Article

Androgenesis—Technology for Obtaining Genetically Stable Breeding Material of *Capsicum annuum* L.

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Abstract: Androgenesis in vitro is a basic method of obtaining haploid plants and DH (doubled haploid) lines of major crops such as potato, rapeseed, tomato, pepper, wheat, maize, and barley, and also many different minor crops and species with lower agricultural impact. Diploid plants appearing among androgenic regenerants are the effect of spontaneous doubling of the chromosome number in haploid cells during an embryo's early developmental stages and are valuable fully homozygous breeding material. The subject of the presented research is spontaneous diploidization occurring in the development of androgenic, haploid pepper regenerants. In the presented experiment, the formation of diploid seeds was observed in the progeny of an androgenic, haploid plant derived in an anther culture of a hybrid (*Capsicum annuum* L. ATZ × *Capsicum annuum* L. 'Corno di toro')F₂. Agromorphological and molecular analyses concerned eight diploid plants being progeny of the anther-derived haploid regenerant. Five of the plants constituted a phenotypically balanced group with valuable agromorphological features. Their genetic homogeneity was confirmed using 10 RAPD markers and 16 ISSR markers. Based on the results, it was concluded that anther-derived haploid plants of *Capsicum* can be the source of diploid, apomictic seeds, and the obtained offspring may constitute genetically stable, valuable breeding material.

Keywords: apomixes; androgenesis; haploid; DH line; diploid; RAPD; ISSR; pepper breeding

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1. Introduction

The biggest challenge of modern agriculture is the intensification of agricultural production while limiting its negative impact on the environment. In plant production, breeding techniques leading to the creation of new varieties are the most effective way to achieve this goal. One of the methods providing a quick opportunity for obtaining valuable breeding materials is the induction of androgenic embryos in vitro [1]. Pepper is a species universal in terms of use, with valuable culinary and health-promoting values. Pepper fruits are a rich source of flavonoids, provitamin A, vitamin E, vitamin C, potassium, calcium, magnesium, and iron. They also contain especially valuable lycopene and capsaicin, which help lower blood pressure and protect against cardiovascular diseases. In addition, phytoactive compounds found in pepper fruits have anticancer and anti-atherosclerotic properties and delay the aging processes. A wide range of existing varieties of peppers with different tastes, colors, and fruit shapes is an invaluable source of genetic variation. The doubled haploid technology provides an opportunity for the rapid genetic stabilization of post-meiotic variability characteristic for hybrid generation [2,3], with induced androgenesis being the most important tool used for this purpose [4]. Increasing the efficiency of this phenomenon is addressed in much of the research. Changes in donor plant culture and their pretreatment are proposed [5,6] to deal with problems such as a lack of an androgenic response or low androgenesis effectiveness. Similarly, different media compositions [7] are frequently suggested solutions. For some genotypes, a higher percentage of embryos is achieved through the shed-microspore approach [8], whereas, in the case of others, a two-step culture system compared to liquid and double-layer culture proved to be more

effective [9]. As a result of the applied procedures, androgenic embryos are obtained that develop into haploid or diploid plants. The androgenic origin of diploid regenerants resulting from the spontaneous doubling of the haploid number of chromosomes is confirmed on the basis of morphological homogeneity in R2 and the subsequent generations [10]. Molecular techniques based on DNA polymorphism are also used to confirm their microspore origin and to describe their genetic variation [11,12]. Haploid regenerants are usually sterile plants, but individual seeds can sporadically form in their fruits. There is no accurate information about the ploidy and genetic status of seeds collected from *Capsicum* anther-derived haploids. Haploid regenerants are usually sterile plants, but individual seeds can sporadically form in their fruits. There is no accurate information about the ploidy and genetic status of seeds collected from *Capsicum* anther-derived haploids. The research presents the morphological and molecular characteristics of the second generation originating from seeds of an anther-derived haploid plant. The aim was to check the level of functional traits of the studied population and to verify the genetic homogeneity of the analyzed materials in the context of their use in the breeding of *Capsicum annuum* L.

