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SHORT REPORT

Androgenic response of *Capsicum* interspecific hybrids and capsaicinoid characteristics of DH lines

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Summary

In the research on induced androgenesis, eight groups of plants, the progeny of interspecific hybrid of *Capsicum frutescens* L. x *C. annuum* L., were used. Half of them were standard hard-flesh forms, while the others have represented *SS or Ss* genotype conditioning the soft flesh of ripe fruit. Anthers from the plants of six groups produced mixoploid callus and the DNA content ranged from 1C to 16C. Three groups formed embryos which converted into plants. The total number of regenerants was 19, included both androgenic haploids (13) and diploids (6). The results did not allow to present the simple relationship between the physiological feature of soft-flesh and the effectiveness of androgenesis. Pungent, soft-flesh genotypes appeared as a poor responsive in anther culture. The R_2 and R_3 generation of two soft-flesh diploids, evaluated in two-year experiments, showed full phenotypic uniformity, proofing the androgenic origin of diploids. These DH lines were different with regard to capsaicinoid profiles. The content of capsaicinoids ranged from 0.056 to 2.170 and from 0.019 to 1.610 $g \cdot kg^{-1}$ for capsaicin and dihydrocapsaicin, respectively. The highest concentration of the compounds was observed in the placenta. Although the androgenic effectiveness was not fully satisfactory, the doubled haploid technology can be used for the rapid genetic stabilization of soft-flesh *Capsicum* spp. recombinants and the special attention should be paid to the spontaneous androdiploids.

Key words: *Capsicum spp., soft-flesh, androgenesis, capsaicinoid, DH line*

INTRODUCTION

Capsicum frutescens L. and *C. annuum* L. are elements of the common gene pool and when crossed one with other, give fertile progeny [1]. This can be an opportunity to broaden the genetic variation by interspecific hybridization. C*apsicum annuum* L., a species with a large number of cultivars, is a source of genes conditioning high yield. *C. frutescens* L. includes forms with a wide variation in fruit shape and capsaicinoids content. Another important feature is a softening of the ripe fruit flesh. Offspring obtained from interspecific hybrids combining the abovementioned properties can offer interesting raw material for the production of nutraceutics [2]. An efficient method facilitating the fast genetic stabilization of hybrid recombinants is the creation of DH lines. The lines are based on haploids most often obtained as a result of induced androgenesis. Effective procedures of anther cultures *in vitro* for standard, hard-flesh *C. annuum* L. genotypes have been developed [3]. The results reported by Supena *et al*. [4] indicate the high effectiveness of two-layer cultures of the shed-microspore type in hot Indonesian cultivars of *C. annuum* L. Highly effective procedures are described for embryogenesis and plant regeneration through isolated microspore for the same species represented by hot cultivar Milyocng – iare (*C. annuum* L.), by Kim *et al*. [5]. They were able to obtain at least four plants for every 8 x 10^4 – 10 x 10^4 microspores cultured, approximately the number of microspores from one flower bud. However, attention should be paid to high specific genotype reaction in regard of androgenic response. In experiments on the improvement of isolated microspore culture carried out by Lantos *et al*. [6] half of six cultivars gave androgenic plantlets only. A different reaction of some *C. frutescens* L. forms is also known [7], however, but no experimental data are available for genotypes being the hybrid progeny of the abovementioned species. The aim of the present research was an attempt to define the applicability of the soft-flesh hybrids obtained as a result of the *C. frutescens* L. and *C. annuum* L. crossing to haploid induction in *in vitro* anther culture as well as the evaluation of capsaicinoid profile of DH lines, the practical effect of investigation.

MATERIAL AND METHODS

The anthers of ten plants of each of six groups of hybrids derived from interspecific crossing of *Capsicum frutescens* L. x *C. annuum* L were the research material in *in vitro* culture. The maternal form of the hybrid was a small-fruited, soft-flesh heterozygotic genotype. A registered ATM1 line served as a pollinator, the initial form of commercial hybrids of an average fruit weight exceeding 200 g. The plants of groups marked 3/21HF, 3/23HF, 2/21HF, 2/24HF, 3/3SF and 3/6SF were phenotypic uniform within each of population. Two groups: 3/18SF and 1/46SF comprised individuals different in regard to plant habit, fruit weight and shape. The letters HF

and SF mean hard or soft of the mature fruit pericarp. Among plants obtained in the anther culture (R_1 generation), the attention has been paid to diploids of 3/18 and 1/46 due to their fruit differences as compared with the donor plant. The fruit of the next generations (R_2 and R_3 DH lines) of mentioned plants were under evaluation of capsaicinoid profile. The donor plants as well as DH lines were grown under plastic film greenhouse. The conditions of culture, fertilization and irrigation were typical for commercial pepper production. Evaluated material is a part of *Capsicum* spp. plant collection maintained in the Department of Plant Genetics, Physiology and Biotechnology, University of Technology and Life Sciences, Bydgoszcz, Poland.

