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Somatic embryogenesis in pepper anther culture: The effect of incubation treatments and different media

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Abstract

The frequency of obtained androgenic plants depends highly on the genotype; therefore the low rate of haploid recovery limits the utility of anther culture in pepper breeding. The need for incubation treatment and adequate nutrition media supplemented with plant growth regulators, especially auxins, are suggested as important factors to obtain somatic haploid embryos in pepper anther culture. The effect of three incubation treatments of the androgenic potential in pepper anther culture on MS, N, LS, NN and CP medium are summarised, and the results demonstrate that:

- by incubating treatment in cold conditions (at 7 °C) in darkness for 7 days, and then transferring the explants to light conditions (12-h photoperiod at 25 °C) for 4 weeks, on LS and NN mediums, anthers produced callus;
- by incubating treatment in heat conditions (at 25 °C) in darkness for 7 days, and then transferring the explants to light conditions (12-h photoperiod at 25 °C) for 4 weeks, on MS and N mediums, anthers produced callus;
- by incubating treatment in heat conditions (at 35 °C) in darkness for 8 days, the next 4 days to light conditions (12-h photoperiod at 25 °C) on CP medium, and then transferring the explants to R_1 medium for 4 weeks, anthers produced embryos.

Keywords: Androgenesis; Thermal shock; Embryo formation; *In vitro*; *Capsicum annuum* L

1. Introduction

The creation of varieties of pepper, corresponding to the modern requirements, is a long-term and labour-consuming process due to the fast genetic degeneration of breeding materials as a result of uncontrolled foreign pollination, necessity of applying certain schemes of maintenance with large space isolations, and a lack of possibility for vegetative reproduction. These difficulties in the conventional breeding process may be overcome through the establishment and introduction of precise and optimised methods for *in vitro* production of haploid regenerants of microspore origin from anther culture and genome diploidisation. The haploid plants are the ideal material for genetic and breeding studies due to the total manifestation of genetic potency and mutations, part of which remain invisible in a recessive state of diploid organisms. The haploid plants are extremely valuable with the heterosis breeding. By the multiplication of their chromosome number, highly isogenic lines are received, the creation of which at the traditional breeding requires a long period of time, accompanied by inbreeding and back-cross fertilisation, by controlled self-pollination and breeding of separate individual plants. The accelerated obtaining of stable homozygous lines is an advantage; it is only breeding that possesses this potential, and it can hardly be achieved with the other methods of breeding.

Haploid and spontaneous diploid plant production from anther culture is a well-developed and useful tool in practical plant breeding as well as in basic research. The first *in vitro* haploid pepper production via anther culture was obtained by Wang et al. (1973). Haploid morphogenesis in *Capsicum* was conducted by George and Narayanaswamy (1973) and Kuo et al. (1973) even though the production of haploid individuals had been very low. Although the first report on pollen embryogenesis in the anther culture of *Capsicum annuum* L.

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was published in 1973 by George and Narayanaswamy, a reproducible anther culture method was developed by Dumas de Valux et al. (1981). Since only a low number of haploid plantlets were regenerated from the excised anthers, further research was carried out, concentrating not only on the composition of the culture medium, but also on other factors affecting the frequency of haploid induction (Mitykó et al., 1995, 1999). Nowadays, the *in vitro* androgenesis – based on nearly 30 years of research – appears to be an effective method for haploid induction.

The production and frequency of haploids can be stimulated by many different techniques, and one of them is shocks at high or low temperature. Although the mechanism of temperature shock in inducing higher androgenesis is not known, one of the possibilities suggested is the reduction of ABA levels in the cultured anthers (Dolcet-Sanjuan et al., 1997). Anthers, immature zygotic embryos, and callus derived from immature zygotic embryos are only types of explants which have formed somatic embryos in pepper *C. annuum* L.

The cold-shock and heat-shock pre-treatments have shown a positive effect on embryo or callus formation from microspores in cultured anthers (Sangwan and Sangwan-Norreel, 1990).

