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DOUBLE HAPLOIDS FROM TWO-EMBRYONIC SEEDS OF PEPPER (*Capsicum annuum* L.)F_1 HYBRID

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ABSTRACT

The results of the investigation proved the gametophytic origin of diploids derived from two-embryonic seeds of pepper *C. annuum* L. hybrid. During the germination of seeds harvested from the red fruited hybrid (*C. annuum* L. ATZ × *C. annuum* L. 'Sono') F_1, additional embryos have been found. Four of diploid twins F_2 generation were different in their phenotype within the pair and/or from the F_1 mother plant. Plants of three pairs: 1A–1B, 2A–2B, 4A–4B were significantly different with regard to the average fruit weight, length and seeds number. Yellow colour of ripe fruit was characteristic for 2A, 3A and 3B plants. In RAPD molecular analysis, twenty three primers were used and six of them enabled polymorphic products to be obtained in reactions. The results of the analysis confirmed phenotypic differentiation of the twins and their parental forms. The phenotypic and molecular analyses proved that spontaneous diploids from a two-embryonic seeds are ready for the production of genetically stable, sexual progeny.

Key words: polyembryony, twin plants, spontaneous diploidisation, phenotypic marker, RAPD analysis

INTRODUCTION

Double-haploids are fully homozygous organisms created as a result of doubling the number of chromosomes. They are a very important initial material in crop improvement and have also been a key feature in establishing chromosome maps for a range of species [Forster and Thomas 2005]. Haploid embryos can be produced by the hybridisation of distantly related species, as a result of chromosome elimination and by *in vitro* or *in vivo* parthenogenesis or androgenesis [Dunwell 2010]. For the genus *Capsicum*, this is the method most commonly used [Forster et al. 2007, Kothari et al., 2010, Germana 2011, Irikova et al. 2011]. The conducted experiments indicate a number of factors determining the effectiveness of this phenomenon, with the genotype of donor plants being mentioned as a key element conditioning the induction and regeneration of androgenic embryos [Kristiansen and Andersen 1993, Dolcet-Sanjuan et al. 1997, Mityko and Fari 1997, Rodeva et al. 2004]. The selection of anthers containing uninucleate and early binucleate microspores is most recommended to improve the effectiveness of this phenomenon [Kim et al. 2004, Lantos et al. 2009]. For genotypes with low androgenesis efficiency, different media compositions and in vitro culture conditions are frequently suggested solutions [Supena and Custers 2011, Para-Vega et al. 2013]. In the case of lack of androgenic response, it is believed that physical or osmotic stress may be the appropriate factors to switch from microspore gametophyte to sporophyte embro stage [Dumas de Vaulks et al. 1981, Dolcet-Sanjuan et al. 1997]. The production of genetically-stable homozygous diploids starts with a haploid plant, which requires diploidisation. Doubling the genome number in haploids by colchicine treatment results in the production of stable diploids, but genetically-unstable mixoploids can also appear. Some of

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the colchicine-treated haploids remain unchanged, but many of them also die [Chauhan and Khurana 2011]. Chromosome mosaicism, chromosomal aberrations, single-gene mutations, DNA amplification and an altered relationship between the nucleus and the cytoplasm can also be observed [Rao et al. 1987, Nikolova and Niemirowicz-Szczytt 1996, Niemirowicz-Szczytt 1997]. Chauhan and Khurana [2011] showed that the frequency of chromosome-doubling following colchicine treatment equalled 48% and about half of the plants died after colchicine treatment. A lower effectiveness was observed in the experiment of *in vitro* colchicine using *Capsicum annuum* L. genotypes. The apical parts of 106 androgenic haploids were placed on the MS medium containing colchicine at the concentration equal 400 mg dm^{-3} for six days. The largest groups of regenerants were mixoploids (29–55%) and haploids (20–50%). For the studied genotypes, an effective diploidisation at the level of 17–27% was noted [Olszewska et al. 2015].

Spontaneous diploidisation should be understood as a process of chromosomes doubling without application of the c-mitotic chemical mutagens. Spontaneous diploids are usually free of disadvantages caused by the exposure of cells to colchicine and are ready to production of DHs lines. In anthers or microspores *in vitro* cultures the spontaneous androgenesis-derived diploids are often observed and their share in the regenerates population depends on donor plant genotype [Forster et al. 2007, Germana 2011, Irikova et al. 2011].