Anther culture was conduced following the method described by Chambonnet [3] for *C. annuum* L. The applicability of plants to induce androgenic haploids was evaluated in two periods. In the summer cycle, starting with the anther display of June 24 through to July 29, all the genotypes were used. In autumn (September 20–25) the research was limited to three groups, one hard-flesh and one soft-flesh which gave an androgenic response in the summer experiment were selected. Additionally a group characterized with abounding florescence occurring in September, was also introduced. The selected flower buds had similar length of sepals and petals. This morphological characteristic of *C. annuum* L. [8] shows that the major part of microspores were in the late uninucleated phase. Microscope observation of anther slides, stained with actetic-carmine, of our plant material confirmed the mentioned criterion for interspecific hybrids. Flower buds were sprayed with 70% ethanol, then surface-sterilized, shaken for 15 min in the 5% solution of calcium hypochlorite, and subsequently, rinsed three times with sterile water. Anthers isolated from two buds were placed on one Petri dish, with their inner part facing the medium. The anthers were cultured on the CP medium containing 0.01 mg·dm^{-3} 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.01 mg dm^{-3}·KIN (kinetin). For the first 8 days, anther cultures were incubated in the darkness at the temperature of 35°C. Then the dishes were exposed to 12-hour photoperiod, at the temperature of 25°C. After 14 days, anthers were transferred onto R1 medium (0.1 mg dm^{-3}·KIN). In all the performed experiments, Gelrite (3 g·dm^{-3}) was used to solidify the media. The embryos occurring in anther cultures were transferred onto the V3 medium without growth regulators. Well developed plants were then planted into the peat substrate and acclimatized in the glasshouse in increased air humidity condition.

The ploidy of the plants obtained in anther cultures as well as callus was determined with the use of flow cytometry, based on the measurements of DNA content in the cells analyzed. The samples for analysis were prepared following Galbraith *et al*. [9] procedure. Plant material was chopped with the razor blade in 1 ml of buffer isolating cell nuclei (0.1 M Tris, 2.5 mM MgCl2 × 6H2O, 85 mM NaCl, 0.1% Triton X-100; pH=7.0), containing DAPI (2 μg·ml^{-1}). The samples were filtered through the 30 μm nylon mesh to remove debris, and then analyzed with the Partec CCA flow cytometer (Partec GmbH, Münster, Germany), equipped with the mercury lamp (High Pressure Lamp HBO-100W). In each sample at least 5000 cell nuclei were analyzed, at the flow ratio was 20 nuclei·s^{-1}. The external standard

used for the measurements was the diploid plant of annual pepper *C. annuum* L. (2n = 2x = 24). The results were collected in a form of histograms and subsequently were analyzed with Partec DPAC V.2.2 software.

The subjects of capsaicinoid analysis were the whole, ripe fruit, pericarp, placenta as well as puree made as a result of mechanical separation of the soft tissue from inedible fruit parts. After washing a few fruit were taken at random from every sample. The placenta with seeds and septa were removed from them. Then pericarp was dried at a temperature of 60°C for 3 days. The remaining fruit in every sample were broken up and rubbed through a sieve. In this way a uniform puree was obtained and the share of product in raw weight means the technological performance. The puree in thin layers was dried in Petri dishes in conditions identical to those of abovementioned samples. In the studies, the content of capsaicin and dihydrocapsaicin were determined. For this aim, the HPLC technique was employed as it was presented by Collins *et al*. [10], with a modification involving the preparation of the samples for soft-flesh material. Every sample was subjected to three HPLC analyses. In order to extract capsaicinoids, ground samples of 1.5 g poured over with 15 ml of acetonitrile were placed in 50 ml glass bottles with teflon-lined lids. The bottles were capped and placed in an 80°C water bath for 4 h and stirred manually every hour. Samples were cooled to room temperature. About 3 ml of supernatant were extracted and filtered (0.45 μm Waters Millex – HN filter unit on a 5-ml disposable syringe) into a glass vial, capped and stored at 5°C, until analysed. A 10 μl aliquot was used for each HPLC injection. Determinations were made with the use of Perkin Elmer, Series 200 HPLC device equipped with an autosampler system and PE Nelson Network Chromatography Interface NC 1900. The amount of capsaicin and dihydrocapsaicin was done by isocratic flow of the analysed solution through the column (Waters S50DS2 4.6´100 mm column) at the rate of 1 ml·min^{-1} for the period of 7 min using a detector set with excitation at 280 nm. The mobile phase was isocratic, with 70% solvent A (100% methanol) and 30% solvent B (10% methanol in water, by volume). Standards of 8-methyl-N-vanillyl-6-nonenamide (capsaicin) and N-vanillylnonanamide (dihydrocapsaicin) were obtained from Sigma-Aldrich. Standard solutions of 1, 0.5, 0.1, 0.05, 0.025, 0.01, 0.005 and 0.001 g·kg^{-1} were prepared in 100% methanol by dilution of a 2 g·kg^{-1} stock solution. Results obtained during experiments were subjected to statistical analysis and are given as two-year means. The data marked by the same letter are not significantly different.

