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IN VIVO POLYEMBRYONY INDUCTION IN SPECIES OF *CAPSICUM*

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Haploid plants after doubling the chromosomes can give rise to diploid homozygote lines, which can be used as DH lines in breeding new varieties or as initial plant material in creating F_1 hybrids. This work studied natural polyembryony and the effect of growth regulators on induction of polyembryonic seeds and haploid embryogenesis in five species of the genus *Capsicum*. Water solutions of the following growth regulators were used: 2,4-D (2,4-dichlorophenoxyacetic acid) and BNOA (beta-naphthoxyacetic acid) at 0.001% used separately or combined with BAP (benzylamino-purine). Twin seed frequency was highest in *C. chinense* and lowest in *C. baccatum* var. *pendulum*. In *C. annuum* the share of twin embryos was highest in the 'Corno di toro' variety; 2,4-D clearly increased the number of twin plants only in 'Corno di toro' seeds. Treatment with combinations of 2,4-D or BNOA with BAP increased the frequency of polyembryonic seeds in the 'ATZ1' line. In *C. frutescens* the frequency of polyembryony increased following application of BNOA with BAP. Of all the seeds tested, seven haploid plants were obtained, representing *C. frutescens*, *C. chacoense* and *C. baccatum* var. *pendulum*. The differences in the frequency of polyembryony in the studied genotypes points to genetic control of this phenomenon. The presence of monoploid plants definitely depends on the genotype, as a high frequency of polyembryony is not always accompanied by a high share of haploid plants. For most genotypes evaluated, the effect of growth regulators was disadvantageous, resulting in a considerable decrease in the share of twin plants among germinated seeds.

Key words: *Capsicum* spp., growth regulators, haploid, pepper, polyembryony.

INTRODUCTION

Obtaining haploid plants of the genus *Capsicum* has been a difficult and complex process. The development of biotechnological methods, especially intensive over the past few years, has enhanced the potential for obtaining haploid plants. Anther culture in vitro makes it possible to obtain haploids by androgenesis (Maheshwari et al., 1982). Research into this phenomenon in pepper (Dumas de Vaulx et al., 1982; Morrison et al., 1986; Gemesne et al., 1998; Venczel et al., 1998) determined the conditions for obtaining plants in cultures in vitro, thanks to which androgenesis is more and more frequently used in many pepper breeding programs (Matsubara et al., 1992; Mitykó et al., 1995a,b). The procedures developed for obtaining haploid plants in *Capsicum annuum* L. (Sibi et al., 1979; Dumas de Vaulx et al., 1981) are most applicable in this species only. The largest number of plants regenerated in anther culture was reported by Mitykó and Fari (1995a), who obtained 102 plants in vitro from 100 'Feherozon' anthers. Gemesne et al. (2001) and Gyulai et al. (2000) researched genotypes representing *C. annuum* species; in that work the percentages of regenerants obtained via androgenesis ranged from a few to the low teens. The literature offers little information on the frequency of androgenesis in other *Capsicum* species and hybrids. The procedures developed for annual pepper are not as effective as in other species.

A major factor affecting the formation of haploid embryos is the maternal plant genotype (Venczel and Mitykó, 1995; Mitykó and Fari, 1997; Matsubara et al., 1998; Venczel and Gemesne, 1998). Other important factors are the microspore development stage and the anther culture method, especially temperature, light, and donor plant growth conditions (Kristiansen and Andersen, 1993). Results reported by Vagera (1990) and Regner (1996) indicate that anthers collected from plants grown in conditions optimal for pepper yield better in vitro results than anthers from plants grown outside the natural vegetation period.

Polyembryony is an alternative source of plants with a reduced number of chromosomes. Additional embryos can be obtained from different cells and tissues of both the sporophyte and the gametophyte,

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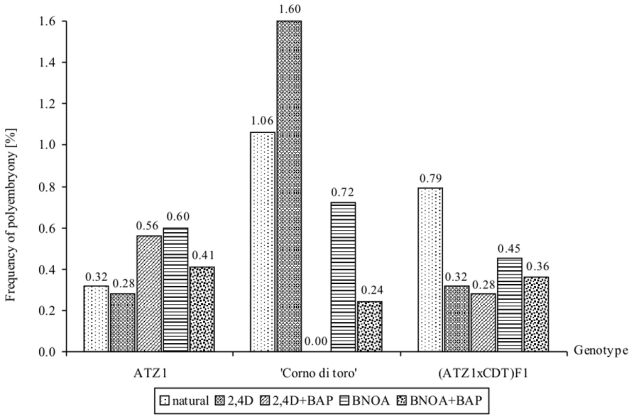

Fig. 1. Frequency of natural and induced polyembryony in *Capsicum annuum* L. genotypes.