In the case of sexual plant reproduction *in vivo,* additional embryos may be established in a single ovule. The seeds developing as a result of such processes comprise two or more embryos and their presence can be detected during seed germination. Barcaccia and Albertini [2013] presented different types of apomixes which could be the source of additional embryos. Adventive polyembryony is regarded a polyembryonic seed source and is defined as the development of an embryo from diploid cells of somatic tissues. Among the agriculturally important species, adventitious embryony (i.e., sporophytic apomixis) has been noted in mango, several *Citrus* species, and orchids. In this case, the new organism is identical to the maternal plant with regard to phenotypic and genotypic traits. Cleavage polyembryony, resulting from the division of a zygote or a proembryo, gives identical organisms. The mentioned sources of polyembryony are not valuable for breeding purposes as they do not provide new genetic variation or are not genetically stable. In the investigations into polyembryony, attention has mainly been paid to haploids accompanying zygotic embryos in two-embryonic seed. Only haploid embryos or spontaneous doubled haploid embryos developed as twins in two-embryonic seed of heterozygous plant may present genetic originality [Naumova 1992].

Polyembryony is observed in species of numerous plant families of dicot and monocot groups. Dunwell [2010] showed many selected examples of twin pairs in which one plant was diploid and the other was haploid. In experiments on tomato [Nowaczyk and Nowaczyk 2006], the objects of research were diploid twins. Special attention was paid to plants which were different within the twin pair with regard to phenotypic markers such as the presence/absence of the so-called green back, and the type of growth, which can be indeterminate or determinate. The different markers within pairs or in relation to the maternal plant indicated the haploid origin of at least one diploid. In the case of genotypes of the genus *Capsicum*, poliembrionia is not a common phenomenon, moreover, its effectiveness depends on the predisposition of the species. For wild pepper genotypes, the frequency of two-embryonic seeds ranged from 0.08% for *C. baccatum* L. to 0.50% for *C. chinense* Jacq. The highest frequency of polyembryony (1.06%) has been observed for the 'Corno di toro' cultivar of *C. annuum* L. [Jedrzejczyk and Nowaczyk 2009].

The identification of diploid twins characterised by an original genotype in relation to the mother plant is important for practical purposes. The main aim of the investigation was analysis of the phenotypic differentiation of twins and confirmation of their DH character on the molecular level using RAPD technique.

MATERIALS AND METHODS

Origin of experimental material. Four pairs of peppers *C. annuum* L. plants, from the population of twins, were used in the experiment into the origin of diploid twin embryos. As a result of the cross-pollination of a red-fruited ATZ breeding line (the maternal form of commercial cultivar 'Stanola F_1') and

a yellow-fruited 'Sono' cultivar, red-fruited F_1 hybrids were created. The F_2 seeds harvested from the mentioned hybrids were the experimental material. During the germination process of six thousand seeds, seventeen seeds were found with additional embryos. The obtained seedlings were the object of ploidy verification. During their growth, four pairs of twins differentiated in their phenotype within the pair and/or from the F_1 plants, which were the source of two-embryonic seeds. The twin pairs under evaluation were marked as 1A *vs.* 1B, 2A *vs.* 2B, 3A *vs*. 3B and 4A *vs*. 4B.

Ploidy analysis of plants. The ploidy of the twin seedlings was determined with the use of flow cytometry, based on the measurements of DNA content in the analysed cells. The samples for analysis were prepared following the procedure reported by Galbraith et al. [1983]. Plant material was chopped with a razor blade in 1 ml of buffer isolating cell nuclei (0.1 M Tris, 2.5 mM $MgCl_2 \times 6H_2O$, 85 mM NaCl, 0.1% Triton X-100; pH = 7.0), containing DAPI (2 µg ml^{-1}). The samples were filtered through a 30 µm nylon mesh to remove debris, and then analysed with the Partec CCA flow cytometer (Partec GmbH, Münster, Germany), equipped with the mercury lamp (High Pressure Lamp HBO-100 W). In each sample, at least 5000 cell nuclei were analysed, at the flow rate of 20 nuclei s^{-1}. The external standard used for the measurements was the diploid plant of *C. annuum* L. (2n = 2x = 24). The results were collected in the form of histograms and were then analysed with Partec DPAC V.2.2 software.