RESULTS AND DISCUSSION

During anther culture, some of the genotypes developed a callus which was exposed to cytometric analysis to determine the amount of DNA in the cell nuclei. All plants representing the soft-flesh groups formed the callus in the summer cycle. A similar reaction was noted in two hard-flesh forms. In the autumn cycle the callus was formed by the anthers of plants of two groups (tab. 1). The callus

of respective genotypes differed in their morphological characteristics. In each soft-flesh form, on average, half of the anthers formed the callus. The largest papules of this tissue were a rare green in colour and showed a compact structure. The content of DNA was particularly interesting and the presence of 1C level cell nuclei was observed (fig. 1). The number of nuclei with the content of 1C DNA in the samples was about four times lower than in those in which 2C or 4C DNA was recorded. The cytometric analysis of the other callus types demonstrated the presence of cells with a higher DNA level. As given in Table 1 all the calluses were mixoploid. For the abovementioned haploid tissue, the source of 1C cells a microspore must have been, while the parts of callus with higher ploidy level were a result of a spontaneous polyploidization, as seen in the callus formed from somatic cells of the anther. A varied reaction of callus formation of the different genotypes was observed earlier in *C. annuum* L. [11, 12]. The practical applicability of tissue depends on its embryogenic potential. Unfortunately, none of the callus kind we obtained were embryogenic under the conditions of the culture. During of the prolonged culture it was dying. In this situation it seems reasonable to attempt to modify further callus culture stages, especially for tissues identified by the presence of the haploid cells. Four out of eight plant groups gave an androgenic response (tab. 1). As a result of embryogenesis a limited number of embryos and plants were observed. The effectiveness of the process was low and only in genotype 2/24 did it exceed the level of 1%, implied as a share of the plants obtained as compared with number of anthers cultured. Despite the lower than satisfactory effectiveness of the procedure this form is distinguished by the positive androgenic reaction during the autumn cycle. According to Ercan *et al*. [13], since 4-month-old donor plants gave the highest embryo yield and it is possible to say that anthers collected from old plants have sufficient embryogenic response when optimum developmental stage is selected. Our autumn cultures were initiated from anthers of plants at the final stage of their vegetation. A definitely higher effectiveness of two-layer cultures of the shed-microspore type was described for *Capsicum annuum* L. by Supena *et al*. [14]. It must be stressed that only 20% of the embryos obtained were converted into normal plants and the results concern a specific Indonesian *C. annuum* L. pepper cultivar. Nearly 70% of plants being the result of embryos conversion were haploids. The ploidy level was defined on a base of 1C DNA content (fig. 1) and its androgenic origin cannot be doubted. The origin of diploid plants may have been similar and it was assumed that the formation of diploid embryos was preceded by the number of chromosomes doubling. Such a suggestion is also justifiable by callus observations: the presence of cells of a varied ploidy level. This means that the culture conditions used enhanced the process of spontaneous diploidization. In addition, the report of Morrison *et al*. [15] on androgenic diploids formation *in vitro* of *C. annuum* L. and *C. chinense* Jacq. hybrids seems to confirm the suggestion. Weighty proof of haploid embryo spontaneous diploidization gave the results of Lantos *et al*. (2009)

