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Study of in vitro anther culture in selected genotypes of genus *Capsicum*

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Abstract: The combined effect of anther incubation time on CP induction medium (12, 14, and 16 days) and 2 concentrations of kinetin in R1 regeneration medium (0.1 and 0.3 mg/L) on the effectiveness of androgenesis was investigated in 17 genotypes of *Capsicum* grown in Poland. Plant material consisted of breeding lines and intraspecific hybrids of *C. annuum*; the species of *C. frutescens*, *C. chinense*, and *C. baccatum* var. *pendulum*; interspecific hybrids F_1 (*C. frutescens* × *C. chinense*) and F_1 (*C. frutescens* × *C. baccatum*); and doubled haploid lines derived from the hybrids. The results of the tested variants of the experiment were compared with the androgenic response of the control anthers cultured according to the standard protocol previously developed for pepper (12 days incubation on CP, 0.1 mg/L kinetin in R1). Under control conditions, androgenic embryos regenerated only from anthers of 3 of the tested genotypes, whereas application of the selected modifications promoted embryo development in an additional 12 genotypes. The highest effectiveness of androgenesis was observed after 16 days of anther incubation on CP medium combined with 0.1 mg/L kinetin in R1 medium. Twelve- and 14-day-long anther incubation was more effective when followed by transferring anthers onto R1 medium supplemented with 0.3 mg/L kinetin.

Key words: Androgenesis, embryo induction, haploid, incubation length, kinetin, pepper

1. Introduction

The genus *Capsicum* covers several dozen species, including breeding forms of *C. annuum* and *C. frutescens*, as well as many wild species that, as a result of crossbreeding, can contribute to a wider genetic variation of *C. annuum*. One approach to stabilize a new variation induced this way is to produce haploid plants through anther cultures of hybrid forms. Homozygous doubled haploid (DH) lines obtained as a result of doubling the number of chromosomes of haploid regenerants are widely used in breeding programs of various crop species and vegetables, including annual pepper (Dumas et al., 1981; Asakaviciute, 2008; Başay and Ellialtıoğlu, 2013), but also less popular forms of *C. frutescens* (Nowaczyk et al., 2006; Gemesne et al., 2009; Irikova et al., 2011).

The majority of the reports on the effectiveness of androgenesis in pepper available to date refer to the cultivated forms of *C. annuum*. According to the previously published results, the effectiveness of in vitro anther culture for selected, highly responsive genotypes of annual pepper can exceed 50% (Dumas et al., 1981; Irikova et al., 2011). However, many authors consider pepper a recalcitrant species, and the reported number of androgenic embryos obtained per 100 cultured anthers often ranges from 0 up to approximately 10 embryos (Ercan and Ayar Şensoy, 2011; Segui-Simarro et al., 2011).

Recent studies on induced androgenesis in the species of family Solanaceae point to a number of factors that significantly affect the success of inducing haploids. Among the most important are the genotype of anther-donor plants (Ercan et al., 2006; Asakaviciute, 2008; Nowaczyk et al., 2009a, 2009b; Başay and Ellialtıoğlu, 2013) and the developmental stage of microspores in the in vitro cultured anthers (Kim et al., 2004; Nowaczyk and Kisiala, 2006). It has been previously proven that the efficiency of haploid embryo induction may vary and depend on the growth conditions of anther-donor plants (Matsubara et al., 1998; Buyukalaca et al., 2004). The exposure of flower buds to high or low temperatures prior to anther isolation can significantly affect the developmental pathway of microspores, changing it from gametophytic to sporophytic (Özkum Çiner and Tıpırdamaz, 2002; Barany et al., 2005; Koleva-Gudeva et al., 2007). The research on effective anther culture conditions for pepper, and especially the optimization of media composition, focuses on analyzing the effect of different types and

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concentrations of exogenous phytohormones, as well as on specific stress factors, such as incubation temperature of the anthers (Özkum Çiner and Tıpırdamaz, 2002; Niklas-Nowak and Nowaczyk, 2009). The aim of such modifications is to induce or enhance androgenic embryo development, mainly by adapting the method to locally cultivated or poorly responsive genotypes.