The objective of the present study was to examine the response of anthers from different genotypes to different media, heat-shock and cold-shock pre-treatments regarding the direct somatic embryogenesis. This report describes the cold thermal shock (at 7 °C in darkness) and heat thermal shock (at 35 °C and at 25 °C in darkness) as a precondition that will stimulate the anther cultures towards direct somatic embryogenesis.

2. Materials and methods

2.1. Anther-donor plant material and donor plant growth conditions

Nine pepper varieties (*C. annuum* L.) were used in the experiment: Feferona (long, hot type), Slatko Luta (long, medium-hot type), Vezena Luta (long, hot type), Sivrija (long, sweet type), Zlaten Medal (sweet, spice type), Kurtovska Kapija (sweet, spice type), California Wonder (bell-shaped, sweet type), Rotund (tomato-shaped, sweet type), and Féherözön (Hungarian wax-bell-shaped, sweet type). Anther-donor plants were grown under greenhouse conditions. Mother plants were used during the 4 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 macrospores was determined in microscopic slides of acetocarmine squashes. The preparation of colour acetocarmine for the determination of the microspore stadium is 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, one to two 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.

2.2. Anther culture conditions

Flower buds were surface sterilised in 70% ethanol for several seconds, then in 5% Ca $(ClO)_2$ + 2–3 drops Tween 20 for 10 min, 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 media employed for anther culture were: MS (Murashige and Skoog, 1962) supplemented with kinetin (Kn) (1.0 mg l^{-1}), 2.4-dichlorophenoxyacetic acid (2.4-D) (0.01 mg l^{-1}) and indole-3-acetic acid (IAA) (0.001 mg l^{-1}); N (Nitch, 1969) supplemented with Kn (1.0 mg l^{-1}), IAA (0.001 mg l^{-1}); LS (Linsmaer and Skoog, 1965) supplemented with Kn (3.0 mg l^{-1}) and IAA (1.0 mg l^{-1}); NN (Nitch and Nitch, 1969) supplemented with Kn (0.01 mg l^{-1}) and 2.4-D (0.001 mg l^{-1}) and CP (Dumas de Valux et al., 1981) supplemented with Kn (0.01 mg l^{-1}) and 2.4-D (0.01 mg l^{-1}).

A different number of anthers from the genotypes under study were cultured on different media under thermal treatments. The anthers cultivated on MS and N media with the supplementary hormones were incubated for 7 days at 25 ± 2 °C in the dark, and then transferred to 25 ± 2 °C with 12 h photoperiod. The anthers cultivated on LS and NN media with the supplementary hormones were incubated for 7 days at 7 ± 2 °C in the dark, and then transferred to 25 ± 2 °C with 12 h photoperiod. The anthers cultivated on CP medium with the supplementary hormones were incubated for 8 days at 35 ± 2 °C in the dark, and then transferred to 25 ± 2 °C with 12 h photoperiod. After 12 days of induction on CP medium, the anthers were transferred each month onto fresh R_1 medium supplemented with Kn (0.01 mg l^{-1}) and simultaneously the perished anthers were removed.

The cultures were observed regularly and the data were recorded every week. The frequency of callus formation and the number of embryos per anther were recorded.

The androgenetic potential was determined from the percentage of embryogenetic anthers according to Mitykó et al. (1995) classification.

2.3. Data analysis

All data on callused anthers and the percentage of embryogenetic anthers were subjected to analysis of variance (ANOVA), and mean values were evaluated at the $p < 0.05$ level of significance using Duncan's Multiple Range Test.

3. Results

The anthers cultured on different media under thermal treatments responded either with callus formation without regeneration (Fig. 1) or with direct embryo formation (Fig. 2). There was considerable variation in the response of studied genotypes to different medium.

