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PAN POLSKA AKADEMIA NAUK ODDZIAŁ W KRAKOWIE

SCREENING OF *ASPARAGUS OFFICINALIS* L. SEEDS FOR OCCURRENCE AND PLOIDY OF TWIN EMBRYOS

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We used germination tests to assess the frequency of polyembryony in 9 asparagus cultivars with a high propensity to produce double embryos with different ploidy levels: Alpha, Andreas, Boonlim, Cipres, Eposs, Helios, Limbras, Ravel and Sartaguda. Twin embryos inside a single seed were found in 3 cultivars: Eposs 2n, Ravel 2n and Sartaguda 2n, at 0.60% frequency (15 seeds with twin embryos out of 2500 seeds). Of 30 obtained seedlings, 14 were separated diploid-diploid twins, 6 were conjoined diploid pairs, 8 were separated diploid-haploid and 2 were diploid-haploid pairs conjoined in the hypocotyl region. Some embryos showed unilateral dominance of one embryo (size and shape). The haploid status of the smallest embryo was confirmed by chromosome number (n=x=10) and flow cytometry (nuclear C DNA amount 1.95 pg). The haploid obtained in this manner possessed enough vegetative vigor to undergo chromosome doubling.

Key word: Polyembryonic seeds, haploid seedlings, *Asparagus officinalis*, flow cytometry, chromosome number, morphological analysis.

INTRODUCTION

Asparagus officinalis L. (Liliaceae) belongs to a large genus containing 200 perennial species. Numerous species are used for ornamental purposes [*A. densiflorus* (Kunth) Jessop cv. Plumosus; *A. densiflorus* cv. Sprengeri and *A. falcatus* L.] but the most important agricultural crop is *A. officinalis* L. (Valdes, 1964). About 100 different edible cultivars are grown in Europe and many genotypes are held in local collections (Knaflewski, 1996) and in a large collection of doubled haploids (Ricardi et al., 2011). *A. officinalis* is a dioecious species with homomorphic sex chromosomes. Female plants are homogametic (XX), whereas males are heterogametic (XY). The high yield and homogeneity of male cultivars (with XY karyotype) makes them particularly desirable; molecular markers have been used to identify them prior to flowering (Gebler et al., 2007). Some morpho-agronomic traits of asparagus as a dioecious and perennial plant have been described, such as the structure of shoots, inflorescences, fruits and seeds (Ellison, 1986; Stajner et al., 2002; Ito et al., 2007). The diploid chromosome number of *A. officinalis* is 2n = 2x = 20 but some cultivars are also tetraploid 2n = 4x = 40 (Randall and Rick, 1945; Kunitake et al., 1998). Breeding programs have created the triploid cv. Hiroshima Green (2n = 3x = 30), which has larger shoots and a longer harvest period than diploid cultivars (Ozaki et al., 2004; Moreno et al., 2006).

Asparagus officinalis is becoming increasingly important in agriculture. Breeding efforts to introduce disease resistance from wild relatives of cultivars are therefore needed (Marcellan and Camadro, 1999). In crop breeding programs the role of haploids in creating homozygotic dihaploids is particularly important (Malepszy, 2009; Riccardi et al., 2011). Many positive features can be captured and valuable traits can be obtained through double haploidization and multiple cross combinations with male asparagus plants (Thevenin, 1968; Ellison, 1986; Kunitake et al., 1998) but such processes are labor-intensive, time-consuming and often inefficient. Haploids created by androgenesis often display greater variability, lower fitness and heterozy-

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TABLE 1. Frequency of polyembryonic seeds in asparagus cultivars screened in germination test

Cultivars	Ploidy	Sex*	Number of seeds	Monoembryony %	Number of seeds with twin embryos
Alpha	2n	♀/♂	425	83.7 ± 9.1b	0
Andreas	2n	♂	640	90.0 ± 6.5	0
Boonlim	2n	♂	510	91.8 ± 6.1a	0
Cipres	2n	♀/♂	435	91.1 ± 6.9	0
Eposs	2n	♀/♂	1500	84.6 ± 7.3	8
Helios	2n	♀/♂	400	88.0 ± 7.6	0
Limbras	2n	♀/♂	800	89.1 ± 8.1	0
Ravel	2n	♂	450	92.4 ± 6.2a	5
Sartaguda	2n	♀/♂	550	90.8 ± 6.6	2
Total			5710		15

Data are means ± SD. Different letters indicate significant differences ($P < 0.05$); *Dioecious or male

gosity, so double haploids of asparagus need to be stabilized by vegetative propagation and by in vitro methods (Doré, 1990; Zhang et al.,1994; Ito et al., 2007; Shiga et al., 2009).

