Bulgarian Journal of Agricultural Science, 21 (No 4) 2015, 806-810
Agricultural Academy

IN VITRO COLCHICINE TREATMENT OF ANTHER-DERIVED PEPPER HAPLOIDS

D. OLSZEWSKA, I. JEDRZEJCZYK*, P. NOWACZYK, S. SENDEL and B. GACZKOWSKA
University of Technology and Life Sciences, Department of Genetics, Physiology and Plant Biotechnology, Faculty of Agriculture and Biotechnology, 85-796 Bydgoszcz, Poland

Abstract

OLSZEWSKA, D., I. JEDRZEJCZYK, P. NOWACZYK, S. SENDEL and B. GACZKOWSKA, 2015. *In vitro* colchicine treatment of anther-derived pepper haploids. *Bulg. J. Agric. Sci.*, 21: 806–810

Capsicum genus covers several dozens of species, which can be used in crossbreeding with annual pepper. The induction of haploid and dihaploid plants through anther cultures of the received hybrids is one of the possibilities of the fast stabilization of the new variability. In this study the following interspecific hybrids were the plant material for the induction of androgenesis: (905 × 'Sono')F_1, (905 × 'Mino')F_1, (405 × 'Luba')F_1, (405 × 'Sono')F_1. Haploid plants obtained from anther cultures were colchicine treatment according to protocol developed for *Capsicum annuum* L. The apical parts of 106 androgenic haploids were placed for six days on the MS medium containing colchicine at the concentration equal 400 mg dm^{-3}. The largest groups of regenerants were mixoploids 29-55% and haploids 20-50%. For the studied genotypes an effective diploidization at the level of 17-27% was observed. Micropropagation was performed on the 23 haploids obtained and the received microcuttings (in the number of 73) underwent again a-six-day and a-nine-day treatment with colchicine on the MS medium containing 200 mg dm^{-3} of colchicine. The effectiveness of the regeneration after the second colchicine treatment was significantly reduced. Single diploid plants appeared among the regenerants (905 × 'Sono')F_1 and (405 × 'Luba')F_1.

Key words: androgenesis, *Capsicum*, diploidization, haploid, interspecific hybrids, flow cytometry

Introduction

Fully homozygotic diploid plants are the primary target of inducing androgenesis. Traditional breeding methods allow such genotypes to be obtained as a result of 5-6 years of inbreeding. An alternative and faster method involves the induction of sporophytic development path of the microspores in anthers or isolated microspores cultures. The research into the induced androgenesis in *Capsicum* genus has enhanced the effectiveness of the process and it's optimising (Irikova et al., 2011; Segui-Simarro et al., 2011; Lantos et al., 2012). Publications concerning the induction of androgenesis in case of other *Capsicum* species and interspecific hybrids are rare to encounter (Munyon et al., 1989; Gemesne et al., 2009). The efficiency of embriogenesis for such genotypes is significantly lower in comparison to the cultivar forms *C. annuum* (Nowaczyk et al., 2009a,b; Olszewska et al., 2014). The characteristic feature of the *Caspicum* is the presence of haploid and diploid plants among the androgenic regenerants. In case of plants in which spontaneous diploidization did not take place it is necessary to apply the factor provoking the duplication of the number of chromosomes and restoring fertility. Available literature offers studies concerning the colchicine treatment of haploid regenerants obtained by androgenesis of *Capsicum annuum* genotypes (Gemesne et al., 2001; Gemesne et al., 2006; Lantos et al., 2009). For other pepper species and interspecific hybrids an effective method of diploidization has not been described so far. In the study an attempt was made to adapt the protocols of colchicine treatment previously described for *Capsicum annuum* genotypes (Gemesne et al., 2001; Gemesne et al., 2006; Lantos et al., 2009) in order to apply it to the interspecific hybrids of *Capsicum*.