The main objective of this work was to investigate the effect of prolonged anther incubation time on CP induction medium, combined with 2 different concentrations of kinetin in R1 regeneration medium, with the aim of optimizing the previously described protocols by adapting them to the locally grown breeding forms of *C. annuum* as well as various noncultivated *Capsicum* genotypes. The developed modifications resulted in considerably higher androgenic response of the tested *Capsicum* genotypes when compared to the standard culture conditions.

2. Materials and methods

2.1. Anther-donor plants

The research material consisted of *C. annuum* L. breeding lines ATZ1, PO, TG, and CDT; intraspecific hybrids F_1 (ATZ × PO), F_1 (ATZ × TG), and F_1 (ATZ × CDT) and DH lines derived from them (respectively AP40, AT4, and AC7); species *C. frutescens* L., *C. chinense* Jacq., and *C. baccatum* L. var. *pendulum*; and interspecific hybrids: F_1 (*C. frutescens* × *C. chinense*) and F_1 (*C. frutescens* × *C. baccatum*) and DH lines derived from them (FCH and FA). All the materials originated from the collection held by the Department of Genetics, Physiology, and Plant Biotechnology at the University of Technology and Life Sciences, Bydgoszcz, Poland. The anther-donor plants were grown in the moderate climate of north-central Poland, with average summer temperatures fluctuating between 15 and 20 °C. To avoid the risk of late-spring frosts, pepper seedlings were originally cultivated in a greenhouse and transplanted to unheated plastic tunnels around mid-May. Common agricultural practices for *C. annuum* were applied during the vegetation season. Multipurpose mineral fertilizer Azofoska [1(N):0.5(P_2O_5):1.4(K_2O); Grupa Inco S.A., Góra Kalwaria, Poland] was applied prior to planting and additionally every 3 weeks during plant cultivation (2.8 kg/100 m^2). Plants were not treated with pesticides or herbicides until the anther-sampling period was over.

2.2. Anther culture conditions

The sampling period lasted for about 1 month, beginning at the end of June, when the first flower buds were visible on the pepper plants. Buds were collected randomly from 20 plants of each of the genotypes and transported to the laboratory in plastic bags. Buds for anther isolation were harvested from healthy plants when the crown petals equaled or were slightly longer than the calyx sepals. Buds of this size contain mostly microspores in the late uninucleate stage and entering the first mitotic division, or in the early binucleate phase, optimal for inducing androgenic embryo development in pepper (Gemesne et al., 1998). Flower buds were rinsed with 70% ethanol, followed by surface sterilization by shaking in 5% solution of calcium hypochlorite ($CaCl_2O_2$) for 10 min, and were finally rinsed 3 times with sterile distilled water.

Anthers cultured following the protocol of Dumas de Vaulx et al. (1981) were used as the control in all the experiments. All the media used in this study (CP, R1, and V3) were prepared as described by Chambonnet (1988). After being incubated for 12 days on CP induction medium, the control anthers were transferred onto R1 regeneration medium supplemented with 0.1 mg/L kinetin. The modifications of the original protocol involved combinations of different anther incubation periods on CP medium (tested variants: 12, 14, and 16 days) and 2 levels of kinetin in R1 regeneration medium (tested concentrations: 0.1 and 0.3 mg/L).

Petri dishes with anthers were maintained in the growth chamber and all the observations were made during the 4 months of the experiment. The first embryos emerging from the anthers were observed after 4 weeks of the culture in progress (Figure 1A). The response of 130 anthers of each genotype was analyzed in each of the experimental combinations, including the control. In total, 2210 anthers were cultured in each of the 6 tested culture variants. The effectiveness of androgenesis, expressed as a percentage share of produced embryos to the total number of cultured anthers, was determined individually for each of the evaluated genotypes. Additionally, the effectiveness of regeneration was defined as the percentage share of regenerated plants to the number of developed embryos. The emerging plants were transferred onto V3 medium and later acclimatized in a greenhouse (Figures 1B and 1C).