Biometric analysis of fruit. The twins were grown in a plastic house. Three typical ripe fruit of each twin were the subject of biometric analysis. The fruit weight, length and seed number were evaluated. In order to compare the individual variation in plants within initial populations, ten plants of the ATZ line, ten 'Sono' cultivars and ten F_1 hybrids (ATZ × 'Sono') were cultured under the same conditions. Three plants of each mentioned genotype were randomly chosen and three typical ripe fruit were subjected to the evaluation shown above. The progeny of twins from F_3 populations was evaluated in the same way. The fruit of cv. Sono and twins 2A, 3A, and 3B were yellow, while the rest of the genotypes were red-fruited.

Molecular analysis and genetic distance evaluation. In the molecular analysis, young and fresh leaves of fully developed plants were used. The extraction of genomic DNA was performed from 100 mg of plant tissue using Sigma GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Germany). The obtained DNA solution was stored at –20°C until analysis. Twenty three RAPD primers were used in the study. The PCR reactions were carried out using the ATC401 Thermal Cycler in a 20 µl reaction volume containing 20ng of genomic DNA as a template, 20 mM $MgSO_4$, 0.25 µM of primer, 200 µM of each dNTP and 0.5 units of *Taq* polymerase (A&A Biotechnology, Poland). The temperature regime was based on the proposal of Ilbi [2003]. The initial denaturation was carried out at 91°C for 1 min, followed by 40 cycles consisting of 15 s at 91°C, 15 s at 42°C and 1 min and 10 s at 72°C. The final extension was done by 5 min at 72°C. The reactions were carried out twice. The separation of reaction products was made by electrophoresis in 1.8% agarose gel stained with ethidium bromide in TBE buffer, running at a constant voltage of 100V for 2 hours. For visualisation and photographing images, the Gel Doc 2000 (Bio-Rad Laboratories, UK) trans illuminator was used. Genetic distant within the twin pairs and between twins and mother plant was (singular or plural) set using the variation coefficient according to Nei and Li [1979].

RESULTS

Ploidy of twin plants. The ploidy of twins has been estimated during the first stage of seedling culture. Among the obtained plants, three types of ploidy were noted: haploid, diploid and mixploid (Fig. 1). Particular attention has been paid to diploid pairs. All of them reached full physiological maturity. Four pairs of diploid plants differing from one another within the pair and/or from F_1 parental hybrid were used in the next stage of investigation.

Phenotypic characteristics of twins. The mean fruit weight and length of the plants of twin pair 3A *vs.* 3B were the same from a statistical point of view (Fig. 2A, 2B). On the other hand, big differences between plants within twins 1A *vs.* 1B, 2A *vs.* 2B and 4A *vs.* 4B have been noted. Plants within the three pairs of twins were significantly different with regard to all traits under evaluation. In the case of pair 3A *vs.* 3B, differences that were significant from a statistical point of view were only concerned with the number of seeds

