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Effect of 2,4-dichlorophenoxyacetic acid pretreatment of *Capsicum* spp. donor plants on the anther culture efficiency of lines selected by capsaicinoid content

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Abstract

Androgenic low-responsive and non-responsive lines obtained as a result of the selection within the hybrid population of *Capsicum frutescens* L. × *C. annuum* L. were used for the determination of the pretreatment effect of donor plant with 2,4-dichlorophenoxyacetic acid (2,4-D) on the anther culture effectiveness. A significant increase in embryogenesis of the explants derived from pretreated plants showed an advantageous effect of 2,4-D application. The number of plantlets obtained was 1 to 18 for control and treated plant, respectively. About one half of the plants produced in the anther culture were diploids, characterized by 2C nuclear DNA content (1C/2C – 9/10). Callus tissues were mixoploid and comprised cells from 1C to 8C or from 2C to 16C of the nuclear DNA content. The microspores were the source of the first group of the callus tissue mentioned above. Spontaneous diploidization could be the reason for diploid plantlets regeneration from microspores. Obtaining regenerants in anther culture of a non-responsive hot line, in the case when donor plants were treated with 2,4-D, is the most interesting result of the experiment.

Key words: DNA content, haploid, diploid, callus, microspores

Introduction

The induced androgenesis allows for the rapid production of genetically stable recombinants being the original initial plant material for the breeding program of the innovative *Capsicum* spp. cultivars. Unfortunately, the pungent genotypes, the source of capsaicinoids, are recalcitrant in the androgenesis. The results of the investigation of the *in vitro* androgenesis of *Capsicum* spp. show genotype properties as particularly important for the effectiveness of the process. Sweet-fruited cultivars are a better source of the microspore-derived haploids. However, a great variation across genotypes occurs among these cultivars (Mityko et al., 1995; Regner, 1996; Rodeva et al., 2004; Dunwell 2010; Irikova et al., 2011). Genotype-dependent differences in organogenesis and somatic embryogenesis of pepper are also improved (Dabauza and Peña, 2001; Kaparakis and Alderson, 2008). In addition, the embryogenesis efficacy is highly influenced by the age of the donor plants (Ercan et al., 2006). Agronomic importance of *C. annuum* L. made this species the objective of experiments on induced androgenic embryogenesis in the *Capsicum* genus. The effective crossing between *C. frutescens* L. and *C. annuum* L. (Silva Monteiro et al., 2011) enables interspecific hybridization to be the source of a new genetic variation explored by androgenesis.

In the effective procedures of culture, a special role is played by 2,4-D as an androgenesis-inducing factor (Dumas de Vaulx et al., 1981; Supena et al., 2006). In the currently published modifications of the androgenic procedures, a special attention is paid to the improvement of media, their composition, and the treatment of explants (Koleva-Gudeva et al., 2007; Lantos et al., 2009). This growth regulator increases the fluency of polyembryony and haploid embryo frequency when plants are treated during florescence (Jędrzejczyk and Nowaczyk, 2009).

The crossing with highly effective forms is suggested to enhance the efficiency of androgenesis of cultivars without androgenic response. Unfortunately, creating new forms in this way causes undesirable changes in the plant genotype. In search of simple and low-cost androgenesis induction methods for non-responsive *Capsicum* spp., attention has been turned to plant material treatment before *in vitro* culture initiation.

The aim of the study was to evaluate the effect of the donor plant pretreatment with 2,4-D before the collection of flower buds, on the androgenic embryogenesis effectiveness in the *in vitro* anther culture of low-responsive and non-responsive lines selected from *C. frutescens* L. and *C. annuum* L. hybrids.

Material and methods

Besides agronomical values, for better characterization of the used plant material in this experiment, an analysis of capsaicinoid content was performed. Certain morphological and physiological properties of 30 fruits of each of the examined accessions were evaluated because of the plant material origin. As described by Collins et al. (1995), HPLC technique was applied for the analysis of capsaicinoid content in the whole fruit. Ground material samples (1.5 g) were separated into 50 ml glass tubes and submerged in 15 ml of acetonitrile. A Perkin Elmer Series 200 HPLC instrument equipped with Waters S5ODS2 4.6 × 100 mm column, autosampler, and PE Nelson Network Chromatography Interface NC 1900 was used. Standards of 8-methyl-N-vanillyl-6-nonenaminde (capsaicin) and 8-methyl-N-vanillyl-nonenaminde (dihydrocapsaicin) were provided by Sigma-Aldrich Co.

The anthers of 3 F7 lines, denoted 335, 342, and 345, derived through individual selection with progeny evaluation among interspecific *Capsicum frutescens* L. (female parent) and *C. annuum* L. hybrids, characterized by soft-flesh pericarp and capsaicinoid content, constituted the research material. Donor plants were grown in a plastic tunnel. Of about 20 individuals of each genotype were treated with 0.1 mg/l 2,4-dichlorophenoxyacetic acid water solution by spraying the whole plant about 12 h prior to bud collection. The buds' calyx and corolla were used and were similar in length, and most of the microspores were at the late-uninucleate stage.