Materials and Methods

Plant material

The plant material used in the experiment was derived from the collection of the Department of Genetics, Physiol-

E- mail: *jedrzej@utp.edu.pl, dorota@utp.edu.pl, nowaczyk@utp.edu.pl

ogy and Plant Biotechnology of the University of Technology and Life Sciences in Bydgoszcz. For the androgenesis induction the following hybrids were used: (905 × 'Sono')F_1, (905 × 'Mino')F_1, (405 × 'Luba')F_1, (405 × 'Sono')F_1. Lines 905 and 405 comprising the parental forms of the crossings were selected from the *Capsicum frutescens* L. × *Capsicum annuum* L. hybrid materials. The presence of dominant alleles determining soft flesh of ripe fruits as well as the capacity for the synthesis of capsaicinoids is their characteristic feature. As pollinators were used 'Sono' and 'Luba' cultivars growing under foil, and 'Mino' – to field growing. The androgenesis induction in anther cultures was performed according to the method developed by Dumas de Vaulx et al. (1981). The androgenesis was induced on the Cp medium supplemented with kinetin (0.1 mg dm^{-3}) and solidified with agar (8 g dm^{-3}) at pH 5.9 (Dumas de Vaulx et al., 1981) with modification involving the prolongation the time of incubation on the anther induction medium CP to 14 days and higher content of kinetin (0.3 mg dm^{-3}) on the R1 regeneration medium (Olszewska et al., 2014).

Determination of ploidy level

The ploidy level of androgenic regenerants was determined using the flow cytometry method by the estimation of a relative DNA content in the youngest leaves. The measurements were performed three times. For the first time the androgenic regenerants were analysed for the haploid plants identification, then the ploidy of regenerants was verified after each colchicine treatment. The samples for cytometric analyses were prepared according to the previously developed procedure for pepper (Jędrzejczyk and Nowaczyk, 2009). The plant material was chopped with a sharp razor blade in a plastic Petri dish containing 1 ml of nucleus-isolation buffer [0.1 M Tris, 2.5 mM $MgCl_2 \times 6H_2O$, 85 mM NaCl, 0.1% (v/v) Triton X-100; pH 7.0] supplemented with fluorochrome DAPI at the concentration of 2 µg cm^{-3}. The nuclei suspension was passed through a 50 µm mesh nylon filter and then analysed using a Partec CCA flow cytometer (Münster, Germany), equipped with mercury UV lamp. At least 5000 cell nuclei were analysed for each sample with the usage of the linear scale. The leaves of diploid species of *C. annuum* were used as a reference standard with known ploidy. Histograms were analysed using the Partec DPAC v.2.2 software.

Diploidization of androgenic regenerants

The experiment was performed in the growth chamber at the temperature of 25°C exposed to 12-hour light. The apical plant part was cut off from 106 haploid plants at the 2-3 leaf height and placed on the MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g dm^{-3}) and solidified with agar (8 g dm^{-3}) at pH 5.8 with the addition of colchicine at the concentration of 400 mg dm^{-3}. After six days the plants were transferred to the MS medium of the basic composition. The DNA ploidy level control was performed after eight weeks. The diploids and mixoploids were acclimatized in the glasshouse, while haploids were cut into fragments containing nodes and placed onto the MS medium not containing any supplements. In that way a single plant provided 1-6 explants demonstrating the plant-developing potential. The total yield of 73 of haploid microcuttings underwent the colchicine treatment for the second time on the MS medium containing 200 mg dm^{-3} of colchicine. Haploid plants were exposed to the effect of colchicine for six and nine days and then regenerated on the MS medium of the basic composition. Eight weeks after the transfer on the MS medium, the final cytometric analyses were made.

The plant regeneration effectiveness after the successive colchicine treatments was calculated against the initial number of the plants treated. The percentage share of the rooted plants was determined in comparison to the number of plants regenerated in respective genotypes.

Results

Four weeks after the first colchicine treatment a regenerate evaluation (Table 1) was conducted. It was found that 79% of the plants were developing and formed green leaves. Most of them also formed roots. The highest percentage of regenerated plants was identified for genotype (405 × 'Sono')F_1 and reached the level of 96%. The plants received in the anther cultures of hybrid (905 × 'Mino')F_1 were found to be rooting best. An inconsiderable percentage of bacterial and fungal infections, which eliminated 12 regenerants was observed in the experiment. Finally, eight weeks after the completion of the first diploidization, samples from 73 regenerants were taken for the cytometric analysis, which equalled 68% of the colchicine-treated plants (Table 1). The most numerous group among the regenerants consisted of mixoploids which share ranged from 29% to 55% depending on the genotype. The second group formed haploids whereas from 20% to 50% of the plants exposed to a six-day colchicine treatment proved not to change their ploidy level. An effective diploidization was observed for genotypes (905 × 'Sono')F_1, (405 × 'Luba')F_1 and (405 × 'Sono')F_1 and reached the 25-27%. As for regenerants (905 × 'Mino')F_1, one diploid plant was received only. The regenerants that remained haploid underwent micropropagation. For genotype (905 × 'Sono')F_1 4 haploids produced 19 microcuttings. For (905 × 'Mino')F_1 3 haploids provided 16 plants and for genotype (405 × 'Luba')F_1 10 haploids produced 22 plants, whereas in case of genotype (405 × 'Sono')F_1 – from 6 haploids 16 plants were regenerated.