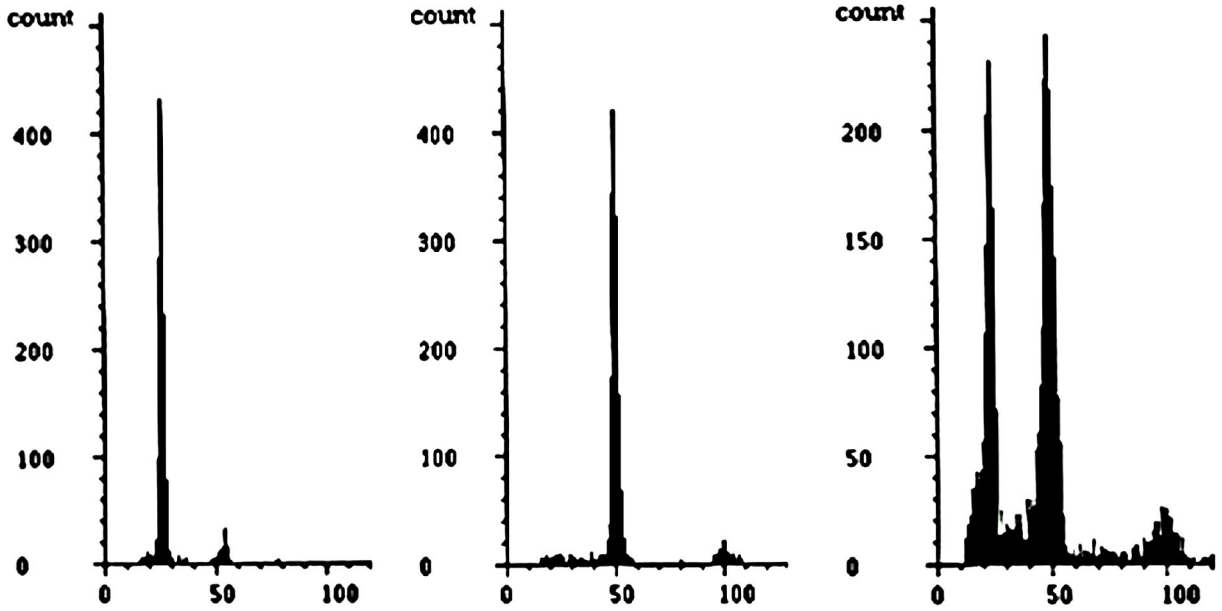

Fig. 1. Histograms with peaks of relative DNA content in cells of the twin plants of pepper *C. annuum* L.; first – 1C DNA (haploid), second – 2C DNA (diploid), third – 1C and 2C DNA (mixoploid)

in fruit (Fig. 2C). No statistical differences with regard to phenotypic traits were observed between plants within pairs of the ATZ line, 'Sono' cultivar or the (ATZ × 'Sono') F_1 hybrid. The progeny of twins were also uniform in their phenotype within F_3 population.

Molecular evaluation. Among the twenty three primers used in the experiment, the following have generated monomorphic products only: OP A02, OP A03, OP A04, OP A05, OP A06, OP A07, OP A08, OP A09, OP A10, OP A12, OP A18, OP A19, OP A20, OP 1A, OP AE11, OP AF07, OP AG01. In the PCR reaction with the remaining starters: OP A11, OP AE10, OP B10, OP E19, OP F05, OP PO09 thirteen polymorphic products were obtained (Table 1). The minimum and maximum percentage of observed polymorphism ranged from 15% to 44% for the OPB10 and OPP09 primers, respectively. The highest number of polymorphic products was created when the last of the primer mentioned above was used. Differences in product sizes were also noted (Fig. 3). Molecular analysis of each twin pair shows that the highest number of specific products was observed for plants marked 2A *vs.* 2B (Table 2). Four primers gave six different products for plants from this pair. In the next pair denoted as 4A *vs.* 4B, four different products from two primers were recorded. The plants from the last pair, 1A *vs.* 1B, were different with regard to three PCR products from three primers. Not a single specific product was generated for the yellow-fruited genotypes 3A *vs.* 3B and the genetic distant between plants of this pair equalled zero. The plants of each twin pair were molecularly different from the maternal F_1 genotype and the genetic distant coefficient ranged from 0.018–0.100.

DISCUSSION

Four pairs of pepper *C. annuum* L. twins derived from the F_1 hybrid were the objects of investigation. Additionally, the hybrid mentioned above and its parents were examined to prove the genetic originality of the twin plants. Each of these initial genotypes was represented by three randomly chosen plants. Flow cytometric analysis of DNA content showed that all twin plants were diploids.

The yellow colour of ripe fruit from three twin plants (2A, 3A and 3B) excluded their origin from sporophytic cells of the hybrids. The obtained results allowed to confirm the hypothesis that all of the twins developed from recombined haploid cells with the subsequent spontaneous diploidisation. The possibil-