Haploids have been obtained in situ parthenogenetically from unfertilized egg cells or after apogamic processes of embryo development from antipodal and synergid cells. The subsequent development of haploid embryos is highly dependent on the endosperm (Zenkteler and Nitzsche, 1984). Monoploids in *Zea* and polyhaploids in *Triticum, Solanum* and *Gossypium* were obtained by apogamy, the suitability of which for breeding was evaluated after double haploidization or stimulation by pollination of pollen inactivated after radiation with UV or γ rays (Malepszy et al., 1989). Gynogenic haploids of *Beta, Helianthus, Oryza* and *Allium* have been successfully produced in breeding programs (Forster et al., 2007). Haploidization also takes place by somatic reduction of chromosomes during mitosis of hybrid cells a few days after double fertilization, which subsequently eliminates male chromosomes from the hybrid embryo and from the endosperm. The main cause of this is loss of balance between the genomes of both partners of distant crosses, as has been well documented in crosses of *Hordeum vulgare* × *Hordeum bulbosum* (Zenkteler and Straub, 1979).

In this study we assessed the frequency of natural polyembryony and the possibility of obtaining haploid plants from screened germination of seeds of asparagus cultivars growing in Polish plantations.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds from 9 randomly selected cultivars of asparagus obtained from the collection of the Department of Vegetable Crops of Poznań University of Life Sciences were analyzed after germination tests for the prevalence of twin embryos (Tab. 1), between 2006 and 2008. The germination data are means ±SD of three replicates.

We made ovule squash trials to find the best screening method and establish the origin of twin embryos. Immature fruits were harvested and three developmental stages of cv. Eposs fruits (equatorial diameter of 0.3, 0.4 and 0.6 cm) compared in order to select the optimal stage for ovule isolation (Fig. 1a–c). Stage 1 (Fig. 1a) proved to be the best size for squashing ovules in 1% acetocarmine. The ovules usually contained a visible single embryo; very rarely we found twin embryos in the same ovule (Fig. 1d).

The germination tests were performed with seeds sown in semi-sterile conditions at 23±2°C, RH 60%, with 1 week of dark incubation. The seeds were surface-disinfected in 70% (v/v) ethanol for 1 min, rinsed five times with sterile distilled water and sown on wet filter paper in 20 cm Ø Petri dishes. The seeds were morphologically screened 14 and 20 days after sowing. The frequency per cultivar and morphology of twin seedlings in one seed were recorded.

ROOTING AND ACCLIMATIZATION

After germination the twin seedlings were separated from seeds, transplanted to plastic pots with a mixture of autoclaved soil with sand and perlite (1:1:0.3/l), then watered and fertilized once a week with 1/2 MS macrosalt solution (Murashige and Skoog, 1962). The potted plantlets were covered with polyethylene bags to maintain high humidity. After 1 week the covers were removed progressively. About 95% of diploid and all haploid plantlets survived and showed normal growth inside the culture room (23°C, 60% RH, 16 h photoperiod). Two