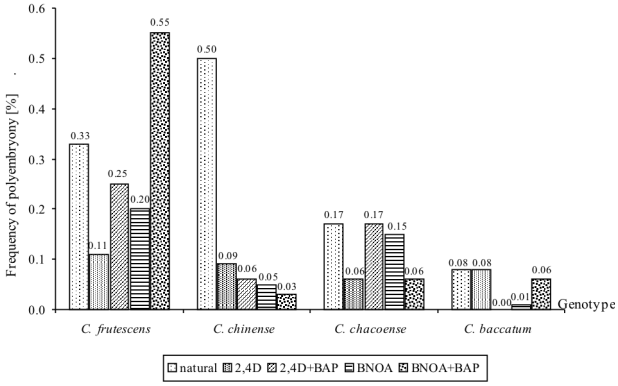

Fig. 2. Frequency of natural and induced polyembryony in other *Capsicum* species.

from haploid, diploid and polyploid cells. They can be the result of a sexual process, or not directly connected with this process. After doubling the number of chromosomes, haploid plants can give rise to diploid homozygote lines which can be used as DH lines for breeding new varieties or as initial plant material for creating F_1 hybrids. Selecting forms especially prone to produce polyembryonic seeds provides another tool for development of varieties. The natural tendency to create polyembryonic seeds is variety- and species-specific (Morgan and Rappleye, 1954).

Some growth regulators increase the number of additional embryos in seeds. The first records on the effect of growth substances on polyembryony frequency concerned *Eranthis hiemalis* L., a species with a distinctive course of embryo development. Embryos are formed only a few months after the seeds reach maturity once they are in the soil. Haccius (1955) obtained a significant increase in the number of polyembryos after treatment with 2,4-D.

The work studied the frequency of polyembryony in selected *Capsicum* genotypes, and evaluated the effect of growth regulators on the frequency of occurrence of twin plants and the share of haploid plants.

MATERIALS AND METHODS

The experimental material comprised seeds of selected *Capsicum* genotypes: *C. annuum* L. – line 'ATZ1', 'Corno di toro' (CDT) variety, and the F_1 hybrid of them (ATZ1 × CDT); and other species – *C. frutescens* L., *C. chinense* Jacq., *C. chacoense* A.T. Hunz. and *C. baccatum* L. var. *pendulum* L.

Growth regulator treatments were made during plant growth in an unheated foil tent. Flower buds of each genotypes were immersed in 0.001% water solutions of growth regulators. Two groups of growth regulators were used: auxins (2,4-D, BNOA) and the cytokinin BAP. The auxins were used separately or combined with BAP. Thirty plants of each genotype were cultivated in a foil tent, and four plants for each genotype and each growth regulator were randomly selected for treatment. The effect of growth regulators on flower buds and on the presence of polyembryonic seeds was evaluated following the methods developed by Nowaczyk et al. (1999). Twin plants were transferred to pots filled with peat substrate and placed in the greenhouse. In the second half of May the plants were transferred to the foil tent. The frequency of polyembryony was calculated as the percentage of twin plants versus the number of germinated seeds.

Ploidy level was evaluated in all the twin plants indicating phenotypic variation, to identify the haploid forms. Indirect methods of evaluating ploidy (length and width measurements of guard cells and pollen grains) were used in addition to the basic method of flow cytometry. The plant material was prepared according to Galbraith et al. (1983) with slight modifications. The external standard was young leaves of diploid *Capsicum* species (2n=2x=24). Leaf fragments were chopped with a razor blade in a Petri dish with 1 ml buffer isolating the nuclei (0.1 M Tris, 2.5 mM $MgCl_2 \times 6H_2O$, 85 mM NaCl, 0.1% Triton X–100; pH 7.0), supplemented with DAPI (4',6-diamidino-2-phenylindole) fluorochrome stain (2 μg/ml). The suspension was filtered through a 50 μm mesh nylon filter. Measurements used a Partec CCA flow cytometer (Münster, Germany) with a mercury UV lamp. Analyses used at least 5000 nuclei, and the results are presented as histograms, analyzed with DPAC V.2.2 software.