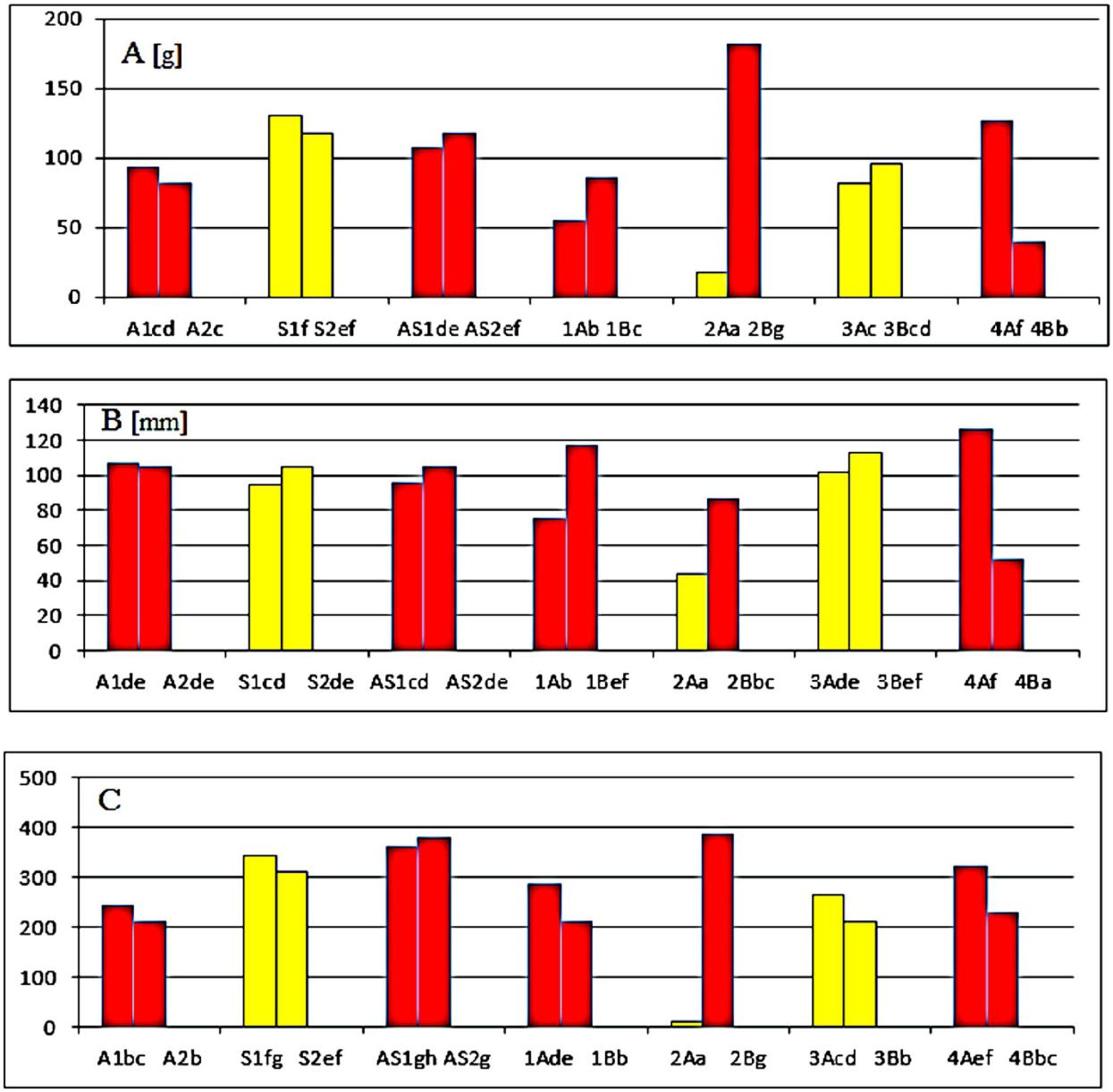

Fig. 2. Mean fruit weight (A), mean fruit length (B), mean number of seed per fruit (C) of two plants of parental forms (A, S), F1 hybrid (AS) and four pairs of twin (1A *vs.* 1B – 4A *vs.* 4B) of pepper *C. annuum* L.; the same little letters by plant symbols show the lack of significant differences (P = 95%); diagram colour like as ripe fruit colour

ity of this method of regeneration is confirmed by the observations of Forester and co-authors [2007].

