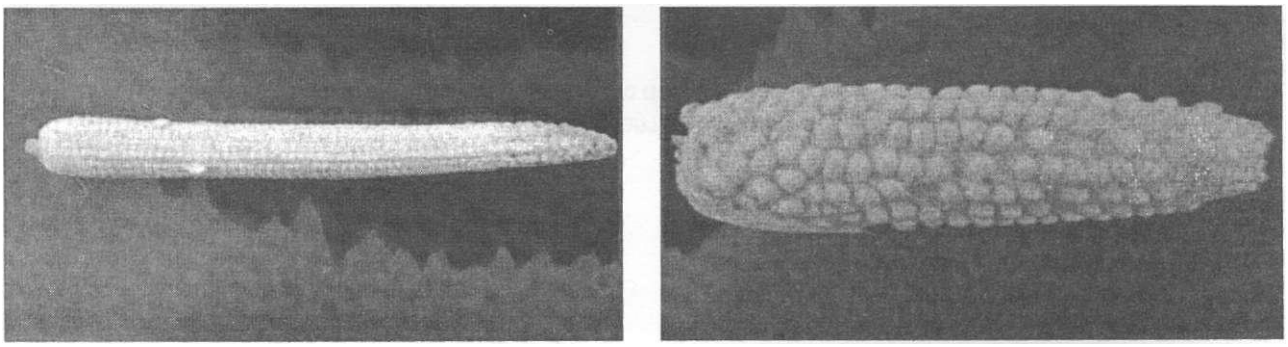
MATERIAL AND METHODS

The experimental work was carried in the Laboratory of Plant biotechnology of the Agricultural University of Plovdiv in 2006. Seeds from the selected maize lines and hybrids were provided by the Corn Research Institute, Knezha. Crosses between these lines and hybrids and the wild species were made at the research fields of CRI and the ears collected at 14 days after pollination (DAP). Immature embryos from the Bulgarian standard hybrid Knezha 509 and the Chinese breeding line CHI 31 were used as controls to which the development of embryos from a mutant line XM 87 136 and the following crosses: XM 94 521 x *Tripsacum dactyloides* (Fig. 1a); Knezha 509 x *Tripsacum dactyloides* (Fig. 1 b) and Knezha 625 x *Tripsacum dactyloides* (Fig. 1c) were compared. Ears from each line and hybrid were surface sterilized with hypochloride (5% solution of commercial bleach) for 30 min and rinsed three times with sterile water. Immature embryos were aseptically excised and cultivated on a basic N6 medium (Chu et al., 1975), supplemented as follows: 1 - control (no hormones added); 2 - supplemented with 0.00022 umol 2,4-D and



а) XM 94 521 x *Tripsacum dactyloides*

б) Кнежа 509 x *Tripsacum dactyloides*
b) Knezha 509 *x Tripsacum dactyloides*



в) Кнежа 625 x *Tripsacum dactyloides*
c) Knezha 625 x *Tripsacum dactyloides*

д) Мутантна линия XM 87 136
d) Mutant line XM 87 136

\f ***Фиг. 1.*** *Получаване на хибридни завръзи от междувидови кръстоски царевица*
Fig. ***1.*** *Initial stage of obtaining interspecific maize hybrids - early grain development*

0.00011 µmol kinetin; 3 – supplemented with 0.00044 µmol 2,4-D and 0.00011 µmol kinetin. After the pH was adjusted to 5.6 media were autoclaved for 25 min at 121°C and 1.5 psi. During the first 20 days embryos were cultivated in the dark at 26°±1C. Afterwards the young plantlets were transferred to the normal conditions of the growing chamber: 16/8 h photoperiod at 25±2°C and light intensity of 2300 lx. Every two weeks after the introduction in vitro explants (and regenerants later on) were transferred to fresh cultivation media. At the day 45 after the initial plating in vitro plantlets that had well developed root system were transferred to cultivation pots, filled with peat-perlite mix for adaptation to greenhouse conditions. Roots were thoroughly washed with distilled water and the plantlets transferred to pots filled with wetted peat-perlite mix. The adaptation of the plantlets that were successfully transferred to pots was achieved through initial cultivation under a plastic cover followed by a gradual extension of the cultivation periods under open air (Fig. 2).