RESULTS

FREQUENCY OF NATURAL POLYEMBRYONY

The frequency of polyembryonic seeds differed between the analyzed genotypes (Figs. 1, 2). The

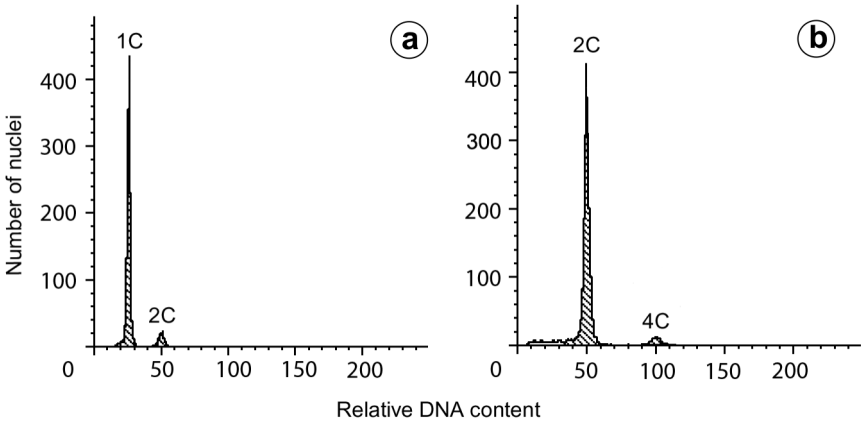

Fig. 3. Nuclear DNA content in leaf cells of twin plants. (**a**) Haploid, (**b**) Diploid.

results for natural polyembryony were the control against which the effect of growth regulators was compared. Of all the species, the frequency of twin seeds was highest for *C. chinense* and lowest for *C. baccatum* var. *pendulum*. For the hybrid (ATZ1 × CDT) F_1 the frequency of occurrence of polyembryonic seeds (0.79%) was between the frequencies of the parental forms. The ATZ1 maternal form yielded half that number of twin seeds, and the pollinator's yield was almost 1.5-fold higher. The 'Corno di toro' variety had the highest share of twin seeds of all the genotypes representing *C. annuum* species (Fig. 1).

EFFECT OF GROWTH REGULATORS ON POLYEMBRYONY FREQUENCY

For most evaluated genotypes the effect of growth regulators was disadvantageous, considerably reducing the share of twin plants among the germinated seeds. 'Corno di toro' increased the number of polyembryonic seeds versus the control in material from plants whose flower buds were treated with 2,4-D. In contrast, the frequency of polyembryonic seeds decreased threefold in *C. frutescens* and fivefold in *C. chinense*. Application of 2,4-D with BAP almost doubled the share of twin forms in the 'ATZ1' line (Fig. 1), but reduced the share of twin forms by eightfold versus the control *C. chinense* (Fig. 2). No polyembryonic seeds were found among the germinating seeds of *C. annuum* var. 'Corno di toro' and *C. baccatum* var. *pendulum*. BNOA almost doubled the share of twins in seeds of line 'ATZ1' versus the control. The combination of BNOA with BAP was most effective for *C. frutescens* (Figs. 1, 2).

TABLE 1. Size (μm) of guard cells and pollen grains in n–n and n–2n twin plants

Genotype	Plant symbol	Guard cell		Pollen grain	
		length	width	length	width
C. frutescens	1a n	8.8	6.2	8.1	6.5
	1b 2n	11.6	8.8	14.5	7.4
C .frutescens	2a n	8.2	5.8	7.6	5.8
	2b 2n	11.4	8.2	14.1	7.2
C. chacoense	1a n	8.9	5.3	7.8	6.5
	1b 2n	13.5	8.9	15.9	8.4
C. chacoense	2a n	9.3	5.6	8.0	7.0
	2b 2n	13.8	9.3	16.1	8.6
C. baccatum var. *pendulum* (2,4-D)	1a n	10.8	6.7	15.0	7.7
	1b n	10.3	6.4	14.6	7.4
C. baccatum var. *pendulum*	2a n	11.1	7.3	14.8	7.6
	2b 2n	11.9	9.2	16.0	8.2

HAPLOID PLANTS

Seven haploid plants were obtained from all the tested seeds. They originated from two-embryo seeds of *C. frutescens*, *C. chacoense* and *C. baccatum* var. *pendulum*. The *C. baccatum* var. *pendulum* seeds produced a single pair of haploid twins, which came from the 2,4-D treatment. The other twin pairs followed a pattern: haploid-diploid, and originating from natural polyembryony. Length and width measurements of guard cells and pollen grains made the initial identification of haploid plants. Table 1 presents the measurements of haploid and diploid twin plant pairs. Flow cytometry established the final identification. Figure 3 gives the histograms of nuclear DNA content in leaf cells of haploid (a) and diploid (b) twin plants of *C. frutescens*.