2. Materials and Methods

2.1. Plant Material

The second generation of hybrids obtained as a result of crossing the *Capsicum annuum* L.: ATZ line and 'Corno di toro' cultivar were the anther donor material used for the experiment. The plants were cultivated under foil tents in accordance with a standard method for *Capsicum annuum* L. culture. Flower buds were collected in the second half of June 2018. The buds were rinsed in 70% ethyl alcohol and were then surface sterilized in 5% calcium hypochlorite (CaCl_2O_2) for 15 min and, next, rinsed three times with sterile distilled water. Anthers isolated from two buds were placed on a Petri dish, with their inner part facing the medium, with a total number of 100. 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, the anther cultures were incubated in the darkness at the temperature of 35°C . Then, the dishes were exposed to a 12-h photoperiod, at the temperature of 25°C . After 14 days, the anthers were transferred onto R1 medium (0.1 mg dm^{-3} KIN). The experiment was carried out for 4 months. The embryos occurring in anther cultures were transferred onto the V3 medium without growth regulators [13]. Well-developing plants were then planted into the peat substrate and acclimatized in the glasshouse.

In order to compare anther-derived haploid and diploid plants, all fruits were considered. In the evaluation process of the sexual progeny of diploid (plant numbers: 1–10) and apomictic, diploid progeny of haploid (plant numbers: 11–18), three fruits of each plant were used. Their phenotypic analysis included the measurement of the most important agromorphological traits of pepper fruits: weight, length, width, pericarp thickness, fresh seed weight, and seeds number. The average values and standard deviation were calculated for the above-mentioned traits. The achieved results were analyzed statistically by means of one-way analysis of variance and Scheffé's test. The results were deemed statistically significant at $\alpha = 0.05$.

2.2. Ploidy Evaluation

The ploidy of the regenerants was determined by means of a Partec CCA flow cytometer equipped with a high-pressure lamp HBO-100W (Partec GmbH, Münster, Germany). In each sample, at least 5000 cell nuclei were analyzed at a flow ratio of 20 nuclei s^{-1} . The samples for analysis were prepared to follow the procedure described by Galbraith et al. [14]. 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 the form of histograms and were analyzed with Partec DPAC V.2.2 software.

2.3. PCR-RAPD, PCR-ISSR Analysis

Young and fresh leaves of fully developed plants were used in the molecular analysis. The extraction of genomic DNA was performed from 100 mg of plant tissue using the Sigma GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Saint Louis, Missouri, USA). On the basis of the previous research in our laboratory, 10 RAPD primers and 16 ISSR primers were used in the study. The progeny of the diploid regenerant (plant numbers: 1–10) and haploid anther-derived regenerant (plant numbers: 11–18) were analyzed with the application of the RAPD technique. ISSR markers were used to assess the genetic stability of the second generation derived from the haploid regenerant. The PCR reactions were carried out using the ATC401 Thermal Cycler (Nyx Technik Apollo, San Francisco, CA, USA) in a 20 µL reaction volume containing 20 ng of genomic DNA as the template, 20 mM MgSO₄, 0.25 µM of primer, 200 µM of each dNTPs, and 0.5 unit of Taq polymerase (A&A Biotechnology, Gdańsk, Poland). The RAPD-PCR reaction was conducted in accordance with the method developed from Ilbi [15] for *C. annuum* L. The initial denaturation was carried out at 91 °C for 1 min and was followed by 40 cycles consisting of 15 s at 91 °C, 15 s at 42 °C, and 1 min 10 s at 72 °C. For ISSR primers, the initial denaturation was also carried out at 91 °C for 1 min and followed by 40 cycles consisting of 30 s at 91 °C, 1 min at 48.5–67.2 °C, and 2 min at 72 °C. The final extension was completed for 5 min at 72 °C for both the RAPD and ISSR techniques. The reactions were carried out twice. The products of the PCR-RAPD and PCR-ISSR reactions were separated in 1.8% agarose gel stained with ethidium bromide. Electrophoresis was carried out for two hours at a constant voltage of 100 mV. The size of the obtained products was determined using the molecular mass marker GeneRuler DNA Ladder Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the computer program GelAnalyzer 2010a (gelanalyzer.com, June 2020). The obtained DNA fragments were visualized and recorded on a Gel Doc 2000 UV transilluminator (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. Cytological and Agromorphological Characteristics of the Progeny of Anther-Derived Plants

In the process of androgenesis induction, individual plants of the hybrid (*C. annuum* L. ATZ × *C. annuum* L. Corno di toro)F₂ were used. In the anther cultures of one of the analyzed donor plants, six embryos were obtained, but only two of them were converted into plantlets (Figure 1).