In the first part of the experiment mixoploids which somatic cells contained from 1C to 8C of DNA comprised a large group of regenerants. Therefore, in the second diploidization treatment the colchicine concentration was decreased by half and the incubation time was extended to six and nine days. Four weeks after the second colchicine treatment the growth disorder was observed, which proved to be directly proportional to the time of explant incubation on the colchicine-containing MS medium (Table 2). After six days of colchicine treatment 38% of the plants regenerated and after nine days their number decreased to 33%. Genotype (405 × 'Sono')F_1 was regenerating the least, only 25% of plants were received. As far as other genotypes are concerned, 37-41% regenerants were obtained. The colchicine treatment also disturbed rooting in more than half of the regenerated plants. The largest group among the regenerants consisted of mixoploid plants (1C-4C), which were obtained in all of colchicine treatment conditions and depending on the genotype constitutes 25-100% of the regenerats (Table 2). A considerable number of regenerants were haploids received for all the genotypes investigated. Single diploid plants were obtained for genotypes (905 × 'Sono')F_1 and (405 × 'Luba')F_1 after six and nine days of colchicine treatment (Figure 1).

Disscusion

The research into the induction of androgenesis in *Capsicum* genus performed since 1973 has allowed both to enhance the effectiveness of that process and to optimise it. Depending on the genotype of the donor plant presence of haploid and diploid forms among the obtained regenerants is to be observed. This phenomenon is characteristic not only for *C. annuum* L. but also for *C. frutescens* or hybrids of the *Capsicum* genus. In case of some genotypes the diploids can outnumber the haploid regenerants (Nowaczyk et al., 2009b; Niklas-Nowak et al., 2012; Luitel and Kang, 2013; Olszewska et al., 2014). The lines of double haploids received in a spontaneous way comprise valuable, fully homozigotic material. In case of the haploid plants it is necessary to apply the conditions aiming at doubling the number of chromosomes in the cells. It is colchicine that is a commonly applied c-mitotic substance disturbing the structures of spindle apparatus and the segregation of sister chromatids (Dhooghe et al., 2011). Traditionally, colchicine is applied to young plants by spraying the plants with a water solution or through a short immersion.

The plant material used for the diploidization can be also provided by seeds, shoots, flower buds, shoot apices, callus

Table 1
The effectiveness of regeneration, rooting and ploidy level of plants obtained after the first colchicine treatment (400 mg dm^{-3} colchicine)

Origin	Number and ploidy of plants after 6 days of colchicine treatment						
	Treated	Regenerated	Rooted	Haploids	Diploids	Tetraploids	Mixoploids
(905 × 'Sono')F_1	31	24 (77%)	20 (83%)	4 (20%)	5 (25%)	1 (5%)	11 (55%)
(905 × 'Mino')F_1	8	6 (75%)	6 (100%)	3 (50%)	1 (17%)	0	2 (33%)
(405 × 'Luba')F_1	41	29 (71%)	21 (72%)	10 (42%)	6 (25%)	1 (4%)	7 (29%)
(405 × 'Sono')F_1	26	25 (96%)	16 (64%)	6 (27%)	6 (27%)	0	10 (45%)
Total	106	84 (79%)	63 (75%)	23 (32%)	18 (25%)	2 (3%)	30 (42%)

Table 2
The effectiveness of regeneration and ploidy level of plants obtained after the second colchicine treatment (200 mg dm^{-3} colchicine)