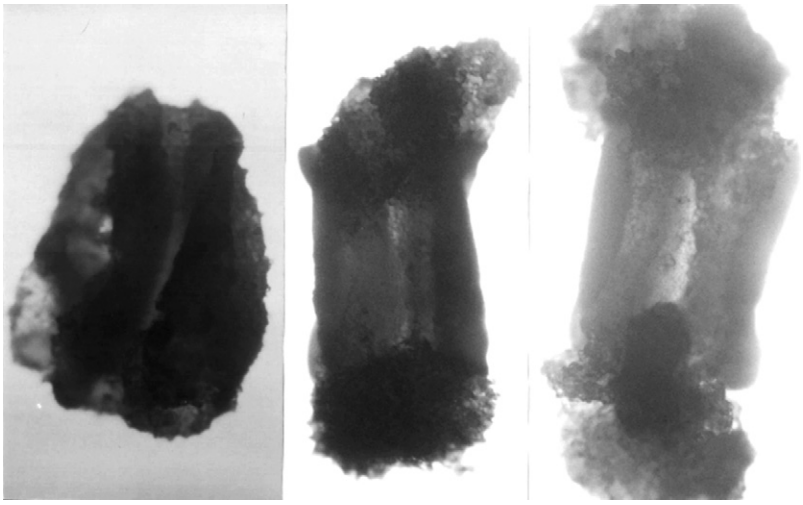

Fig. 1. Callus induction in anther culture of pepper (*Capsicum annuum* L.).

3.1. Effect of different genotypes of pepper, media and thermal pre-treatments on callus induction

The anthers from the pepper varieties Feferona, Slatko Luta, Vezena Luta, Sivrija, Zlaten Medal, Kurtovska Kapija, California Wonder, Rotund and Féherözön cultured on the MS, N, NN, LS and CP media with the supplementary hormones responded with callus induction (Table 1, Fig. 1).

The anthers of the genotype Feferona showed the highest callus induction when they were cultivated on N medium at 25 °C as compared to all the other genotypes. Feferona anthers did not respond when were they were cultivated on CP medium and pre-treated with a 35 °C heat shock. The anthers of Slatko Luta and Zlaten Medal responded with callus induction on all four media and pre-treatments, but the MS medium at 25 °C gave the highest callus induction, 48.90% and 14.90, respectively. California Wonder showed callus induction of 30.66% when the anthers were cultivated on N medium, at 25 °C, which is significantly different in comparison with the other media and thermal treatments. Rotund was the only

