Androgenesis Efficiency in Anther Culture of Pepper (*Capsicum annuum* L.)

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Abstract

The frequency of obtained androgenic plants depends highly on the genotype, therefore the low rate of androgenic embryo recovery limits the utility of anther culture in pepper breeding. In the present study, the effect of genotype of induced androgenesis in in vitro anther culture of 21 different pepper genotypes was investigated. The aim of this study was establishment of effective in vitro technology for induction of embryogenesis in pepper anther culture; development of the embryos into regenerants; successful adaptation and acclimatization of regenerants from sterile to greenhouse conditions and future study of androgenic plant regenerants. The anthers were cultured according to the method developed by Dumas de Valux et al. (1981) where heat pre-treatment was applied. The experiment showed that the effectiveness of androgenesis depends on pepper genotype and the conditions for anther culture maintenance. The direct embryogenesis resulted in embryo formation that developed into plantlets. After successful acclimatization of the regenerants, firstly in growth chamber and after in greenhouse conditions, seed material from four pepper genotypes was collected: Piran, Kurtovska kapija SR, Zlaten medal SR and Féherözön. The collected seeds is excellent basis for further breeding processes, at cytogenetic and other molecular level research.

INTRODUCTION

The methods of biotechnology such as androgenesis introduce new possibilities for faster creation of new pepper varieties or at least faster development of genotypes with desirable traits which can be used in further breeding and development of improved pepper varieties (Koleva-Gudeva, 2007; Kim et al., 2004).

Androgenesis is a method that opens possibilities for development of haploids and spontaneous dihaploids plants in anther culture. Haploid morphogenesis of *Capsicum* species was studied by George and Narayanaswamy (1973) and Kuo et al. (1973); although the production of haploid plants was very low. The first successful reproductive method for pepper androgenesis was developed by Dumas de Valux et al. (1981). In the past twenty years several different protocols were developed and haploid embryogenic regenerants in pepper were obtained (Dumas de Valux et al., 1981; Mytikó et al., 1995, 1997; Dolcet-Sanjuan et al., 1997; Bárány et al., 2005). Mytikó and Gémes (2006) reported great improvement of in vitro haploid induction and genome duplication methods routinely used for resistance breeding in sweet and spice peppers.

A number of factors influence the effectiveness of androgenesis in pepper, some of the most important ones are the donor plant genotype (Nowaczyk and Kisiala, 2006; Mytikó and Fári, 1997) the developmental stage of the microspores (Kim et al., 2004; Ökum and Tripirdamaz, 2002); the condition of culture maintenance and the source of plant material (Kintzos et al., 2000). According to Irikova and Rodeva (2004) and Koleva-Gudeva et al. (2007) the effect of different media and different incubation

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treatments showed that induction of embryogenesis in anther culture is possible with heat treatment of 35°C on CP medium (Dumax de Valux et al., 1981) in majority of the genotypes under investigation. The objective of the present work is the effect of different pepper genotypes on androgenesis efficiency in anther culture to be studied.

MATERIAL AND METHODS

Anther-Donor Plant Material and Anthers Culture Conditions

Twenty-one pepper genotypes were used as anther-donor plants. The pepper genotypes used in the experiment are with different origin of production and different genotype characteristics. Anther-donor plants were planted in polyethylene pots in greenhouse by the end of April. They were used during the four weeks after the first flower buds had appeared. The flower buds were harvested when the corolla was of the same length as the calyx or slightly longer. The developmental stage of the microspores was determined in microscopic slides of acetocarmine squashes.