Origin	Number and ploidy of plants after 6 days of colchicine treatment					Number and ploidy of plants after 9 days of colchicine treatment				
	Treated	Regenerated	Haploids	Diploids	Miksoploids	Treated	Regenerated	Haploids	Diploids	Miksoploids
(905 × 'Sono')F_1	9	3 (33%)	1 (33%)	1 (33%)	1 (33%)	10	4 (40%)	1 (25%)	-	2 (50%)
(905 × 'Mino')F_1	8	4 (50%)	1 (25%)	-	2 (50%)	8	2 (25%)	-	-	2 (100%)
(405 × 'Luba')F_1	10	4 (40%)	2 (50%)	-	1 (25%)	12	5 (42%)	2 (40%)	1 (50%)	1 (50%)
(405 × 'Sono')F_1	7	2 (29%)	1 (50%)	-	-	9	2 (22%)	-	-	-
Total	34	13 (38%)	5 (39%)	1 (7.7%)	4 (31%)	39	13 (33%)	3 (23%)	1 (7.7%)	5 (39%)

and embryos. The first reports on the application of colchicine in pepper breeding concern the induction of tetraploid and octoploid plants after the use of 0.3% of the colchicine solution transferred onto apical meristems of diploid seedlings (Panda et al., 1984). Chambonet (1988) presented a similar method for pepper haploids which involved topping the plants to abandon the apical dominance and removing lateral buds. Instead, agar paste with 5% colchicine added was placed; the treatment was repeated twice at few-hour intervals. As a result of such procedure diploid shoots were received. Applying colchicine treatment *in vivo,* Mityko and Fari (1997) received about 50% diploid plants. Gemesne et al. (2001) evaluated the effectiveness of diploidization by applying colchicine in *in vitro* culture. The authors showed that the growth of young *Capsicum annuum* L. plants on the medium supplemented with colchicine at the concentration of 400 mg dm^{-3} for six days resulted in an effective diploidization of 50-95% explants depending on the genotype.

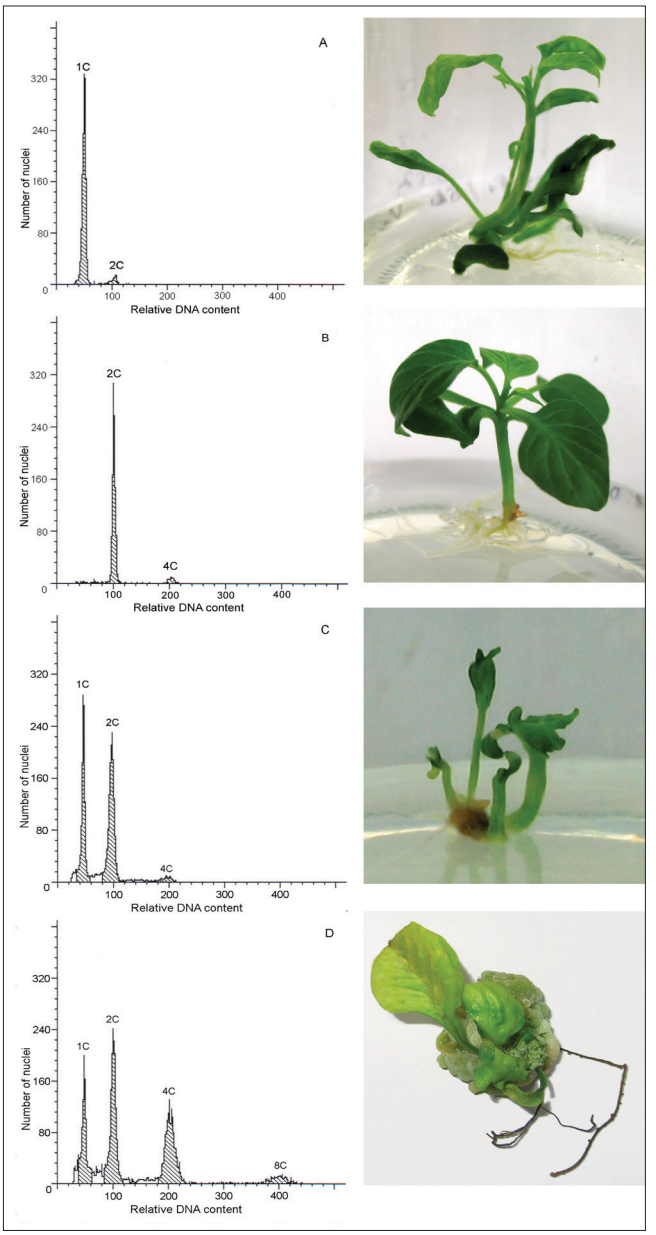

Fig. 1. Histograms of ploidy level of plants obtained after colchicine treatment: A – haploid (1C); B – diploid (2C); C – mixoploid (1C-4C); D – mixoploid (1C-8C)