RESULTS AND DISCUSSION

1. XM 94 521 x *Tripsacum dactyloides* L. cross

Crossing of the line XM 94 521 through applying pollen from the wild relative *Tripsacum dactyloides* L. resulted in a very efficiency of hybrid formation. After pollinating 5 ears only a single developing grain was obtained (Fig. 1a), that could be grown till the time for isolating and transferring to artificial media would come (14 days after pollination – DAP). The putative hybrid embryo was successfully transferred for cultivation on media 2 and was transferred in the following passages on the same media. At the end of the 45 day *in vitro* experiment this single embryo has grown very little and no modification of its color was observed.

2. Knezha 509 x *Tripsacum dactyloides* L. cross

This interspecific cross resulted in obtaining a number of putative hybrid embryos, which were transferred and grown on the three modifications of the N6 medium, studied in these experiments. The embryos transferred to media 2 and 3 demonstrated good development with green leaves and intensively growing stems and roots. In most of the cases these roots penetrated the cultivation media and branched normally. Embryos grown on N6 medium without hormones had reduced viability, expressed in lack of actively growing shoot and green parts. However intensive risogenesis could be observed that produced highly branched and vigorously increasing in length rooting system. Regular transfers to a fresh medium did not stimulate the growth and development of the excised embryos (Fig. 3a). Transferring of embryos to a hormone-free medium affected positively the development of the shots. While not very intensive (and sometimes accompanied by appearance of necrotic tissues) the transfer to such media often resulted in shoot development (Fig. 3b).

а) върху перлит
a) on perlite

б) върху торово-почвена смеска
b) on peat-manure mix

Фиг. 2. Адаптация на регенерантите от междувидовите кръстоски
Fig. 2. *Adaptation of the regenerants from the interspecific crosses*

а) интензивен ризо- и калусогенезис при безсменно отглеждане върху среда 3
a) intensive rhizo- and calusogenesis after continuous cultivation on medium 3

б) развитие на прораст след прехвърляне върху безхормонална среда
b) shoot development after transplanting on hormone-free medium

Фиг. 3. Ефект на хранителната среда върху развитието на ембриони от кръстоската Кнежа 509 x Tripsacum dactyloides L.
Fig. 3. *Effect of the nutritive media on the embryo development from the cross Knezha 509 x Tripsacum dactyloides L.*

3. Knezha 625 x *Tripsacum dactyloides* L. cross

Seven embryos have been obtained from this cross that were successfully isolated and cultivated on media 2 and 3. The results demonstrated apparent differences in embryo development, the emergency and the speed of forming of shoots and the intensity of root formation in different treatments with hormones, supplemented to N6 medium. Relatively higher stimulating effect on the organogenesis processes was recorded in embryos, cultivated on medium 4 that contained 0.00044 μmol 2,4-D. For this genotype the application of higher phytohormone levels is an efficient measure with a positive effect on the development of young plantlets.

4. Line CHI 31

In about 50% of the cultivated embryos from the line CHI 31 formation of organs (roots and shoots) was observed where no significant differences were recorded between the three tested modifications of N6 medium. Cultivation of this genotype in the presence of phytohormones in tested concentrations does not produce any differences in the capacity to induce regeneration process.

5. Line XM 87 136

The results from testing the effect of cultivation media 2 and 3 on the development of self-pollinated embryos from the mutant line XM 87 136 demonstrated growth and development suppression of the embryos when cultivated on medium 2. Both processes progressed uninterrupted towards successful regeneration of embryos when medium 3 was used for cultivation. Our observations in this case were that the increase of 2,4-D concentration to 0.00044 μmol provides for higher frequency of embryo development induction and therefore – to increasing the possibilities for successful plant regeneration.

Overall our results demonstrate that successful regeneration of immature embryos from interspecific crosses is more likely to be achieved when cultivation medium is supplemented with moderate levels of phytohormones than if hormone-free medium is provided. These results correspond with data from other studies (Fransz and Schel, 1990; Kemper et al. 1996) where embryos from pure lines and intraspecific crosses were cultivated in vitro. The application of the embryo rescue technique proposed here provides the opportunity for simplified obtaining of interspecific hybrids through eliminating laborious and technically challenging techniques like isolation under microscope (Laurie et al., 1999; Gruis et al., 2007). Therefore our method is easier to execute thus baring the potential fro wider adoption in breeding programs.

CONCLUSIONS

As a result from this study the following conclusions can be made:

1. Embryo rescue can be an effective procedure in interspecific hybridization for improving maize.
2. The proposed simplified procedure for embryo cultivation and plant regeneration allows for eliminating the labor-intensive manipulations that were used in other studies. This opens further opportunities for wider application of interspecific hybridization.

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