Flower buds in first pollen division stage were surface sterilized in 70% ethanol for several seconds, then in 5% $Ca(ClO)_2$ + 2-3 drops Tween 20 for 10 minutes, and rinsed three times in sterile distilled water. After the removal of the filaments, anthers from three flower buds were placed in Petri dish (6 cm diameter), with the concave face down, touching the culture medium. The method of Dumas de Valux et al. (1981) was used for induction of androgenesis. According to the method, the anthers were cultivated on CP medium + 0.01 mg/L 6-furfurylamino purin (KIN) + 0.01 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) with incubation of 8 days in darkness at 35±2°C, the following 4 days the anthers were transferred to growth chamber at 25±2°C with photoperiodism 12 h light/12 h dark. Afterwards, the anthers were subcultured on R_1 medium + 0.01 mg/L KIN and placed in a growth chamber at 25±2°C with photoperiod of 12 h light/12 h dark. Young shoots emerging from the anthers were transferred onto hormone free V_3 media in order roots to be formed. The plantlets were planted in polyethylene pots on sterile mixture of perlite : peat : sand (1:1:1) and acclimatized in growth chamber and after placed in greenhouse under acrylic cover in order crosspollination to be barred. When transferred to the greenhouse the plants were under regular fertilization and pest protection control. The fruits from each plant were harvested separately; seeds were collected and stored appropriately in the gene bank of the faculty.

Determination of the Microspore Stadium

The determination of the microspore stadium was observed microscopically in a fresh anthers squash stained with color acetocarmine. The preparation of color acetocarmine for the determination of the microspore stadium was done as follows: 1 g of carmine was dissolved in 45 ml glacial acetic acid, followed by the addition of 55 ml of distilled water. The solution was left to boil for 5 min. When the boiling time was over, the solution was left to cool and filtrate. At the next stage, 1–2 drops of iron hydroxide was added for colour intensification. A drop of acetocarmine was placed on the isolated anthers. After a few minutes the anthers were macerated on the glass microscopic slide, the slide was placed under the microscope, and the observation of the microspore growth stadium was performed (Fig. 2A). Microspores are observed also in the anther culture, after onset on the medium, where microspore nucleus division could be seen (Fig. 2B).

Determination of Chromosome Number

Certain number of seeds from each plant was used for determination of chromosome number of each androgenic plant. The seed material was germinated in Petri dishes and the number of chromosome was counted in root tip meristems. The tissue was prepared according to Tjio and Levan (1950) cytological technique, as well as standard "squash" method. The root tips were pretreated with 2 mM 8-hydrohyquinoline for 12–24 hours, fixed in acetic alcohol solution (1:3), hydrolysed with HCl 1N at 60° for 9 minutes and stained with 1–1,5% Gomori's hematoxylin (Konstantinov et al., 1985).

Data Analysis

All data on percentage of embryogenic anthers and number of embryos per 100 anthers were subject to analysis of variance (ANOVA), and the mean values were evaluated at the $p < 0.05$ level of significance using Duncan's Multiple Range Test.

RESULTS AND DISCUSION

Not all genotypes under investigation were able to produce embryos from anther culture (Table 1, Fig. 2C, D). The responsive anthers from studied genotypes reacted with direct embryo formation or callus formation without regeneration. Once callus was initiated the induction of somatic embryos did not occur, which is similar with the results reported by Binzel et al. (1996). After the induction period on CP medium for 12 days the anthers were subcultured on R_1 medium, where since the beginning the embryos showed totipotency, progression in development, growth and shoot formation. The experiment showed that the highest percentage of embryogenic anthers was given by Féherözön (17.39%) and Tura (17.05%), while 9 genotypes did not show embryo formation at all (Table 1). The percentage of embryogenic anthers of the other pepper genotypes varied from 9.23% (Pritavit F_1) to 2.43% (Slatko luta). Rodeva et al. (2004) demonstrated considerably genotypic differences in in vitro response of anthers in investigated six lines, six varieties and four hybrids, where anthers reacted mostly with direct embryogenesis. The shoots proliferated from anthers when incubated on R_1 medium continued the development on V_3 medium, where in absence of phytohormones young plants were formed (Fig. 2E). The rooting was also on V_3 medium and well rooted shoots were transferred on sterile mixture of sand : perlite : peat in ration 1:1:1. In this stage the plants were ready for adaptation and acclimatization in greenhouse conditions (Fig. 2F).