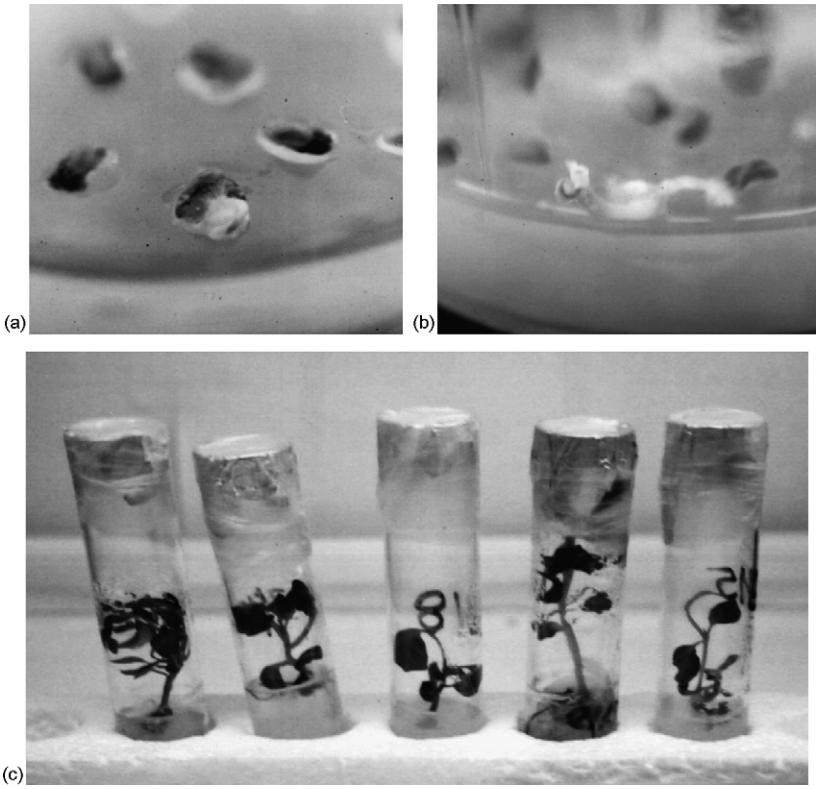

Fig. 2. Embryogenesis and plant regeneration in cultured anthers of pepper (*Capsicum annuum* L.): (a) direct embryo formation from cultured anthers of pepper; (b) microplant regenerant via anther culture of pepper; (c) pepper plantlets with leaves subcultured in growing media.

Table 1
Anther callus induction (%) of different pepper (*Capsicum annum* L.) genotypes when cultured on different media under cold and heat pre-treatments

Treatment	Genotypes								
	Feferona	Slatko Luta	Vezena Luta	Sivrija	Zlaten Medal	Kurtovska Kapija	California Wonder	Rotund	Féherözön
No. of induced anthers	396	428	360	405	297	352	429	331	301
MS, 25 °C	36.49 a	48.90 a	30.98 a	11.42 a	14.91 a	14.91 a	21.10 b	10.06 b	4.94 bc
N, 25 °C	58.88 a	34.00 b	9.84 b	14.42 a	9.47 bc	8.04 b	30.66 a	9.26 b	4.78 bc
LS, 7 °C	26.24 bc	34.40 b	33.33 a	18.03 a	6.66 c	15.22 a	13.67 b	17.35 a	13.44 a
NN, 7 °C	19.37 c	17.44 c	5.03 b	18.25 a	11.40 b	9.33 b	14.96 b	9.28 b	11.30 ab
CP, 35 °C	0.0 d	6.8 c	27.85 a	14.23 a	7.33 c	8.26 b	15.13 b	19.00 a	3.92 c

Each value is mean of three measurements. Mean within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test.

Table 2
Androgenic response of different pepper genotypes (*Capsicum annuum* L.) incubated on CP medium (+35 °C, in darkness)

Varieties	Number of anthers cultured	Embryogenic anthers (%)	Number of embryos per 100 anthers	Embryogenic response
Feferona	79	–	–	No
Slatko Luta	140	2.43 ± 0.20 b	3.33 ± 0.57 c	Poor
Vezena Luta	83	–	–	No
Sivrija	104	–	–	No
Zlaten Medal	94	3.31 ± 0.24 b	3.66 ± 0.57 c	Poor
Kurtovska Kapija	120	1.55 ± 0.50 b	2.66 ± 0.57 c	Poor
California Wonder	151	6.16 ± 0.28 b	5.66 ± 0.57 b	Fair
Rotund	109	–	–	No
Féherözön	130	33.66 ± 6.02 a	55.36 ± 1.00 a	Excellent

Each value is mean of three measurements. Mean within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test.

genotype that demonstrated the highest callus induction (19.00%) when the anthers were cultured on CP medium under a heat-shock treatment. The anthers of genotype Féherözön gave the lowest callus induction (3.92%), significantly different compared to the other media and the pre-treatments, when the anthers were cultivated on CP medium under a heat-shock treatment of 25 °C.

3.2. Effect of CP medium and heat-shock pre-treatment on embryo induction

Induction of haploid embryos was achieved only when anthers were cultured on a CP medium according to Dumas de Valux et al. method (1981). Moreover, the cultured anthers from the genotypes of Feferona, Vezena Luta, Sivrija and Rotund did not respond with embryogenesis (Table 2). On the other hand, the callus formation with this method is negligible in comparison with the other two induction treatments. The anthers of Feferona genotype, which is the hottest variety of all in the experiment, neither formed callus nor haploid embryos.