The marker phenotypic trait of fruit colour is the definitive evidence of the gametic origin of yellow-fruited plants. The twins 3A and 3B were statistically identical with regard to the measured traits, such as fruit weight, length and seed number per fruit. The lack of polymorphic products of the PCR reactions showed the molecular identity of the plants in question. The origin of these twins was probably the result of the zygotic embryogenesis of recombined haploid cells, and the division of diploid proembryo. This method of

Table 1. Effectiveness of RAPD primers in generating the polymorphic products in molecular analysis of pepper *C. annuum* L. twin pairs

Primer	Number of generated products	Number of polymorphic products	Percentage of polymorphism
OPA11	7	2	29
OPAE10	6	2	33
OPB10	13	2	15
OPE19	6	1	17
OPF05	7	2	29
OPP09	9	4	44

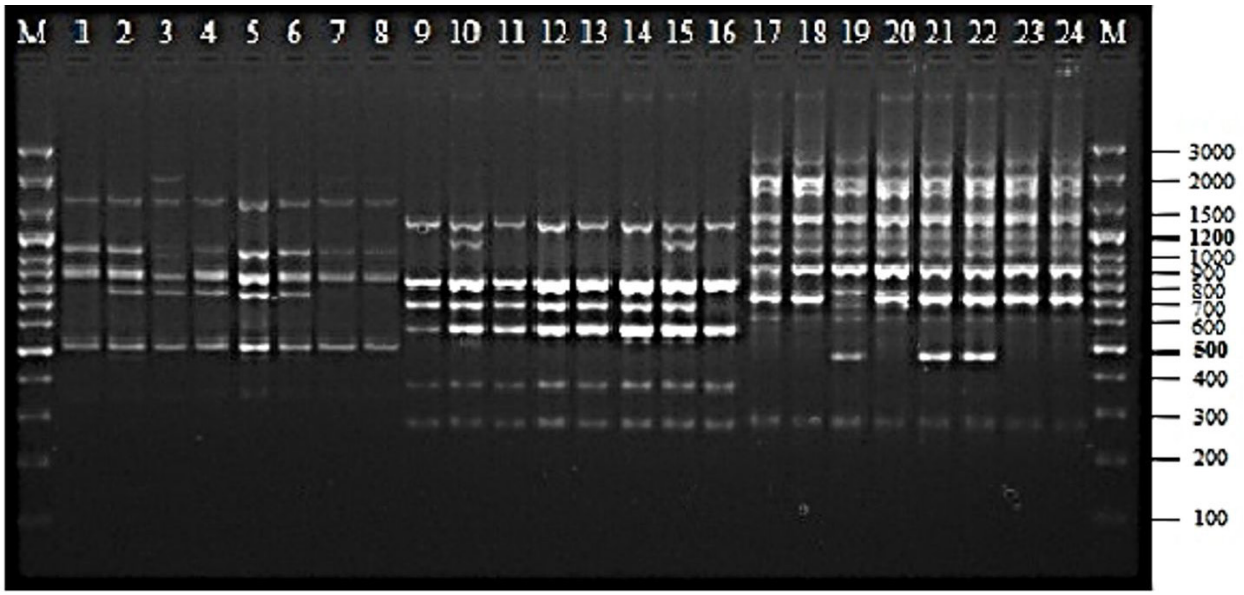

Fig. 3. Electrophorogram of PCR reaction with OPP09 (1–8) OPA11 (9–16), OPB10 (17–24) primers for twin pairs: 1A (1, 9, 17), 1B (2, 10, 18); 2A (3, 11, 19), 2B (4, 12, 20); 3A (5, 13, 21), 3B (6, 14, 22); 4A (7, 15, 23), 4B (8, 16, 24); M – marker

embryo formation and a number of other possibilities of haploids formation and spontaneous diploidsation are presented in the abovementioned article by Forester and co-authors [2007].