Figure 1. In vitro anther culture of peppers: (A) anther donor plants of (*Capsicum annuum* L. ATZ × *Capsicum annuum* L. Corno di toro)F₂, (B) embryo conversion of morphological anomalies, (C) deformed cotyledons, (D) callogenesis on the leaves, and (E) androgenic regenerant.

The cytometric analysis of the regenerants showed that one plant was haploid, and the other was diploid (Figure 2).

Figure 2. Anther-derived plants of (*C. annuum* L. ATZ × *C. annuum* L. ‘Corno di toro’) F_2 : diploid on the left and haploid on the right.

As a result of the culture of haploid and diploid anther-derived plants, ripe fruits were harvested (Figure 3). In ten fruits of the haploid plant, eight normal-looking seeds were found, and all of them gave diploid seedlings. All of the eight plants reached full physiological development and formed fruits with seeds. In the case of the diploid progeny of andro-haploid regenerant (plant numbers: 11–18), the big variation of the considered traits should be emphasized. Plants marked with numbers 12, 17, and 18 differed from the other five in terms of fruit weights and lengths (Table 1). In their case, all analyzed fruit traits had the lowest values among those recorded in the experiment. The average fruit weight of plants 12, 17, and 18 was 83 g, and the fruit length and width were 103 mm and 53 mm, respectively. However, other plants constituting the diploid progeny of andro-haploid regenerant marked with the numbers 11, 13, 14, 15, and 16, were characterized by high values of the tested utility traits. Their fruits had a weight of 152 g, length 170 mm, and width of 60 mm. At the same time, it was a group of regenerants most aligned in terms of the analyzed phenotypic features. From this group of plants, the progeny with the best utility parameters was selected for further analysis to examine the stability of the studied traits and to verify the genetic homogeneity in the next generation. The sexual progeny of the anther-derived diploid plants (marked 1–10) had fruits weighing 143 g, and their length and width equaled 134 mm and 62 mm. In terms of most of the assessed morphological features, they were equal to the plants marked 11, 13, 14, 15, and 16, constituting the diploid progeny of andro-haploid regenerant. The fertility of the plants, measured as the seed number per fruit, as well as the seed weight and wall thickness, was similar in both assessed groups of plants: 1–10 and 11–18.

Figure 3. DNA content in cells of two anther-derived plants of (*C. annuum* L. ATZ × *C. annuum* L. Corno di toro)F₂: haploid (1C DNA) histogram and fruits on the left and diploid (2C DNA) histogram and fruits on the right.

Table 1. Variation of fruit traits of the sexual progeny of diploid (plants 1–10) and apomictic progeny of haploid (plants 11–18) *C. annuum* L.

Fruit Trait:	Progeny of Diploid Plant	Progeny of Haploid Plant	
	Plants: 1–10 Mean ± SD	Plants: 11, 13, 14, 15, and 16 Mean ± SD	Plants: 12, 17, and 18 Mean ± SD
Weight (g)	143 ^a ± 21	152 ^a ± 18	83 ^b ± 28
Length (mm)	134 ^b ± 27	170 ^a ± 10	103 ^c ± 34
Width (mm)	62 ^a ± 7	60 ^{ab} ± 6	53 ^b ± 8
Wall thickness (mm)	4.96 ^{ns} ± 1.47	4.81 ^{ns} ± 0.57	4.24 ^{ns} ± 1.60
Wet seed weight (g)	3.24 ^{ns} ± 1.08	3.30 ^{ns} ± 0.39	3.06 ^{ns} ± 1.10
Seeds number	220 ^{ns} ± 60	239 ^{ns} ± 75	197 ^{ns} ± 66

* a–c Statistically homogeneous groups; ^{ns} not significant.

3.2. Molecular Analysis of Progeny of Anther-Derived Plants

Pepper plants constituting the progeny of diploid androgenic regenerant (plants 1–10) and progeny of haploid androgenic regenerant (plants 11–18) were analyzed with the application of 10 RAPD primers (Table 2). As a result of the reactions, a total of 83 products were obtained, and 10 (12%) of them were polymorphic bands (Figure 4).

The number of bands generated per primer varied from 9 (A06) to 16 (A17 and B10). The approximate size of the amplified products ranged from 102 to 2591 bp, while the percentage of polymorphism varied between 0% (A10, A17, and A19) and 25% (A11), with an average of 12%. No polymorphic products were observed between ten plants of the progeny of anther-derived diploid (marked 1–10). In the same analysis of the apomictic progeny of the anther-derived haploid, ten differences were found as a result of the reactions carried out with primers A06 (1032 bp.), A11 (482 bp. and 674 bp.), A14 (359 bp. and 863 bp.), AE10 (442 bp.), AE11 (519 bp.), AE19 (784 bp. and 956 bp.), and B10 (818 bp.). The observed differences applied to plants 12, 17, and 18.