The fruit of each pair was analyzed for total yield, number and mean weight, length, width and fertility (number of seeds per fruit) (Tab. 2). There were clear differences for all parameters in haploid-diploid pairs. Haploid fruit weight did not exceed one gram and the seeds collected contained no embryos. The *C. frutescens* 2a haploid plant did not

TABLE 2. Yield, fertility and morphological characteristics of n–n and n–2n twin plant fruits

Genotype	Plant symbol	Total yield [g]	Number of fruits	Fertility	Weight of fruit [g]	Length of fruit [mm]	Width of fruit [mm]
C. frutescens	1a n	2	3	1	0.8	21	12
	1b 2n	926	23	275	45.1	68	48
C. frutescens	2a n	Fruits absent					
	2b 2n	240	13	68	18.5	38	34
C. chacoense	1a n	1	5	–	0.3	9	6
	1b 2n	80	67	55	1.2	29	11
C. chacoense	2a n	18	188	5	0.2	10	7
	2b 2n	402	409	61	1.0	28	11
C. baccatum var.*pendulum* (2,4-D)	1a n	21	97	158	0.3	36	6
	1b n	40	173	130	0.4	35	6
C. baccatum var. *pendulum*	2a n	20	44	48	0.5	31	5
	2b 2n	678	162	98	4.2	94	17

set fruits, while the haploid-haploid twin pair had many fruits with embryoless seeds. Haploids had smaller flowers and leaves, and the whole plant was light green and smaller than the twin diploid partner.

DISCUSSION

The results add details to our understanding of natural and induced polyembryony in *Capsicum* species. Of all the analyzed genotypes, the share of polyembryos was highest in the 'Corno di toro' variety, representing *C. annuum*, and lowest in *C. baccatum* var. *pendulum*. Our results for genotypes of *C. annuum* species were similar to those reported in literature (Novak and Betlach, 1969; Nowaczyk and Nowaczyk, 1989). Morgan and Rappleye (1954) suggest that the differences in polyembryony frequency between varieties indicate genetic control of it, as supported by their studies of *C. frutescens*. In some varieties of it they observed variation in the frequency of polyembryos from 0.06% to 0.65%. In our study the frequency was 0.33% for this species. Besides diploid twin seedlings, haploid twin as well as haploid/diploid twins were found.

The literature seems to offer no information on the frequency of occurrence of polyembryonic seeds in wild species of *C. chinense*, *C. chacoense* and *C. baccatum* var. *pendulum*, so it was difficult to verify our results for these genotypes. To evaluate the shares of polyembryos in these species we compared them with data reported for *C. annuum* and *C. frutescens*, in view of the relationship between with wild species and these genotypes. The frequency of twin seeds for *C. chinense* was high and similar to that reported in literature for *C. annuum*. The results for the five studied genotypes support the conclusion that the tendency to polyembryony may be genetically based.

In hybrids the frequency of polyembryony probably can be controlled by selecting the parents used for crossing. Proper selection of forms showing a tendency to polyembryony should yield hybrids with a high share of polyembryonic seeds.

Type and concentration of growth regulators were selected based on information from the literature on polyembryony in *C. annuum* and *Lycopersicon esculentum* Mill. (Nowaczyk, 1990; Nowaczyk and Nowaczyk, 1996); 2,4-D had a considerable effect in increasing the share of twin forms in those species. A similar effect is also noted for BNOA. These auxins are used in culture to stimulate an increase in callus tissue in vitro. In eggplant and pepper species, Matsubara et al. (1992, 1998) found that treatment with 2,4-D combined with kinetin increased callus tissue and boosted embryo development. In anther and microspore cultures of *Capsicum* species, 2,4-D is a component of CP induction medium (Dumas de Valux et al., 1981).

The genotypes differed in their response to the growth regulators used. 2,4-D clearly increased the number of twins in 'Corno di toro' seeds. The frequency of polyembryony increased versus the control value in 'ATZ1' line seeds following the application of 2,4-D combined with BAP, BNOA alone, and BNOA combined with BAP, while in the (ATZ1 × CDT) F_1 hybrid all the growth regulators reduced the share of polyembryonic seeds. In *C. frutescens* the frequency of polyembryony rose only in the BNOA/BAP treatment, to 0.55% (control: 0.33%). For the other genotypes the share of polyembryonic seeds in treatments was less than that of the control. Such reactions in the tested wild species must have been due to inappropriate concentrations of regulators.