The origin of the three remaining twin pairs can probably be explained as a consequence of the embryogenesis of recombined haploid cells and spontaneous diploidisation. Significant differences in the phenotypic traits and molecular dissimilarity within the pairs and from the hybrid mother plant show that each embryo has developed from different recombined haploid cells. The parthenogenetic embryogenesis of egg cells or synergids from the different embryo sacs could be the source of such twins. However, the androgenesis *in vivo* and spontaneous diploidisation cannot be excluded. This phenomenon was described many years ago by Campos and Morgan [1958] in *C. frutescens* L. This method enabled phenotypic markers to prove the androgenic origin of culture-derived diploids of *Capsicum* spp. [Nowaczyk et al. 2015]. Unfortunately, this

Table 2. RAPD primers specific for twin pairs of pepper *C. annuum* L. and coefficient of genetic distant

Twin plants	Primers generating specific products; number of products in parenthesis	Coefficient of genetic distant	
		A *vs*. B	F_1 maternal hybrid *vs.* A or B twin plant
1A *vs.*1B	OPA11 (1), OPF05 (1), OPP09 (1)	0.114	*vs.* A 0.079 *vs.* B 0.028
2A *vs.* 2B	OPB10 (2), OPE19 (1), OPF05 (1), OPP09 (2)	0.127	*vs.* A 0.100 *vs.* B 0.048
3A *vs.* 3B	lack of specific products	0.000	*vs.* A 0.038 *vs.* B 0.038
4A *vs.* 4B	OPA11 (2), OPAE10 (2)	0.048	*vs.* A 0.018 *vs.* B 0.067

mode of evaluation is limited by the small number of monogenic traits conditioned by the alleles of single genes. Additionally, phenotypic marker traits are usually visible in the stage of full plant maturity. From this point of view, the molecular analysis of twins seems to be better because the genetic variability of twins may be stated in the seedling phase of plants.

CONCLUSIONS

The *in vivo* seed-derived spontaneous doubled haploids of pepper *Capsicum annuum* L. should be given more attention because they are ready for the production of genetically stable, sexual progeny. The RAPD technique is suitable for the detection of genetic differences between twins as well as between twins and parental plants, and can provide information about the origin of additional pepper embryos.

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REFERENCES

Barcaccia, G., Albertini, E. (2013). Apomixis in plant reproduction: a novel perspective on an old dilemma. Plant Reprod., 26, 159–179, DOI: 10.1007/s00497-013-0222-y.

Campos, F., Morgan, D.,T., Jr. (1958). Haploid pepper from a sperm. Androgenetic haploid of *Capsicum frutescens* L. J. Heredity, 49, 135–137, DOI: 10.1093/oxfordjournals.jhered.a106786.

Chauhan, H., Khurana, P. (2011). Use of doubled haploid technology for development of stable drought-tolerant bread wheat (*Triticum aestivum* L.) transgenics. Plant Biotech. J., 9, 408–417, DOI: 10.1111/j.1467-7652.2010.00561.x.

Dolcet-Sanjuan R., Claveria E., Huera, A. (1997). Androgenesis in *Capsicum annuum* L.: effect of carbohydrate and carbon dioxide enrichment. J. Amer. Soc. Hort. Sci., 122, 468–475, DOI: 10.21273/JASHS.122.4.468.

Dumas de Vaulx, R., Chambonnet, D., Pochard, E. (1981). Culture in vitro d'anthéres de piment (*Capsicum annuum* L.) amélioration des taux d'obtention de plantes chez différents génotypes par des traitements á +35EC. Agronomie, 1, 859-864. Dunwell, J.M. (2010). Haploids in flowering plants: origins and exploitation. Plant Biotech. J., 8, 377–424.

Forster, B.P., Thomas, W.T.B. (2005). Doubled haploids in genetics and plant breeding. Plant Breed. Rev., 25, 57–88, DOI: 10.1002/9780470650301.ch3.

Forster, B.P., Heberle-Bors, E., Kasha, K.J., Touraev, A. (2007). The resurgence of haploids in higher plants. Trends Plant Sci., 12, 368–375, DOI: 10.1016/j.tplants.2007.06.007.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., Firoozabady, E. (1983). Rapid flow cytometry analysis of the cell cycle in intact plant tissues. Science, 220, 1049–1051, DOI: 10.1126/science.220.4601.1049.

Germana, M.A. (2011). Anther culture for haploid and doubled haploid production. Plant Cell Tiss. Organ Cult., 104, 283–300, DOI: 10.1007/s11240-010-9852-z.

Ilbi, H. (2003). RAPD markers assisted varietal identification and genetic purity test in pepper *Capsicum*

annuum L. Scientia Hortic., 97, 211-221, DOI: 10.1016/S0304-4238(02)00158-9.