According to the classification of Mityko and Fari (1997) for identification of androgenic potential according to the percentage of anthers that give embryos, pepper types are classified into: poor androgenic potential - less than 5% embryogenic anthers; fair androgenic potential - 5.1 - 15% embryogenic anthers; good androgenic potential - 15.1 - 30% embryogenic anthers; excellent androgenic potential - over than 30% embryogenic anthers. The results from our research showed that embryos are formed on CP medium with heat temperature stress (+35°C) which is in concord with the findings of Dumas de Valux et al. (1981). From all 21 genotypes, 12 showed ability for embryo formation, while 9 pepper genotypes were non-responsive (Table 1).

The number of acclimatized androgenic plants from different pepper genotypes (Féherözön, Kurtovska kapija SR, Piran and Zlaten medal SR) used in the experiment is shown in Table 2 and Figure 1. The genotype Féherözön showed good embryogenic response with 32.60 embryos per 100 anthers, but only 11 plants were fully regenerated and adopted to greenhouse conditions. In the case of Kurtovska kapija SR and Piran, although the embryogenic response was poor, 9 and 8 plants, respectively, were regenerated and acclimatized to greenhouse conditions. The genotype Zlaten medal SR showed fair embryogenic response, but the number of 4 regenerated and acclimatized plants is the lowest as compared to other two genotypes with poor embryogenic response.

Not all acclimatized plants were able to produce fertile fruits. There were four fertile plants of the genotype Féherözön from eleven regenerated, in genotype Kurtovska kapija SR three plants were fertile, while in Zlaten medal SR and Piran only two plants from each genotype were fertile (Fig. 1A–D, Table 1). Furthermore, the seeds from each of four genotypes were used for determination of chromosome number. The caryotype analysis showed that acclimatized and fertile androgenic plants of Féherözön, Kurtovska kapija SR, Piran and Zlaten medal SR and are dihaploids with 2n=24 (Fig. 2G, H) as was expected for *Capsicum annuum* L. genotypes (Lanteri and Pickersgill, 1993).

Further caryotype analysis are needed to prove ploidy level of plants that were unfertile and even better, plants before adaptation to greenhouse conditions to be analyzed for ploidity level in order diploisation of haploids to be conducted. The collected seed material is good base for further cytogenetic and molecular research and involvement in process of pepper breeding.

CONCLUSION

Since pepper is a recalcitrant species, moderate results can be achieved in tissue culture, and in vitro anther culture seems to be the only exception under these conditions. The results lead to the conclusion that from 21 pepper genotypes under investigation, 12 possessed potential for direct somatic embryo formation. The potential of pepper genotypes that responded with embryogenesis varied from good to poor and non-responsive genotypes. Not all embryos emerging from anthers were able to regenerate in fully developed plants and even more not all fully developed and acclimatized plants in greenhouse conditions were fertile.

The caryotype analysis showed that acclimatized and fertile androgenic plants of all 4 pepper genotypes (Féherözön, Kurtovska kapija SR, Piran and Zlaten medal SR) are dihaploids (DH). According to Dumas de Valux (1999), generally, the DH lines are homogeneous and stable (with some exceptions) and allow practical use. The lack of seed fertility may result from inbreeding effects. Haploid and DH lines are now commonly used by breeding laboratories.

Still very little is known about the mechanism behind the change of pathways from gametophytic to embryonic (Bárány et al., 2005; Mitykó et al., 1999; Binzel et al., 1996) and how the genetic structure of certain genotype is responsible and involved in activation or repression of microspore developmental process in response to a stress treatment, nutrient media composition and developmental stage of microspores. It is still an open question why not all genotypes react with embryogenesis when the growth conditions for the anther-donor plants and androgenesis conditions are the same for all genotypes.

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