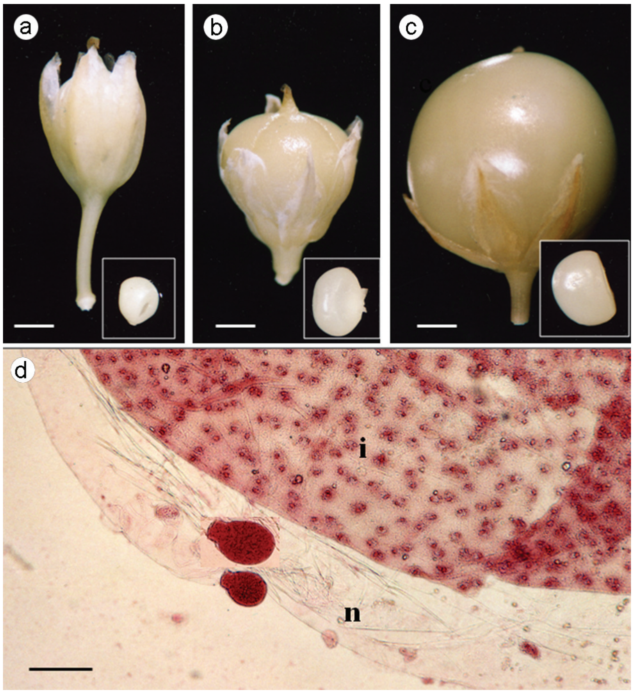

Fig. 1. (**a–c**) Developmental stages of fruits and subsequent ovules of *Asparagus officinalis* cv. Eposs, (**d**) Twin globular embryos outside squashed ovule (fruit of stage a) cv. Eposs. n – nucellus; i – inner integument. Bars = 2 mm in a–c, 30 μm in d.

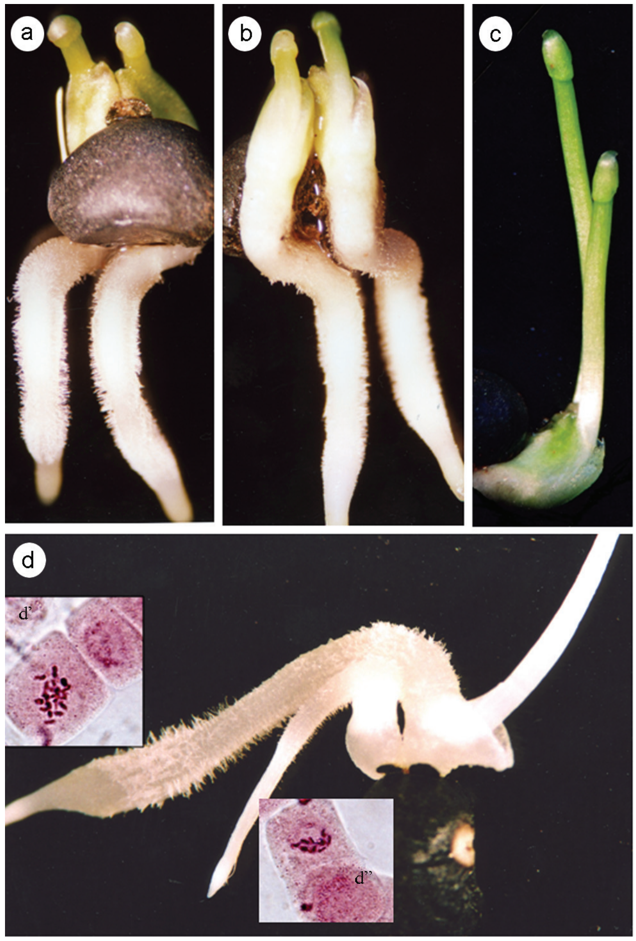

Fig. 2. (**a, b**) Front and rear view of separated diploid/diploid twin seedlings germinated from single seed of cv. Ravel, (**c**) Young phylloclades of diploid/diploid twin pair conjoined at hypocotyl region, cv. Ravel, (**d**) Roots of twins with conjoined phylloclades, cv. Eposs. Mitotic chromosomes of root tip cells of the diploid (d,d' with 20 chromosomes), and haploid (d,d'' with 10 chromosomes). 10×.

months after planting in garden conditions, one spear was removed from each acclimatized plant for flow cytometry (FCM) analysis.

NUCLEAR DNA CONTENT

Juvenile phylloclades collected from twin seedlings were assessed by flow cytometry for identify the haploids and their nuclear DNA amount. *Asparagus* samples together with an internal standard of *Pisum sativum* (2C value 9.11 pg) were chopped with a razor blade in an isolation buffer according to Doležel et al. (1989). After filtering the suspension through 30 μm nylon mesh, measurements were made with a Partec PAS flow cytometer (Partec GmbH, Germany). The UV spectrum excited with an HBO 100W/2 lamp was measured with a GG 435 long-pass filter. Partec software (DPAC V2.1.) was used to determine the G_1/G_0 peaks of the samples.