investigation. In isolated microspore cultures of six annual pepper (*Capsicum annuum* L.) cultivars, 12 well-developed spontaneous diploids and the three diploid (after colchicine treatment) plantlets were gained. By the way the number of regenerants in the quoted experiment was similar to those that we obtained. The number of the tested anthers from each genotype facilitates the definition of the preliminary conclusion concerning the effect of genetic factors on the androgenic response and the effectiveness of androgenesis in progeny originated from an interspecific hybrid. The results of a lot of research suggest the important relationship between the donor plant genotype and the effectiveness of androgenesis [6, 16, 17]. The differences of evaluated genotypes concerned not only the fruit morphology but also the physiological character, namely the ripe pericarp texture. Due to its monogenic inheritance as the activity of polygalacturonase [18], the potential existence of such a relationship should be easy to identify. However, the results do not allow the relationships to be demonstrated between the androgenic response and the allelic form of the gene responsible for the structure of the pericarp tissue. A similarly simple way of inheritance is characteristic for the pungency of the fruit conditioned by capsaicinoids. In this case a relationship was found between the physiological character and the androgenic response. The research on hot and sweet genotypes of *C. annuum* L. [12] led to the conclusion that hot cultivars of the genus *Capsicum* are poor or non-responsive genotypes compared to sweet and bell cultivars. Therefore, the presented investigation confirmed that the problems in induction of androgenesis are not characteristic onlyfor standard hard-flesh genotypes. The results of other research [19] give the new view on the problem. The specific reaction of the individuals within the heterozygotic population let to select the genotype with satisfactory androgenic response.

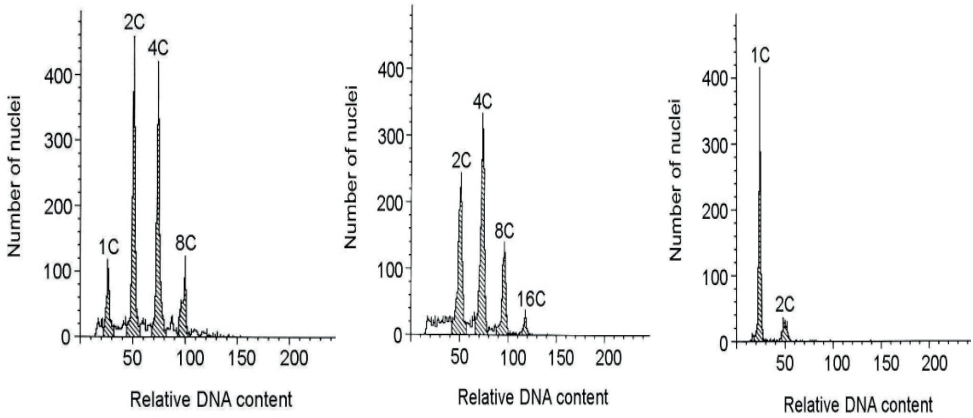

Figure 1.

Histograms of DNA content in the nuclei of green callus (the first), other callus (the second) and in haploid plantlet (the third)

Table 1.

Androgenic response of hybrids derived from interspecific crossing of *Capsicum frutescens* L. and *C. annuum* L.

Group of plants	Number of anthers displayed	Callus DNA content [C]	Number of plantlets and DNA content [C]
Summer cycle			
3/21 (HF)	500	2,4,8,16	0
3/23 (HF)	500	0	0
2/21 (HF)	500	0	4-1C; 2-2C
2/24 (HF)	500	2,4,8,16	4-1C; 2-2C
3/18 (SF)	600	2,4,8,16	1-1C; 1-2C
1/46 (SF)	600	1,2,4,8,16	1-1C; 1-2C
3/3 (SF)	600	2,4,8,16	0
3/6 (SF)	600	2,4,8,16	0
Autumn cycle			
2/24 (HF)	1000	0	2-1C
1/46 (SF)	400	1,2,4,8,16	0
3/6 (SF)	400	2,4,8,16	1-1C

HF – hard-flesh, SF – soft-flesh: 0 – callus and plantlet not observed

The progeny of two diploid plants marked 1/46/1 and 3/18/1 were evaluated in the further years as a generation R_2 and R_3. General characteristics of DH lines fruit is presented in table 2. Full phenotype uniformity, indicated here by low level of standard deviation, of observed populations confirms the androgenic origin of diploid plants obtained in *in vitro* culture. The statistical differences between DH lines of mean fruit, weight and technological performance were observed. Analysis of capsaicinoids content showed the great difference between the lines (tab. 3). In each of analyzed fruit part capsaicin and dihydrocapsaicin content were many times higher in 3/18/1 line than in the second one. The same observation concerned the puree obtained as a result of the mechanical separation of soft pericarp tissue from placenta, seed and skin. For comparison, the capsaicinoids content in whole fruit of very pungent Habanero' variety reached a level of about 12 $g \cdot kg^{-1}$ [20] and in the placenta [21] was higher than 60 $g \cdot kg^{-1}$. In experiments quoted above and in work of Ayuso *et al*. [22] as well as in the material under investigation, capsaicin content was higher than dihydrocapsaicin. From the practical point of view the capsaicinoids concentration should be low because of its irritant properties. Therefore, taking into account the abovementioned feature and also a technological performance, the fruit of 1/46/1 line may be recognized as an interesting raw for the nutraceutics or biologically active food production. The results