From the anthers of the nine genotypes cultured on a CP medium according to Dumas de Valux et al. method (1981), five showed embryo-formation ability: Slatko Luta—low androgenic potential, Zlaten Medal—low androgenic potential, Kurtovska Kapija—low androgenic potential, California Wonder—average androgenic potential and Féherözön—excellent androgenic potential (Table 2, Fig. 2). The evaluation of the androgenic response was performed according to Mitykó et al. (1995). The results showed that the highest percentage of embryogenic anthers gave the variety Féherözön (33.66%) which was significantly different compared to the other varieties. The analysis of the number of formed embryos per 100 anthers demonstrated that the Féherözön variety yielded the highest number of formed embryos (55.36), while Kurtovska Kapija (2.66) yielded the lowest. California Wonder showed an intermediated number of embryos compared to the others.

4. Discussion

There are several factors affecting androgenesis in many species, such as genotypes (Mitykó et al., 1995; Rodeva et al., 2004), growth of donor plants, pre-treatments of anthers (Ökum and Tripirdamaz, 2002; Koleva-Gudeva, 2003; Ashok Kumar et al., 2003), composition of medium (Irikova and Rodeva, 2004; Koleva-Gudeva and Spasenoski, 2001; Dolcet-Sanjuan et al., 1997) and the source of plant material (Kintzos et al., 2000). The response of anthers cultured on different media under certain thermal treatments can go in two directions, callus induction or embryo formation. The mechanism of cold- and heat-shock treatment for induction of somatic embryogenesis has been explored and discussed by many authors (Dolcet-Sanjuan et al., 1997; Dumas de Valux et al., 1981; Matsubara et al., 1998; Munyon et al., 1989). The studies on somatic embryogenesis of pepper (*C. annuum* L.) are in the domain of androgensis: George and Narayanaswamy (1973), Dumas de

Valux et al. (1981), Mitykó et al. (1995,1999), Dolcet-Sanjuan et al. (1997) and Irikova and Rodeva (2004). According to the literature, the heat thermal stress (+35 °C) has greater effect than the cold one (+7 °C) in the process of stimulation of macrospore division of pepper (Bárány et al., 2005; Kim et al., 2004). These findings are in agreement with the results obtained in the present study.

Thermal stress incubation pre-treatments have influence on the stimulation of the androgenic processes in pepper. The cold-shock pre-treatment, for LS and NN media, and the heat-shock pre-treatment, for the CP medium, significantly decreased the anthers callus formation compared to MS and N media, where the anthers were incubated at 25 °C.

The results regarding the process of embryo formation on different media under different thermal conditions showed that the formation of haploid embryos occurred only in the CP medium exposed to heat thermal stress (+35 °C), which is in concord with the findings of De Valux et al. (1981). However, Irikova and Rodeva (2004) reported no embryos formation for the same medium and cultivation conditions. They reported the callus formation was negligible.

From the nine different genotypes researched, only five showed the ability for embryo formation on a CP medium at 35 °C: Slatko Luta, Zlaten Medal, Kurtovska Kapija, California Wonder and Féherözön. Mitykó and Fáry (1997) concluded that bell-shape varieties have the highest androgenic ability, while the rest showed very low or no androgenic activity, which is consistent with our results, where the bell-shape varieties Féherözön and California Wonder showed a higher potential for embryogenesis compared to the hot and the sweet ones. The anthers of Féherözön variety showed low callus formation, but the androgenic ability was the highest on the same medium.

In general, once the callus was initiated, the induction of somatic embryos did not occur, which is similar with the results reported by Binzel et al. (1996).

5. Conclusion

Since pepper is a recalcitrant species, moderate results can be achieved in tissue culture. *In vitro* anther culture seems to be the only exception under these conditions (Mitykó and Fáry, 1997). Bell peppers were generally characterised by a fair (5.1–15.0%) or good (15.1–30.0) androgenic response (Mitykó and Fáry, 1997), which is also in concord with the results of this experiment. The results from this research are similar to those of Dumas de Valux et al. (1981), who reported five to more than 40% androgenetic response in large-fruited bell pepper cultivars. These observations lead to the conclusion that hot cultivars of the genus *Capsicum* are poor or non-responsive genotypes compared to sweet and bell cultivars.

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