CHROMOSOME COUNTING

Root tips of asparagus seedlings 0.5 cm in length were fixed in cool (+4°C) AA solution (96% ethanol and glacial acetic acid, 3:1) for 24 h, then hydrolysed for 15 min in 1N HCl at 60°C and stained with 1% acetocarmine (w/v) for 25 min. Meristematic tissues were squashed and the chromosomes in the metaphase plate was counted and photographed under a Carl Zeiss Axiostar microscope with a digital camera.

RESULTS

ANALYSIS OF SQUASHED OVULES

Only 3 of the 500 immature ovules of cv. Eposs (stage 1, Fig. 1a) that we squashed, acetocarmine-stained and checked by LM contained twin pairs of embryos. In the majority of ovules the embryo sacs contained a single embryo; only 0.6% of the ovules contained double embryos in the same embryo sac.

TABLE 2. Morphological characteristics of asparagus twin seedlings

Number of seeds	Seedling No.	Separated	Conjoined	Fasciated	Equal-sized	Different-sized	Two diploid	Two haploid	Diploid/ haploid	2C DNA amount (pg)
1	Ep 1	+			+		+	–		3.71
	Ep 2	+	–	–	+	–	+	–	–	3.70
2	Ep 3	+			+		+	–		3.69
	Ep 4	+	–	–	+	–	+	–	–	3.68
3	Ep 5	+				+		–	H	1.95
	Ep 6	+	–	–	–	+	–	–	D	3.72
4	Ep 7		+		+		+	–		3.68
	Ep 8	–	+	+	+	–	+	–	–	3.68
5	Ep 9	+				+		–	D	3.73
	Ep 10	+	–	–	–	+	–	–	H	1.91
6	Ep 11		+		+		+	–		3.74
	Ep 12	–	+	+	+	–	+	–	–	3.73
7	Ep 13		+			+		–	D	3.71
	Ep 14	–	+	–	–	+	–	–	H	1.90
8	Ep 15	+			+		+	–		3.77
	Ep 16	+	–	–	+	–	+	–	–	3.74
1	Sart 1	+			+		+	–		3.75
	Sart 2	+	–	–	+	–	+	–	–	3.73
2	Sart 3	+			+		+	–		3.71
	Sart 4	+	–	–	+	–	+	–	–	3.69
1	Rav 1		+		+		+	–		3.75
	Rav 2	–	+	–	+	–	+	–	–	3.76
2	Rav 3	+				+		–	D	3.75
	Rav 4	+	–	–	–	+	–	–	H	1.89
3	Rav 5	+			+		+	–		3.78
	Rav 6	+	–	–	+	–	+	–	–	3.76
4	Rav 7	+			+		+	–		3.72
	Rav 8	+	–	–	+	–	+	–	–	3.71
5	Rav 9	+			–	+		–	H	1.94
	Rav 10	+	–	–	–	+	–	–	D	3.71
Total 15	30	11	4	2	10	5	10	0	5	

Ep – cv. Eposs; Sart – cv. Sartaguda; Rav – cv. Ravel

The frequency of ovules containing no embryo at all (although their endosperm developed normally) was 19%. From the globular twin embryos (three pairs) obtained from immature fruits, two pairs were of equal size and one pair had an embryo that was larger than the other (Fig. 1d). All of the twin embryos were smaller than the normal single ones that developed at the same time.

MORPHOLOGICAL ANALYSIS

The seedlings obtained from germination tests were screened in Petri dishes. The seeds of only three cultivars (Eposs, Ravel, Sartaguda) had some double seedlings (15 twins from 2.500 seeds, 0.6%). Diploids constituted 83.3% of all the plants derived from twin seedlings, and the remainder (16.7%) were haploids. Twin seedlings are shown in Figure 2a–d. In the 450 screened seeds of cv. Ravel (Fig. 2a,b) only 5 pairs of double seedlings were found. The number of double seedlings was highest in cv. Eposs; many fewer were found in Ravel and Sartaguda, and none in the remaining cultivars (Tab. 1). Eleven of the pairs were separated (each plantlet had its own cotyledon and root), 3 pairs were conjoined in the hypocotyl region, and 1 pair had a common hypocotyl and shared one primary root (Fig. 2c). Two equal-sized pairs were fasciated at 3–5 cm along the length of their young phylloclades (Tab. 2). After 6 weeks the conjoined diploid twins separated from each other.