Anther cultures of *C. annuum* L. were conducted in the way as described elsewhere (Dumas de Vaulx et al., 1981). The flower buds were surface-sterilized first by spraying with 70% ethanol and later by shaking in 5% solution of calcium hypochlorite (15 min). Next, they were rinsed thrice with sterile water. Anthers without filaments, isolated from two buds (on average 12 anthers), were placed on a Petri dish, with their inner parts facing the medium. For each genotype and treatment, an average of 75 Petri dishes (900 ± 10 anthers) was used. The anthers were cultured on a CP induction medium containing 0.01 mg/l 2,4-D and 0.01 mg/l KIN (kinetin). For the first 8 days, anther cultures were incubated in the darkness at 35 °C. Then, the dishes were exposed to a 12-h photoperiod at 25 °C. After 14 days, the anthers were transferred onto a R_1 regeneration medium (containing 0.1 mg/l KIN). In all the experiments, Gelrite (3 g/l) was used to solidify the media. The embryos that occurred in anther cultures were transferred onto V3 (Dumas de Vaulx et al., 1981) medium without growth regulators. Well-developed plants were then planted into the peat substrate and acclimatized in a plastic tunnel at increased air humidity.

The ploidy of plants, derived from anther culture and from callus, was assessed by the analysis of nuclear DNA content using flow cytometry. Samples were prepared following the Galbraith et al. (1983) procedure. The samples were analyzed with Partec CCA (Partec GmbH, Münster, Germany) flow cytometer equipped with a mercury lamp (High Pressure Lamp HBO-100W). The external standard used for cytometer calibration was a diploid plant of annual pepper *C. annuum* L. ($2n = 2x = 24$).

The results of donor plant evaluation and the effectiveness of anther cultures were the subject of statistical analyses. The values of the Least Significant Difference were established using Tukey's test at $p = 0.05$.

Results and discussion

When planning the experiment, an important methodology problem was the determination of 2,4-D concentration and application period. Considering the protective effect of corolla petals, which make the growth regulator penetration difficult, a ten-time higher 2, 4-D concentration was used as adequate for the induction medium in the experiment. In addition, it was assumed that providing the donor plants with the growth regulator a dozen-or-so-hours in advance (when compared to the explant collection time) will be justifiable in terms of the length of microsporogenesis and the applicability of buds for anther culture. In other words, it was assumed that disturbing the hormonal balance at the beginning of that process *in vivo* can enhance the effectiveness of androgenesis in *in vitro* culture.

The pungent, soft-flesh, and genetically stable forms used in the experiment, produced as a result of the se-

Table 1. Characteristics of donor plant fruit of *Capsicum* spp. lines

Feature	Line		
	335	342	345
Mean weight [g]	38.0c	15.3b	8.5a
Length [mm]	115b	70a	63a
Width [mm]	40b	24a	18a
Wall thickness [mm]	2.63b	2.20a	3.00b
Soluble solids [°Brix]	8.0a	7.4a	8.1a
Dry matter [%]	12.1b	9.0a	8.6a
Capsaicinoid content [$mg \cdot g^{-1}$]			
Capsaicin	0.46b	0.09a	1.25c
Dihydrokapsaicin	0.13a	0.08a	0.57b
Technological performance [%]	71c	63b	46a

Data denoted by the same letter for feature are not significantly different

Table 2. Effectiveness of callus and embryo formation in anther *in vitro* culture of *Capsicum* spp. lines according to 2,4-D donor plant treatment

Line and treatment	Callus		Embryos	Plants	Number of 1C/2Cplants
	Number per 100 anthers	DNA content	Number per 100 anthers		
335	3.66bc	2C – 16C	0.22a	0.22a	1/0
335 (2.4D)	4.67cd	1C – 8C	0.89b	0.67b	3/3
342	0.03a	1C – 8C	0.0a	0.0a	0
342 (2.4D)	1.22ab	2C – 16C	0.67b	0.67b	1/5
345	4.11c	2C – 16C	0.22a	0.0a	0
345 (2.4D)	6.67d	2C – 16C	0.67b	0.67b	4/2

Data denoted by the same letter for feature are not significantly different

lection within interspecific hybrid population, differed in their morphology and physiology (Table 1). As for the technological characteristics, special attention was paid to the capsaicinoid content, as it is common knowledge that pungent pepper genotypes are very low androgenic-responsive. The created genotypes are initial material suitable for the next breeding step, and from this perspective, their androgenic response should be further evaluated.