Irikova, T., Grozeva, S., Rodeva, V. (2011). Anther culture in pepper (*Capsicum annuum* L.) *in vitro*. Acta Physiol. Plant., 33, 1559–1570, DOI: 10.3906/BIY-1506-79.

Jedrzejczyk, I., Nowaczyk, P. (2009). *In vivo* polyembryony induction in species of *Capsicum.* Acta Biol. Cracoviensia, 51(1), 55–60.

Kim, M., Kim, J., Yoon, M., Choi, D.I., Lee, K.M. (2004). Origin of multicellular pollen and pollen embryos in cultured anthersof pepper (*Capsicum annuum* L.). Plant Cell Tissue Organ. Cult., 77, 63–72, DOI: 10.1023/B:TICU.0000016506.02796.6a.

Kothari, S.L., Joshi A., Kachhwaha, S., Ochoa-Alejo, N. (2010). Chilli peppers-areview on tissue culture and transgenis. Biotechnol. Adv., 28, 35–48, DOI: 10.1016/j.biotechadv.2009.08.005.

Kristiansen, K., Andersen, S.B. (1993). Effects of donor plant, temperature, photoperiod and age on anther culture response of *Capsicum annuum* L. Euphytica, 67, 105–109, DOI: https://doi.org/10.1007/BF00022732.

Lantos, C., Gemes, A.J., Somogyi, G., Otvos, K., Vagi, P., Mithaly, R., Kristof, Z., Somogyi, N., Pauk, J. (2009). Improvement of isolated microspore culture of pepper (*Capsicum annuum* L.) via co-culture with ovary tissues of pepper or wheat. Plant Cell Tissue Organ Cult., 97, 285–293, DOI : 10.1007/s11240-009-9527-9.

Mityko, J., Fari, M. (1997). Problems and results of doubled haploid plant production in pepper (*Capsicum annuum* L.) via anther and microspore culture. Acta Hortic., 447, 281–287, DOI: 10.17660/ActaHortic.1997.447.57.

Naumova, T. (1992). Apomixis in angiosperms: nucellar and integumentary embryony. CRC Press, Boca Raton, 1–144, DOI: 105586/asbp.1981.034.

Nei, M., Li., W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA, 76, 5269–5273, DOI: 10.1073/pnas.76.10.5269.

Niemirowicz-Szczytt, K. (1997). Excessive homozygosity in doubled haploids – advantages and disadvantages for plant breeding and fundamental research. Acta Physiol. Plant., 19, 155–167, DOI: 10.1007/s11738-997-0032-7.

Nikolova, V., Niemirowicz-Szczytt, K. (1996). Dihaploidization of cucumber (*Cucumis sativus* L.) haploids by colchicine treatment. Acta Soc. Bot. Pol., 65, 311–317, DOI: 10.5586/asbp.1996.048.

Nowaczyk, L., Nowaczyk, P. (2006). Genetic analysis of tomato (*Lycopersicon esculentum* Mill.) twin forms. Acta Biol. Cracoviensia, 48(1), 53–58.

Nowaczyk, L., Nowaczyk, P., Olszewska, D. (2015). Genetic analysis of anther culture-derived diploids of *Capsicum* spp. J. Hortic. Sci. Biotech., 90(6), 747–752, DOI: 10.1080/14620316.2015.11668741.

Olszewska, D., Jedrzejczyk, I., Nowaczyk, P., Sendel, S., Gaczkowska, B. (2015). *In vitro* colchicine treatment of anther-derived pepper haploids. Bulgarian J. Agri. Sci., 21(4), 806–810.

Rao, R.K.G., Harini, I., Kumar, A.O. (1987). Colchicine induced chromosome mosaicism in chili pepper (*Capsicum annuum* L.). Proc. Indian Acad. Sci., 97, 55–61, https://www.ias.ac.in/article/fulltext/plnt/097/01/0055-0061.

Rodeva, V., Irikova, T., Todorova, V. (2004). Anther culture of pepper (*Capsicum annuum* L.): comparative study on effect of the genotype. Biotechnol. Equip., 3, 34–38, DOI: 10.1080/13102818.2004.10817117.