The growth and survival of diploid plantlets derived from twins were high, yielding 20 strong seedlings. The haploid seedlings grew less vigorously and represented their characteristic root and shoot phenotype. Two haploid and some diploid seedlings were lost during acclimatization from pots to the garden.

NUCLEAR DNA CONTENT

Relative nuclear DNA content as revealed by the position of the DNA distribution peaks indicated nuclei in the G_1 phase of the cell cycle. The peaks depended on the cultivar analyzed. FCM revealed that different-sized pairs had different ploidy levels, reflected in plantlet morphology. The DNA content of 10 haploid/diploid seedlings ranged from 3.71 to 3.75 pg in the largest seedlings and from 1.89 to 1.95 pg in the smallest. The majority of seedlings derived from equal-sized twin embryos had nuclear DNA content (2C) ranging from 3.68 to 3.77 pg. The two diploid twins did not differ in ploidy level (Tab. 2).

SEEDLING CHROMOSOME NUMBER

Fifteen polyembryonic seeds of asparagus gave rise to thirty twin seedlings (Tab. 2). Only five of the pairs had mixed ploidy level (diploid and haploid), and the rest were diploid only. Metaphase plate analysis of root meristem cells revealed that both (diploid and haploid) chromosome sets were medium-sized, uniform, without morphological differences between them. The majority of double seedlings were diploids with somatic chromosome number 2n = 2x = 20 (Fig. 2d,a; Tab. 2). Only shorter and thinner roots from small seedlings were found upon cytological examination to be essentially haploids containing n = x = 10 chromosomes in their somatic cells (Fig. 2d,b; Tab. 2).

DISCUSSION

In this evaluation of the possibility of obtaining haploid *Asparagus* plants by separating them from natural polyembryonic seeds we found the frequency of twins to be cultivar-dependent. Our results are much lower than those obtained in lemon by Perez-Tornero and Porras (2008) (1–3 embryos per seed), tangerine (6–10 embryos) and orange (10–15 embryos), whose seeds are up to 43% polyembryonic (Moreira et al., 1947). Broad germination screening of seeds has also been performed in the *Allium* genebank for 92 species; polyembryony was detected in 26 species and tended to be species-specific. High rates of twin seedlings, up to 32%, have been found in *Allium splendens* 2n = 4x = 48. In the genus *Allium* the tendency for twin embryo formation may be higher because of the high ploidy level of the species (Specht et al., 2001).

The number of twin embryos per seed was highest in cv. Eposs (8/1500, 0.50%). Germination tests of cv. Mary Washington 500W (Uno et al., 2002) revealed 0.34% double embryos (34/9925). Other authors have reported frequencies of twins obtained from various asparagus genotypes of 0.13–3.54% (Randall and Rick, 1945) 0.22%, and 1.79% in a haploid (Thevenin, 1968). Those authors concluded that *Asparagus officinalis* has only a moderate tendency to produce polyembryonic seeds (Webber, 1940; Randall and Rick, 1945). Very few haploid plants have been obtained by this method. Their poor viability and high mortality present a major obstacle in breeding efforts.

In a comprehensive review of polyembryony in *Dactylis*, *Gossypium*, *Nicotiana*, *Phleum*, *Poa*, *Solanum* and *Triticum*, Webber (1940) stated that there are three origins of polyembryony: nucellar, monozygotic and dizygotic. Nucellar polyembryony is a well known phenomenon in *Citrus* (Frost, 1926; Moreira et al., 1947). The process starts outside the embryo sac: a few nucellar cells next to the micropyle undergo mitotic divisions and give rise to adventitious embryos, and the embryos develop into plants genetically identical to the mother plant. Such a reproductive strategy assures the preservation and wide distribution of the maternal plant genotype (Zenkteler and Guzowska, 1967). This phenomenon would have important evolutionary significance as a mechanism of adaptation and speciation (Ito et al., 2007).