2.3. Determination of ploidy level

The ploidy level of the plants obtained in anther cultures was determined with the use of flow cytometry, based on the measurements of DNA content in the youngest leaves of the regenerants. The samples for cytometric analyses were prepared as described previously by Sliwinska (2003). 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/mL. The nuclei suspension was passed through a 50-µm mesh nylon filter and then analyzed using a Partec CCA flow cytometer (Partec GmbH, Münster, Germany), equipped with a mercury UV lamp (High Pressure Lamp HBO-100W). For each sample at least 5000 cell nuclei were

analyzed at the flow rate of 20 nuclei/s. The reference standard used for the measurements was the diploid plant of annual pepper (2n = 2x = 24). The results were collected in a form of histograms and analyzed with Partec DPAC V.2.2 software.

3. Results

In the present work we investigated the effect of different incubation times on CP induction medium (12, 14, and 16 days) in combination with 2 kinetin concentrations in R1 regeneration medium (0.1 and 0.3 mg/L) on the effectiveness of in vitro androgenesis induced in anther cultures of selected pepper (*Capsicum* spp.) forms. The results of the performed experiments are presented in Table 1 and Figure 1. A varied androgenic response of the tested genotypes was identified depending on the combination of the applied factors. Under the control conditions (12 days on the induction medium and 0.1 mg/L kinetin in the regeneration medium), androgenic embryo development was observed only in 3 out of the 17 tested *Capsicum* genotypes. Selected combinations of induction period length and kinetin concentration allowed us to successfully induce embryo development in anther cultures of the additional 12 of the 17 pepper genotypes. The control treatment resulted in the highest androgenic response only in the case of the *C. annuum* PO breeding line (3.85%) when compared to the results of the other culture variants analyzed for this genotype. Only anthers of 2 wild species, *C. chinense* and *C. baccatum* var. *pendulum*, did not form androgenic embryos in any of the examined experimental conditions.

After culturing anthers on CP medium for 12 days, a significant increase in the number of responding genotypes and developing embryos was observed as the effect of the increased kinetin level in R1 medium. These conditions turned out to be particularly favorable for the ATZ1

Table 1. The effect of anther incubation period (12, 14, and 16 days) on CP medium combined with kinetin level (0.1 and 0.3 mg/L) in R1 medium on androgenic response of selected *Capsicum* genotypes.

Genotypes cultured**	12				14				16			
	0.1*		0.3		0.1		0.3		0.1		0.3	
	Embryo formation											
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
ATZ1	0	-	5	3.85	2	1.54	3	2.31	1	0.77	0	-
PO	5	3.85	1	0.77	2	1.54	0	-	0	-	0	-
TG	1	0.77	0	-	0	-	2	1.54	0	-	0	-
CDT	0	-	1	0.77	0	-	0	-	1	0.77	0	-
F_1 (ATZ × PO)	0	-	1	0.77	0	-	0	-	6	4.62	5	3.85
F_1 (ATZ × TG)	0	-	1	0.77	1	0.77	7	5.38	4	3.08	0	-
F_1 (ATZ × CDT)	1	0.77	1	0.77	0	-	1	0.77	0	-	0	-
AP40 DH line	0	-	2	1.54	1	0.77	2	1.54	1	0.77	1	0.77
AT4 DH line	0	-	1	0.77	1	0.77	1	0.77	5	3.85	2	1.54
AC7 DH line	0	-	3	2.31	2	1.54	0	-	8	6.15	0	-
C. frutescens	0	-	0	-	0	-	0	-	2	1.54	1	0.77
C. chinense	0	-	0	-	0	-	0	-	0	-	0	-
C. baccatum	0	-	0	-	0	-	0	-	0	-	0	-
F_1 (*C. frutescens* × *C. chinense*)	0	-	0	-	0	-	1	0.77	5	3.85	4	3.08
F_1 (*C. frutescens* × *C. baccatum*)	0	-	0	-	0	-	1	0.77	0	-	3	2.31
FCH DH line	0	-	4	3.08	1	0.77	0	-	1	0.77	0	-
FA DH line	0	-	3	2.31	1	0.77	3	2.31	1	0.77	2	1.54
Total	7	0.32	23	1.04	11	0.50	21	0.95	35	1.58	18	0.81

*: Control conditions according to Dumas de Vaulx et al. (1981).