Gemesne et al. (2006) as well as Lantos et al. (2009) reported on similar results by applying the method of a-six-day incubation *in vitro* on the medium containing 400 mg dm^{-3} of colchicine, both for rooted plants and for apical plant parts. In the present experiment in the first cycle of colchicine treatment, despite the same concentration of the compound and the treatment time, the diploidization effectiveness ranged from 17% to 27%. One supposes that the obtained results were due to the specific nature of the genotypes which are derived from the *Capsicum frutescens* × *Capsicum annuum* L. hybrid materials. In the citations on *C. annuum* L., the authors clearly point to the genotypes as the factor determining the success of the research. Supena et al. (2006) also highlighted that the effectiveness of polyploidization considerably depends on the type of the explant and they suggest the application of colchicine at the early phases of androgenesis, also in anther cultures. One can assume that for the genotypes evaluated in the experiment there was captured no optimal explant development stage for the induction of diploidization.

Another essential mater is the concentration of colchicine and the time of its effect. Low doses are often ineffective, while those which are excessively high – show a lethal effect and so the concentration is determined experimentally for each species and tissue type. Treating the plants with high doses can also result in the appearance of plants with a higher ploidy than desired. After the first colchicine treatment some regenerants were observed with changes in the leaf shape, twisting and the callus tissue which appeared on the shoots and leaves. A considerable percentage of mixoploids with the nuclear DNA content from 1C to 8C can suggest that for the genotypes investigated colchicine was used at an excessively high concentration. For that reason in the second colchicine treatment cycle the concentration was decreased by half and the time of its effect was prolonged. As a result, two diploid regenerants were received after a-six-day and a nine-day of incubation on the medium with colchicine. Most haploids exposed to colchicine treatment for the second time died. Strong lethal influence of colchicine observed in the second cycle of the experiment proves that the double usage of the compound

is not purposeful. Further investigation into the diploidization of the haploid regenerants of pepper shall focus on the proper selection of colchicine concentration and the time of a single exposure, as application of the procedures described for *C. annuum* L. does not provide equally good results for the interspecific hybrids of *Capsicum*.

Conclusion

The procedure of colchicine treatment developed for *C. annuum* L. for the following androgenic regenerants: (905 × 'Mino')F_1, (905 × 'Sono')F_1, (405 × 'Luba')F_1 (405 × 'Sono') F_1 caused successful diploidization in case of 17-27 % of the treated explants. The second colchicine treatment of the androgenic haploids strongly reduced their regeneration. Both after the first and the second colchicine treatment the most numerous group among regenerants comprised haploid and mixoploid plants.

References

Chambonnet, D., 1988. Production of haploid pepper plant. *Bulletin Interne de la Station d'Amelioration das Plantes Maraicheres d'Avigon*, Montfavet, France, pp.1-10.

Dhooghe, E., K. Van Laere, T. Eeckhaut, L. Leus and J. Van Huylenbroeck, 2011. Mitotic chromosome doubling of plant tissues in vitro. *Plant Cell, Tissue and Organ Culture,* **104**: 359-373.

Dumas de Vaulx, R., D. Chambonnet and E. Pochard, 1981. In vitro anther culture in red pepper (*Capsicum annuum* L.): improvement of the rate of plant production in different genotypes by treatments at 35 deg. C. *Agronomie,* **1**: 859-864.

Gemesne, J. A., M. Petus, G. Venczel, L. Zatyko, G. Gyulai and M. Cseplo, 2001. Genetic variability of anther donor versus spontaneous double haploid descendants and colchicine induced double haploid sweet pepper (*Capsicum annuum* L.) lines. *Acta Horticulturae,* **560**: 149-152.