Asparagus frequently has been the subject of research on the nature and origin of twin haploid-diploid embryos (Ellison, 1986; Kunitake et al., 1998; Randall and Rick, 1945; Uno et al., 2002). We can only speculate about the origin of additional embryos in polyembryonic seeds of *Asparagus*. Because polyembryony occurs at low frequency it has been extremely difficult to target the earliest stage of twin embryo development by microtome ovule sectioning. In our attempt to locate embryos at an immature stage of ovule development by the squash method we aimed to avoid the risk of early abortion or starvation of the haploid during competition with the second, larger embryo. We do not know whether these were mono- or dizygotic twins. It is not easy to distinguish morphologically nucellar from zygotic seedlings.

In asparagus, sex is determined by heterozygous Mm (male) or homozygous recessive mm (female) genes. The progeny of selfed andromonoecious plants segregate 1:2:1: mm (female), Mm (male) and MM (supermale of great importance for its yield, longevity and resistance to diseases) (Riccardi et al., 2011). When haploids are obtained from microspores, mm and MM plants can be formed and the hybrids between them are also male (Doré 1990). When haploids are extracted from seeds with twin embryos only female lines can be created.

Following the results of others (Thevenin, 1968), we suggest that twins may originate (*i*) from a fertilized egg cell that split (2n/2n), (*ii*) one from the egg cell and the second as an adventitious embryo from the nucellus or integument (2n/2n), or (*iii*) from the fertilized reduced egg cell and simultaneously from an apogamously developing element of the embryo sac (2n/n). A special version of twin embryo formation results from meiotic defects which often give rise to unreduced gametes which may be involved in polyembryony. When chromosome pairing and recombination during meiotic prophase are disrupted, some meiotic mutants with unreduced gametes are produced (Brownfield and Köhler, 2011). Such gametes may participate in fertilization and lead to an attempt at embryo multiplication.

Another possibility for twin formation is early cleavage at the proembryo stage, resulting in identical monozygotic plantlets, as in *Theobroma cacao* (Martinson, 1972). An extremely rare case is the formation of two gametophytes within single ovules, leading to dizygotic development of twins differing in genotype when the twin embryos arise by additional fertilization of synergids or antipodal cells (in *Allium*: Specht et al., 2001). Two-embryonic seeds were induced chemically by treatment of pepper flowers with 2,4-D or IBA (0.001% water solutions) separately or combined with BAP; both growth regulators had a marked effect (1.41% twins) on the increase in frequency of additional embryos (Nowaczyk and Nowaczyk, 1996; Jędrzejczyk and Nowaczyk, 2009). Ascorbic acid (Asc) injected into ovaries (50 μl on the first two days after pollination) promotes proembryo cell division and regulates cell polarity, giving rise to polyembryony and polycotyly in *Nicotiana tabacum* cv. Xanthi; the high level of dehydroascorbate reductase (DHAR), which recycles Asc, induces monozygotic twinning (Chen and Gallie, 2012).

The female gametophyte of asparagus conforms to the eight-nucleate Polygonum type. The mature embryo sac is asymmetrical. The micropylar end, which contains a three-celled egg apparatus, is much larger than the chalazal end, which is almost filled by the three antipodal cells (Webber, 1940). It is the antipodal cells which often give rise to the additional embryos in the early stage of polyembryonic development in the isolated embryo sac of *Allium tuberosum* (Specht et al., 2001). Twin progeny, one haploid embryo and the other diploid, developed simultaneously in the ovules of lily and tobacco hybrids; twins were found arising from a zygote and from a synergid stimulated to parthenogenetic divisions and to haploid plantlet development (Cooper, 1943).

Nowaczyk and Nowaczyk (2006) made an original attempt to explain the origin of twin embryos in *Lycopersicum*. Their experiments were based on segregation of marker characters in the F_1 and F_2 generations of hybrids. The presence of different recessive characters in each of the twins revealed adventitious embryo formation and splitting in tomato polyembryony. Most of the gross structural alterations involved in cleavage probably occurred during seed germination, although the process may also start in earlier stages of embryo development.