**: One hundred and thirty anthers were cultured in each of the tested treatments for each genotype.

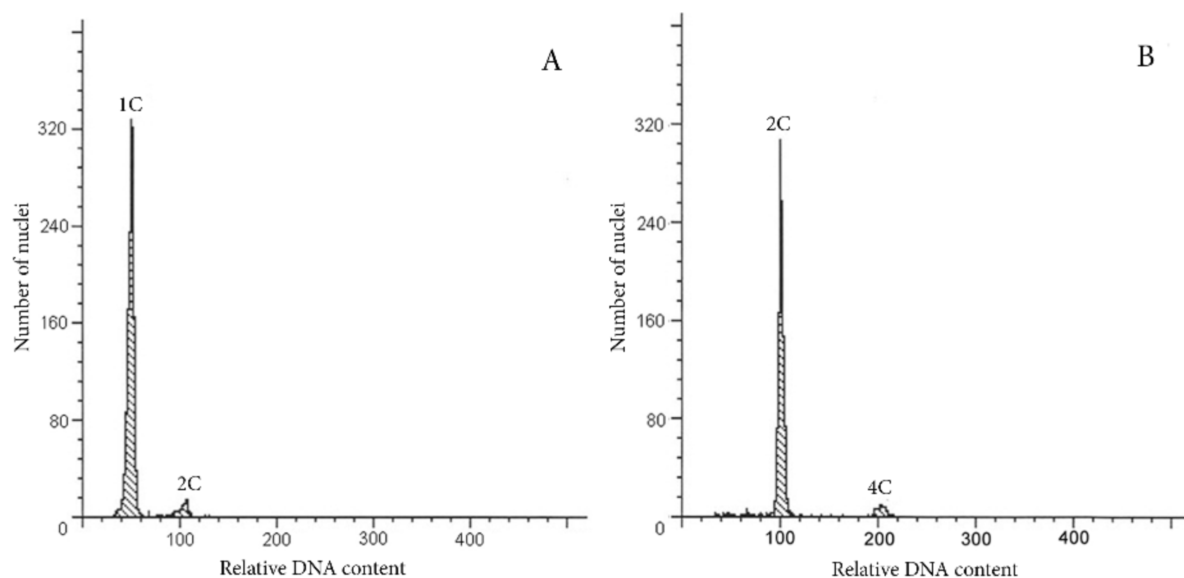

Figure 2. Flow cytometric analysis of the anther-derived pepper regenerants. A) haploid (1C) and B) diploid (2C) genome size.

breeding line (3.85% obtained embryos), and the DH-derived FCH line (3.08%). Extending anther incubation time on CP medium for an additional 2 days (up to 14 days in total) resulted in a similar dependence of anther response to kinetin level in R1; namely, a significantly higher number of embryos was obtained after transferring anthers onto R1 medium supplemented with higher level of kinetin (0.3 mg/L). These particular conditions were the most effective for the hybrid F_1 (ATZ × TG; 5.38% obtained embryos) and the breeding line TG (1.54%). Two of the tested DH lines, AP40 and FA, responded to the increased kinetin level in R1 medium with the same frequency of emerging embryos after being transferred from CP both after 12 days, as well as after 14 days of incubation.

The highest androgenic response was noted for 6 genotypes after the 16-day incubation period on CP medium. Moreover, the combination of a 16-day-long incubation time on CP medium with 0.1 mg/L kinetin in the R1 medium yielded the highest total number of induced embryos when compared with all the other tested variants. The highest androgenic response in this variant of the experiment was observed for 2 DH lines, AT4 (3.85%) and AC7 (6.15%), and 2 hybrids, F_1 (ATZ × PO, 4.62%) and F_1 (C. *frutescens* × *C. chinense*, 3.85%). The anthers of F_1 (*C. frutescens* × *C. baccatum*) responded most positively (2.31%) when transferred to R1 supplemented with 0.3 mg/L kinetin. Additionally, in the case of the species *C. frutescens*, 16-day-long incubation on CP medium was the only variant that yielded androgenic embryos, independent of the applied kinetin concentration.