Gemesne, J. A., G. Venczel, Z. S. Sagi, L. Gajdos, Z. Kristof, P. Vagi and L. Zatyko, 2006. Production of doubled haploid breeding lines in case of paprika, eggplant, cucumber, zucchini and onion. *Acta Horticulturae,* **725**: 845-854.

Gemesne, J. A., Z. Kristof, P. Vagi, C. Lantos and J. Pauk, 2009. *In vitro* anther and isolated microspore culture as tools in sweet and spice pepper breeding. *Acta Horticulturae*, **829**: 61-64.

Irikova, T., S. Grozeva and V. Rodeva, 2011. Anther culture in pepper (*Capsicum annuum* L.) *in vitro*. *Acta Physiologiae Plantarum,* **33**: 1559-1570.

Jędrzejczyk, I. and P. Nowaczyk, 2009. In vivo polyembryony induction in species of *Capsicum*. *Acta Biologica Cracoviensia, Botanica* (series), **51**: 55-60.

Lantos, C., A. G. Juchasz, G. Somogyi, K. Otvos, P. Vagi, R. Mihaly, Z. Kristof, N. Somogiy and J. Pauk, 2009. Improvement of isolated microspore culture of pepper (*Capsicum annuum* L.) via co-culture with ovary tissues of pepper or wheat. *Plant Cell, Tissue and Organ Culture,* **97**: 285-293.

Lantos, C., A. G. Juhasz, P. Vagi, R. Mihaly, Z. Kristof and J. Pauk, 2012. Androgenesis induction in microspore culture of sweet pepper (*Capsicum annuum* L.). *Plant Biotechnology Reports,* **6**: 123-132.

Luitel, B. P. and W. H. Kang, 2013. *In vitro* androgenic response of minipaprika (*Capsicum annuum* L.) genotypes in different culture media. *Horticulture, Environment and Biotechnology*, **54** (2): 162-171.

Mityko, J. and M. Fari, 1997. Problems and results of doubled haploid plant production in pepper (*Capsicum annuum* L.) via anther and micro spore culture. *Acta Horticulturae*, **447**: 281-287.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Acta Physiologia Plantarum*, **15**: 473-497.

Niklas-Nowak, A., D. Olszewska, A. Kisiała and P. Nowaczyk, 2012. Study of individual plant responsiveness in anther cultures of selected pepper (*Capsicum* spp.). *Folia Horticulturae*, **24** (2): 141-146.

Nowaczyk, P., A. Kisiala and D. Olszewska, 2006. Induced androgenesis of *Capsicum frutescens* L. *Acta Physiologia Plantarum,* **28**: 35-39.

Nowaczyk, P., L. Nowaczyk, D. Olszewska and A. Krupska, 2009a. Androgenic response of genotypes selected from *Capsicum annuum* L. x *C. chinense* Jacq. hybrids. *Acta Physiologia Plantarum*, **31**: 877-879.

Nowaczyk, P., D. Olszewska and A. Kisiala, 2009b. Individual reaction of *Capsicum* F_2 hybrid genotypes in anther cultures. *Euphytica*, **168**: 225-233.

Olszewska, D., A. Kisiala and P. Nowaczyk, 2011. The assessment of doubled haploid lines obtained in pepper (*Capsicum annuum* L.) anther culture. *Folia Horticulturae,* **23**: 93-99.

Olszewska, D., A. Kisiala, A. Niklas-Nowak and P. Nowaczyk, 2014. Study of *in vitro* anther culture in selected genotypes of genus *Capsicum*. *Turkish Journal of Biology,* **38**: 118-124. doi:10.3906/biy-1307-50

Panda, R. C., O. Aniel Kumar and K. G. Raja Rao, 1984. Cytomorphology of induced octoploid Chili pepper (*Capsicum annuum* L.). *Theoretical Applied Genetics*, **68**: 567-570.

Segui-Simarro, J. M., P. Corrala-Martinez, V. Parra-Vega and B. Gonzalez-Garcia, 2011. Androgenesis in recalcitrant Solanaceus crops. *Plant Cell Reports*, **30**: 765-778.

Supena, E. D. J., W. Muswita, S. Suharsono and J. B. M. Custers, 2006. Evaluation of crucial factors for implementing shed-microspore culture of Indonesian hot pepper (*Capsicum annuum* L.) cultivars. *Stientia Horticulturae*, **107**: 226-232.