Table 2. Qualities of the RAPD primers applied in the molecular analyses.

Primer	Sequence (5'-3')	Number of Products	Range of Product Size	Polymorphic Products	Percentage of Polymorphism
A06	GGTCCCTGAC	9	167–1382 bp	1	11.1
A10	GTGATCGCAG	13	123–1421 bp	-	0
A11	CAATCGCCGT	14	240–1487 bp	2	25
A14	TCTGTGCTGG	10	251–1295 bp	2	20
A17	GACCGCTTGT	16	234–2591 bp	-	0
A19	CAAACGTCGG	13	156–1681 bp	-	0
AE10	CTGAAGCGCA	15	212–2154 bp	1	6.7
AE11	AAGAACGGGA	14	106–2040 bp	1	14.3
AE19	ACGGCGTATG	13	295–2482 bp	2	15.4
B10	CTGCTGGGAC	16	102–1841 bp	1	6.3
Total	10	83	102–2591 bp	10	12%

Figure 4. Products of the RAPD-PCR with primers: AE10, AE11, and B10; M- marker, 1–10 progeny of anther-derived diploid of (*C. annuum* L. ATZ × *C. annuum* L. Corno di toro) F_2 , 11–18 progeny of anther-derived haploid of (*C. annuum* L. ATZ × *C. annuum* L. Corno di toro) F_2 .

The ISSR technique was used for the genetic analysis of the second generation of the diploid plants derived from the andro-haploid regenerant (Table 3). As a result of the analyses carried out with 16 ISSR primers, the total number of 97 monomorphic products were obtained, and their sizes ranged from 123 to 1832 bp. The number of bands generated per primer varied from four (I 11) to nine (I 9). All the amplified products showed the genetic homogeneity of the second generation of the diploid plants derived from the andro-haploid regenerant.

Table 3. Qualities of the ISSR primers applied in the molecular analyses.

Primer	Sequence (5′–3′)	Number of Products	Range of Product Size	Monomorphic Products
I 1	(GA) ₈ YC	5	221–562	5
I 2	(GACA) ₄ A	6	212–1005	6
I 4	(AG) ₈ YC	5	502–1326	5
I 5	(CTC) ₄ YC	7	167–1010	7
I 7	(TGAG) ₄	6	408–1184	6
I 9	(GAG) ₃ GG	9	397–1623	9
I 10	(GA) ₈ YT	6	203–896	6
I 11	(CT) ₈ GC	4	508–843	4
I 14	(AG) ₈ T	5	217–777	5
I 26	(CA) ₈ AT	6	345–832	6
I 27	(GA) ₈ CT	5	189–989	5
I 28	(CA) ₈ G	5	225–536	5
I 33	(GA) ₈ CTC	8	228–1497	8
I 56	(GA) ₈ G	7	180–1832	7
I 63	(CCCT) ₄	7	396–1122	7
I 65	(GA) ₈ C	6	123–964	6
Total	16	97	123–1832 bp	97