When the growth regulators increased the frequency of twin seeds they also reduced the number of seeds per fruit, as in other reports on *Capsicum*

species (Nowaczyk and Jędrzejczyk, 1999; Jędrzejczyk and Nowaczyk, 2002), tomato and cucumber (Nowaczyk and Nowaczyk, 2000a,b).

In studies of polyembryony is it difficult to keep the plants obtained from polyembryonic seeds alive. Out of 386 twin pepper seedlings, Christensen and Bamford (1943) cultivated 242 plants (62%) to flowering. In *C. frutescens*, Morgan and Rappleye (1950) brought 58% of their twin seedlings to physiological maturity. We obtained 804 pairs of twin plants and 10 triplets; 325 pairs of twins and 2 triplets survived for further research. The seedling survival rate was 40% on average. Such low viability must have been due to high competition among the embryos for reserve materials. The additional embryos are smaller and more delicate than the single-embryo seeds, and many seedlings withered. Of all the twin pairs obtained, seven plants were haploid.

Although *C. frutescens*, *C. chacoense* and *C. baccatum* var. *pendulum* showed a low frequency of polyembryony, haploid plants were still found. The share of them was low due to lower viability, in turn due to poorer vigor, smaller organs and thus poorer growth. Such plants had much less chance to survive in the environment than diploids. The research conditions are also important, mostly in the first period of the growing season when haploids are at the seedling stage. Success also hinges on the quality of the tested seeds, which should be free of fungal diseases. The frequency of monoploid plants definitely depends on the genotype, as a high frequency of polyembryony is not always accompanied by a high share of haploid plants, as confirmed by Novak and Betlach (1969), Nowaczyk and Nowaczyk (1989) and our results.

Haploid plants obtained from control seeds accompanied n–2n sexual embryos. There was also a twin pair in which both embryos were haploid. They represented *C. baccatum* var. *pendulum* and appeared among seeds originating from flowers treated with 2,4-D. It was the only such pair in all the genotypes studied, so an effect of the regulator on induction haploid embryogenesis seems doubtful in this case. The appearance of the n–n twin pair may have been accidental. In research reported by Nowaczyk and Nowaczyk (1989), however, it was this auxin that gave haploids in varieties of *C. annuum* species. Its effectiveness must depend on the genotype.

In determining the origin of haploids one can refer to literature reports. Dumas de Valux (1977) claims that n–2n twin pairs can originate from a synergid and fertilized egg cell, whereas twins with both plants haploid can be formed from a synergid and unfertilized egg cell or as a result of division of the synergid. Morgan and Rappleye (1950, 1954) state that haploids in twin pairs are formed from unfertilized elements of the embryo sac, probably from the synergid. All the haploids produced in our experiments were sterile, since the seeds found in fruits did not have embryos. Novak and Betlach (1969) suggest that, as in most other species, the sterility of pepper haploids is conditioned by inadequate meiosis. Chromosome division is severely disturbed so that the gametes contain an aneuploid number of chromosomes.

Still limiting the use of pepper haploids in genetic research and in breeding is their low number and fertility before and after chromosome doubling. The length of the period needed to produce haploid plants, and the labor and costs involved, are also significant.

Among the diploid pepper plants there may have been plants produced by spontaneous diploidization of haploid plants. This phenomenon was observed and described in *Brassica* species in vitro. Investigating broccoli (*Brassica oleracea* L. var. *italica*), Keller and Armstrong (1983) obtained 53% haploids among the regenerants, accompanied by diploid and tetraploid plants; they suggested that the diploids that appeared may have been produced by spontaneous diploidization of haploid microspores during anther culture. In androgenic broccoli plants, Farnham (1998) reported haploids, diploids, triploids, tetraploids, octoploids and aneuploids. Most of the regenerant population was diploid (53–56%). Kaminski et al. (2004, 2005) had similar findings in culture of Brussels sprouts (*Brassica oleracea* L. var. *gemmifera*) anthers, and Gu et al. (2003) noted 70% diploids among *Brassica rapa* ssp. *chinensis* regenerants from microspore culture. Such double haploids as well as tetraploids can be used in breeding *Brassica* plants.

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