Depending on the genotype, 0% to 75% of the androgenic embryos regenerated into healthy, young plantlets that subsequently were acclimatized in greenhouse conditions (Table 2; Figure 1). We were able to obtain androgenic plantlets from the embryos of all but 2 (TG and CDT breeding lines) of the evaluated pepper genotypes. Cytometric analysis of the ploidy level of the acclimatized plants revealed comparable numbers of haploids and diploids among the regenerants (Table 2, Figures 2A and 2B).

4. Discussion

Medium composition, especially type and concentration of growth regulators, as well as in vitro culture conditions, are among the crucial factors determining the success of haploid embryogenesis in anther culture of *Capsicum* (Özkum Çiner and Tıpırdamaz, 2002; Germana, 2011). According to Dumas de Vaulx et al. (1981), supplementation of R1 medium with 0.1 mg/L kinetin effectively stimulates sporophytic development in pepper androgenic embryos. However, Gemesne et al. (1998) showed that a higher kinetin concentration (0.2 and 0.3 mg/L) in R1 medium results in a further increase in the effectiveness of induced androgenesis in *Capsicum*. Prolonging anther incubation time on the induction medium is yet another factor that modifies androgenic embryo production. The previously published results indicate a positive androgenic response of certain *C. frutescens* genotypes when cultured on CP medium for 14 days instead of 12 days (Nowaczyk et al., 2006).

In the presented work, we tested the effect of 6 selected combinations of the 2 above factors on the effectiveness of androgenesis in a variety of *Capsicum* genotypes. The results of these experiments support the earlier reports on

Table 2. The effectiveness of regeneration and ploidy level of anther-derived plants.

Genotypes cultured	Embryo formation (number)	Plant formation		Ploidy level of regenerated plants	
		(number)	(%)	Haploids	Diploids
ATZ1	11	5	45.5	2	3
PO	8	2	25.0	1	1
TG	3	0	0	0	0
CDT	2	0	0	0	0
F_1 (ATZ × PO)	12	6	50.0	2	4
F_1 (ATZ × TG)	13	4	30.8	2	2
F_1 (ATZ × CDT)	3	1	33.3	1	0
AP40 DH line	7	5	71.4	2	3
AT4 DH line	10	1	10.0	0	1
AC7 DH line	13	9	69.2	3	6
C. frutescens	3	1	33.3	1	0
C. chinense	-	-	-	-	-
C. baccatum	-	-	-	-	-
F_1 (*C. frutescens* × *C. chinense*)	10	3	30.0	2	1
F_1 (*C. frutescens* × *C. baccatum*)	4	3	75.0	2	1
FCH DH line	6	1	16.7	0	1
FA DH line	10	5	50.0	3	2
Total	115	46	40.0	21	25

Figure 1. Development of androgenic regenerants through in vitro anther cultures of *Capsicum* spp. A) Embryos emerging from anthers of hybrid genotype F_1 (ATZ × PO) cultured on R1 medium. B) Young plantlet on V3 medium. C) Acclimatized androgenic plant grown in a greenhouse.

the favorable effect of increased kinetin level in regeneration medium on the effectiveness of pepper anther cultures. Moreover, for most of the tested genotypes, we were able to obtain comparable or better results by extending anther incubation period on the induction medium. Under the control conditions, androgenic embryos were obtained for only 3 out of the 17 genotypes, while optimization of the incubation time on CP medium and kinetin concentration in R1 medium allowed for an increase in the number of the responding genotypes up to a total of 15. In general, 12- and 14-day-long incubation periods on CP were more effective when combined with the higher of the 2 tested kinetin levels (0.3 mg/L). Anthers incubated for 16 days produced more embryos when transferred onto R1 medium supplemented with 0.1 mg/L of kinetin. To the best of our knowledge, this is the first detailed analysis of

the effect of prolonged anther incubation on CP medium combined with different concentrations of kinetin in R1 medium on the androgenic reaction of diverse *Capsicum* genotypes. Concluding from the presented results, the beneficial effects of extended anther incubation time, in correlation with different kinetin levels in the regeneration medium, are worth further investigation, especially due to the fact that many *Capsicum* genotypes still remain nonresponsive to in vitro induced androgenesis.