4. Discussion

Androgenesis in in vitro anther cultures has been used for several decades to induce haploid and dihaploid pepper regenerants. Despite the considerable number of studies conducted with pepper anther culture, the reported effectiveness of the process is still low. Additionally, even if there are androgenic embryoids, they do not always regenerate into fully developed plants. The authors also noted the presence of a number of aneuploid plants among the regenerants. In the experiment, growth disorders and morphological anomalies were observed in four androgenic regenerants that did not undergo acclimatization to in vivo conditions. These disorders included the formation of thickened deformed cotyledons, the lack of proper leaf formation, or the development of callus on the stems and leaves, which limited the further proper growth of the plants. Ultimately, one haploid and one diploid regenerant developed into plants that reached full physiological maturity and formed fertile fruits. The presence of diploids among androgenic regenerants is characteristic of peppers [16]. In a study on *C. annuum* L. conducted by Al Remi et al. [17], the rate of spontaneous doubled haploids was identified as 6%, and in the experiment carried out by Gemesne Juhász et al. [18] the share of diploid regenerants equaled 29.8%. Niklas-Nowak et al. [19] reported that 49% of plants obtained through anther culture were diploid both in the anther cultures of the second generation of *C. annuum* L. hybrids and in the case of interspecies hybrids (*C. frutescens* × *C. chinense*)F₂. Additionally, the research conducted for the first generations of the *C. annuum* hybrids and interspecies hybrids (*C. frutescens* × *C. chinense*)F₁ and (*C. frutescens* × *C. baccatum*)F₁ revealed comparable numbers of haploids and diploids among the regenerants [20]. As it is emphasized in the professional literature, in the case of diploid regenerants, molecular techniques are used as the standard for their identification and for the precise analysis of genetic diversity, with the RAPD method being considered an effective, rapid, and cheap solution [21]. Bhadrageoudar and Patil [22] conducted studies on 45 genotypes of *C. annuum* L. and obtained 63% of products of a differentiating nature. Tilahun et al. [23], assessing 30 genotypes of *C. annuum* L., obtained 83% of the polymorphic products. Polymorphism at the level of 26% was observed in the study of Shapturenko et al. [24] in the analysis of 10 *C. annuum* L. lines. In the presented experiment, in which the progeny of two androgenic regenerants obtained in hybrid anther cultures (*C. annuum* L. ATZ × *C. annuum* L. ‘Corno di toro’)F₂ were studied, 10 RAPD primers were used. In the case of sexual offspring of the diploid regenerant, only monomorphic products were obtained, which confirmed the thesis about the spontaneous doubling of chromosomes and the androgenic origin of the anther-derived diploid. In contrast, seven

of the used primers generated 10 polymorphic products that showed genetic diversity within the progeny of the haploid androgenic regenerant. The professional literature provides no information about seed formation by anther-derived haploids. In the experiment, eight seeds were collected from ten fruits of the haploid regenerant obtained in anther cultures of (*C. annuum* L. ATZ × *C. annuum* L. Corno di toro)F₂. They all germinated and developed into eight plants that reached full physiological maturity. Morphological and molecular analyses allowed to distinguish two groups among them. One consisted of three plants with reduced utility parameters, for which PCR-RAPD reactions showed the presence of 10 polymorphic products, whereas the other five plants constituted a homogeneous group of molecular and phenotypically valuable utility parameters. The progeny of these plants was analyzed in the following year using the ISSR marker system to check their genetic homogeneity. The analysis of ISSR polymorphism was successfully used by Lijun and Xuexiao [25] to assess the genetic diversity of genotypes of *C. annuum*, *C. chinense*, *C. baccatum*, and *C. pubescens*. In the study by Ahmed [26], the polymorphism generated by the ISSR primer enabled the identification of hybrids of *C. annuum* and *C. frutescens*. Thul et al. [27], in a study of the diversity of 22 genotypes of *C. annuum*, *C. baccatum*, *C. chinense*, *C. eximium*, *C. luteum*, and *C. frutescens* species alongside RAPD primers, used ISSR markers as an alternative. The polymorphism generated by both primer groups did not differ significantly, with 67% for RAPD markers and 62% for ISSR. However, the authors indicated the ISSR technique was more effective. Similar observations were made by Shapturenko et al. [24]. As a support for the RAPD technique, the authors used the ISSR marker system. The obtained polymorphism remained approximately level (29%); however, similar to the results of Thul et al. [27], the second method was found to be more effective. In the presented experiment, 16 ISSR primers were used, and 97 monomorphic products were obtained, which showed genetic homogeneity of the offspring of anther-derived haploid regenerant. This is valuable information, because it extends the use of androgenic pepper regenerants in breeding programs and proves that anther-derived haploid plants of *Capsicum* can be the source of diploid and apomictic seeds, and the obtained offspring may constitute genetically stable breeding material.

5. Conclusions

It can be concluded that androgenesis in anther cultures in vitro is an effective method of stabilizing the genetic variability characteristics of hybrid generations of donor plants. Moreover, the induction of androgenic embryos significantly shortens the time necessary to achieve fully homozygous breeding material. In the case of peppers, these can be both spontaneously regenerated diploids, as well as haploid regenerants. On the basis of the obtained results, it was shown that apomictic offspring of haploid pepper regenerants are able to produce diploid seeds from which plants of DH lines develop constituting genetically stable material. The genetic homogeneity of the plants was confirmed by the ISSR molecular technique, while their utility value was demonstrated on the basis of the agro-morphological characteristics. The breeding material selected during the conducted experiment is currently under evaluation at The Research Center for Cultivar Testing.

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