obtained in the frame of project on genetic improvement of plant raw material for nutraceutics production led to conclude that spontaneous androgenic diploids, derived without colchicine treatment, from *in vitro* anther culture appeared as the source of *Capsicum* spp. DH soft-flesh lines different with regard to capsaicinoid profile. From this point of view the induced androgenesis may be recognized as a method for the rapid genetic stabilization within the material of interspecific, soft-flesh hybrids.

Table 2.

Fruit characteristics of *Capsicum* spp. soft-flesh DH lines

Feature	DH lines	
	1/46/1	3/18/1
Mean weight [g]	12.5a±0.3	9.8b±0.2
Wall thickness [mm]	2.35a±0.15	2.45a±0.14
Dry matter content [%]	10.2a±0.4	9.90 a±0.3
Technological performance [%]	61a±2	57b±2

Data within each feature with the same letter are not significantly different at p=95%.

Table 3.

Capsaicin (CAP) and dihydrocapsaicin (DHC) content [g·kg^{-1}] in fruit and puree of soft-flesh DH lines *Capsicum* spp.

Analyzed material	DH lines					
	1/46/1			3/18/1		
	CAP	DHC	CAP+DHC	CAP	DHC	CAP+DHC
Whole fruit	0.099 b	0.045 b	0.144 b	1.010 b	0.910 b	1.920 b
Pericarp	0.056 a	0.019 a	0.075 a	0.215 a	0.180 a	0.395 a
Placenta	0.138 c	0.118 c	0.256 c	2.170 c	1.610 c	3.880 c
Puree	0.096 b	0.051 b	0.147 b	1.245 b	1.000 b	2.245 b

Data within each column with the same letters are not significantly different at p=95%

CONCLUSION

Although the androgenic effectiveness was not fully satisfactory the doubled haploid technology can be used for rapid genetic stabilization of soft-flesh *Capsicum* spp. Recombinants. Special attention should be paid for the spontaneous androdiploids.

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ODPOWIEDŹ ANDROGENICZNA MIESZAŃCÓW MIĘDZYGATUNKOWYCH *CAPSICUM* I CHARAKTERYSTYKA KAPSAICYNOIDOWA LINII DH

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S t r e s z c z e n i e

W badaniach nad indukowaną androgenezą wykorzystano osiem grup roślin będących potomstwem mieszańców międzygatunkowych *Capsicum frutescens* L. x *C. annuum* L. Połowa z nich była standardowymi formami twardoowocowymi, podczas gdy pozostałe reprezentowały fenotyp miękkiego miąższu warunkowany przez dominujący allel *S*. Pylniki z sześciu grup roślin wytwarzały miksoploidalny kalus, a zawartość DNA wahała się w zakresie od 1C do 16C. Trzy grupy formowały zarodki, które przeszły skuteczną konwersję w rośliny. Ogólna liczba regeneratów wyniosła 19, wliczając w to zarówno haploidy (13), jak też diploidy (6). Uzyskane rezultaty nie pozwoliły na zaprezentowanie zależności między fizjologiczną cechą miękkiego miąższu a efektywnością androgenezy. Ostre formy typu soft-flesh okazały się mało wydajne w kulturze pylników. Pokolenia R_2 i R_3 dwóch diploidów soft-flesh, ocenione w dwuletnich badaniach wykazały całkowite wyrównanie fenotypowe, potwierdzając tym androgeniczne pochodzenie. Pokolenia te były zróżnicowane pod względem profili kapsaicynoidowych, a zawartość wymienionych metabolitów wahała się między 0,056–2,170 a 0,019–1,610 $g \cdot kg^{-1}$ odpowiednio dla kapsaicyny i dihydrokapsaicyny, przy czym największą koncentrację stwierdzono w łożysku. Chociaż obserwowana efektywność androgenezy nie była w pełni satysfakcjonująca, to technologia podwojonych haploidów może być wykorzystywana do szybkiej stabilizacji genetycznej rekombinantów soft-flesh *Capsicum* spp. Szczególną uwagę należy poświęcić spontanicznym androdiploidom.

Słowa kluczowe: *Capsicum spp., soft-flesh, androgeneza, kapsaicynoidy, linia DH*