The effectiveness of anther culture was low for the studied genotypes, mostly due to the genetic properties of plant material (Table 2). In total, the number of 19 acclimatized regenerants obtained by us, and 15 derived by Lantos et al. (2009) from microspore culture of 6 hot pepper cultivars, seems to be similar. Depending on the cultivar, the mean number of plants per Petri dish in the experiment ranged from 0% to 1.25%. Koleva-Gudeva et al. (2007) presented the results of the embryogenesis induction on the CP medium similar to one that was used in the present experiment and reported androgenic effectiveness ranging from 0% to 55% (measured as a ratio between the number of embryos and the number of anthers). There were no embryos produced when a pungent donor plant was used. Similar results were recorded by Mityko and Fari (1997) in the experiment with "Serrano" chili pepper. The same physiological pepper type was also included in the present research. Investigating individual reactions of F2 plants of inter- and intra-specific hybrids within the *Capsicum* genus emphasize the effect of the particular genotype on andro-

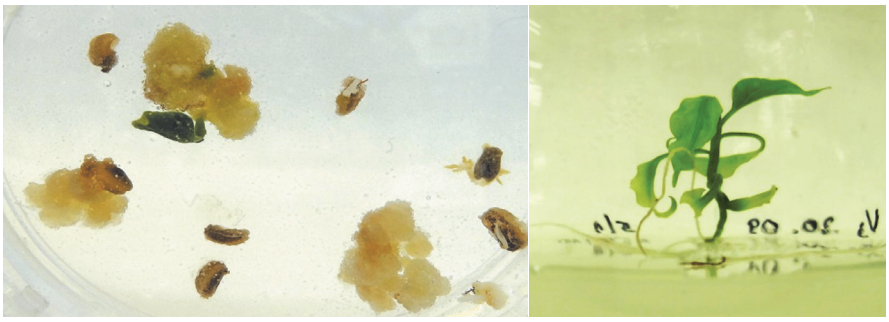

Fig. 1. Callus papules on anthers (R_1 medium) and plantlet (V3 medium) of *Capsicum* spp.

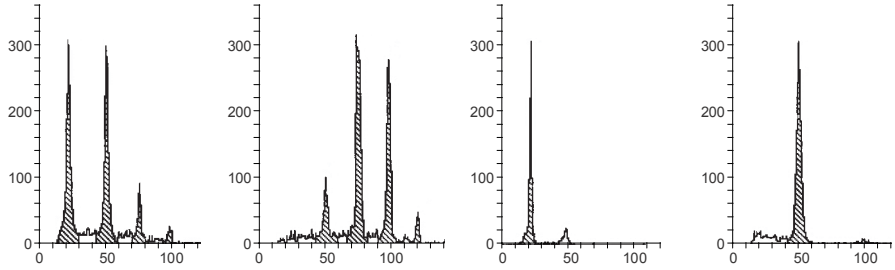

Fig. 2. Histograms of *Capsicum* spp. DNA content in callus cells of 335 line: 1C – 8C (first), 2C – 16C (second); in plantlets cells of 345 line: 1C (third), 2C (fourth)

genesis induction (Nowaczyk et al., 2009). Among other factors that differentiate the androgenic response, the type of explant appears to be important. The high androgenic response can be obtained by shed-microspore cultures. This was confirmed in the studies by Supena et al. (2006) and Kim et al. (2008).

For each of the genotypes studied here, it was noted that explants from the treated donor plants demonstrated a higher androgenic response than untreated ones, suggesting an advantageous impact of 2,4-D. This applies to both callus tissue and androgenic structures. As an effect of the pretreatment of anthers *in vivo*, the number of embryos in anther culture *in vitro* increased. However, the results should be treated with caution before those tendencies are confirmed using a greater number of genotypes and applying different plant treatments.

The conversion of an embryo to a plant is the crucial point of embryogenesis. In the experiment described by Supena et al. (2006), an effective conversion was only observed in some of the embryos. In their latest work on refinement of shed-microspore culture protocol for hot *Capsicum annuum* L., Supena and Custers (2011) reported a high percentage of "regular-looking" embryos. Unfortunately, in the experiment, only one genotype was used, and there is no information about the effectiveness of the embryo conversion. A considerable conversion success in the present experiments seems promising, however, limited by the number of accessions under investigation (Table 2, Fig. 1). The flow cytometry analysis showed the presence of nuclei with 1C or 2C DNA (Fig. 2). The number of diploids was surprisingly high and made the classification of the embryogenesis type difficult. The origin of the somatic tissue of diploid plantlets cannot be excluded. However, on the contrary, a spontaneous diploidization of androgenic embryo is also possible. A polyploidization was noted in the callus tissue. Histograms show a high diversification of 335 line DNA content. Similarly, a spontaneous diploidization was observed by Lantos et al. (2009) in microspore cultures. There were regenerated 15 diploid plants, and only three of them were produced as a result of the application of colchicine. The others, accounting for 80% of the population of regenerates, were spontaneous diploids of androgenic origin. Parra-Vega et al. (2013) confirmed a gametophytic origin of all tested embryos derived from *Capsicum annuum* L. anther culture.

The histogram (Fig. 2) demonstrating the presence of nuclei with 1C-8C DNA content in callus suggests a microspore origin of the tissue. A spontaneous polyploidization is the result of the creation of the cells with

a higher DNA content. A similar situation was typical for callus developed from the somatic cells of the anther wall, and in this case, the DNA content ranged from 2C to 16C. The chromosome number doubling in *Capsicum annuum* L. microspores *in vitro* culture described by Lantos et al. (2009) confirms the androgenic origin of diploids. Evaluating the results, one shall consider the simplicity and low cost of the explant pretreatment. The effects call for further experiments on 2,4-D concentration and the time of plant pretreatment.

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