The effectiveness of androgenesis observed in our work ranged from 0% to 6.15%, depending on the genotype. This allowed us to obtain a total of 115 embryos and, consequently, 46 regenerated plants. The presented values are comparable with the results of the previously published research. The effectiveness of androgenesis presented by Ercan and Ayar Sensoy (2011), who analyzed the androgenic response of 11 *Capsicum* genotypes, ranged from 0% to 7.69%, giving in total 12 regenerated plants. The highest effectiveness of androgenesis observed in the experiment of Koleva-Gudeva and Trajkova (2009) reached approximately 17%; however, they were able to regenerate only 32 plants for 21 analyzed pepper cultivars. According to the literature data, isolated microspore cultures often result in considerably higher effectiveness of embryo induction (Supena et al., 2006; Lantos et al., 2012), but again, it is not always followed by a high number of regenerated plantlets, the most important parameter for breeding practice.

The environmental conditions during cultivation period, in particular the optimal temperature at the beginning of blooming, are also reported as an additional factor highly affecting the androgenic response of pepper (Buyukalaca et al., 2004; Ercan et al., 2006). The most common method of growing pepper in Poland is in plastic tunnels, which does not support stable temperature maintenance during the vegetation period. Meanwhile, it seems that *Capsicum*, a genus native to tropical and subtropical climate zones, presents a better androgenic response when plants are cultivated in the optimal environmental conditions (Supena et al., 2006). We assume that environmental factors, such as unpredictable temperature fluctuation during the donor plant growth, can be a reason for the generally lower effectiveness of androgenesis observed in this work when compared with some of the previously published data. However, our protocol has proven that even in the moderate Polish climate it is not necessary to grow anther donor plants exclusively in controlled environmental conditions. Instead, it is possible to enhance the androgenic response of locally grown pepper genotypes by modifying in vitro culture conditions. The possibility of using plants cultivated in a traditional way as the anther donors is of great importance, as it considerably reduces the cost of androgenic regenerant production.

Cytometric analysis revealed both haploid and diploid plants among the obtained androgenic regenerants. It has been reported before that the share of plants with different ploidy levels often depends on the donor plant genotype (Gyulai et al., 2000; Gemesne et al., 2001). Mityko and Fari (1997) observed a higher ratio of spontaneous diploid plants for large-fruited *Capsicum* cultivars, while haploid plants prevailed among the regenerants of the chili pepper forms. The literature data on the ploidy level of *Capsicum* androgenic regenerants suggest that the diploid plants produced both in anther and isolated microspore cultures are most often formed as a result of spontaneous chromosome doubling at the initial stages of haploid embryo development (Mityko et al., 1995; Dolcet-Sanjuan et al., 1997). Based on the visual observation of morphology and growth characters of the regenerated plants, we concluded that spontaneous diploidization of almost half of the young haploid embryos occurred also in our work. All the androgenic regenerants obtained in this study had formed inside the anther, and at the early growth stages both haploids and diploids were characterized by similar morphological appearances (data not presented).

In conclusion, no single culturing variant was found to be optimal for all the genotypes studied in the presented work. However, selected combinations of the length of induction period on CP medium and kinetin concentration in R1 medium resulted in a successful development of androgenic embryos in the case of 15 out of the 17 morphologically different *Capsicum* genotypes. The strong effect of the genotype on androgenic response, reported previously (Dolcet-Sanjuan et al., 1997; Nowaczyk et al., 2009b) and also observed in this study, suggests a need to adapt anther culture conditions to particular species, cultivars, or even breeding lines. As for the *C. chinense* and *C. baccatum* species, since an effective method of androgenic embryos induction has not been developed yet, it seems reasonable to search for other modifying factors that could stimulate in vitro anther responsiveness.

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