Interspecific hybrids of *Carthammus palaestinus* and *C. tinctorius* showed genetic linkage between stem fasciation and twin embryo seed development in F_1 (Singh et al., 2010). Mutation of the *TWN1* gene disrupts suspensor differentiation and development in early embryogenesis in *Arabidopsis*. Developmental suspensor mutants give rise to a high percentage of seeds containing twin embryos (Vernon et al, 2001).

Ovules with an unequal pair of embryos contain two ontogenetically different types of embryo, one more developed and the other smaller (Fig. 1d). In early development one of the twins competes less well for space and nutrients, and as a result becomes smaller and less vigorous. Some evidence from the squashed ovules indicates that twin embryo sacs may produce dizygotic twin seedlings. This, along with an analysis of the combinations of chromosome numbers and the relative lengths of twin seedlings, indicates that the remaining ones probably originate from cleavage of a single initial embryo.

As determined by FCM, the DNA content of haploids was half that of somatic cells in diploids. Analysis of the nuclear DNA content of plantlets derived from twin embryos revealed differences in content between diploid and haploid embryos. The values found for haploids (1.95 pg) in this study suggest that low DNA content is a property of the genus *Asparagus*.

In this study we obtained haploid plantlets by germinating twin embryo seeds of asparagus, and confirmed their haploid state by morphological comparison, chromosome counts and genome size analysis. Some of the haploids grew well after acclimatization but did not develop flowers, so we could not confirm their sex. Haploid progeny of cultivars such as Ravel and Sartaguda can provide an important source of breeding material.

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REFERENCES

CHEN Z, and GALLIE DR. 2012. Induction of monozygotic twinning by ascorbic acid in tobacco. PLoS ONE 7/6 : 1–12.

BROWNFIELD L, and KÖHLER C. 2011. Unreduced gamete formation in plants: mechanisms and prospects. *Journal of Experimental Botany* 65(5): 1659–1668.

COOPER DC. 1943. Haploid-diploid twin embryos in *Lilium* and *Nicotiana. American Journal of Botany* 30: 408–413.

Doležel J, Binarova P, and Lucretti S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. F1 Hybrids. *Biologia Plantarum* 31: 113–120.

DORÉ C. 1990. *Asparagus Anther Culture and Field Trial of Dihaploids and Biotechnology in Agriculture and Forestry*, 323–333, vol. 12. Springer Verlag, Berlin Heidelberg.

ELLISON JH. 1986. *Asparagus* breeding. In: Basset MJ [ed.], *Breeding Vegetable Crops*, 521–569. AVI Publishing Company Inc., Westport.

FROST HB. 1926. Polyembryony, heterozygosis and chimeras in citrus. *Hilgardia* 1 : 365–402.

FORSTER BP, HEBERLE-BARS E, KASHA KJ, and TURAEV A. 2007. The resurgence of haploids in higher plants. *Trends in Plant Science* 12: 368–375.

GEBLER P, WOLKO Ł, and KNAFLEWSKI M. 2007. Identification of molecular markers for selection of supermale (YY) asparagus plants. *Journal of Applied Genetic* 48: 129–131.

ITO T, ASHIZAWA T, SHIMODATE T, FUKUDA T, KAMEYA T, and KANNO A. 2007. Production and analysis of reciprocal hybrids between *Asparagus officinalis* L. and *A. schoberioides* Kunth. *Genetic Resources of Crop Evolution* 54 (5): 1063–1071.

JĘDRZEJCZYK I, and NOWACZYK P. 2009. In vivo polyembryony induction in species of *Capsicum. Acta Biologica Cracoviensia Series Botanica* 51/1: 55–60.

KNAFLEWSKI M. 1996. Genealogy of asparagus cultivars. In: Nichols M and Swain D [eds.], Proceedings VIII Int. Asparagus Symp.: 87–91.

KUNITAKE H, NAKASHIMA T, MORI K, and TANAKA M. 1998. Somaclonal and chromosomal effects of genotype, ploidy and culture duration in *Asparagus officinalis* L. *Euphytica* 102: 309–316.

MALEPSZY S, NIEMIROWICZ -SZCZYTT K, and PRZYBECKI Z. 1989. *Biotechnologia w Genetyce i Hodowli Roślin*, 151–196. PWN, Warszawa.

MALEPSZY S. 2009. Rośliny w zaspokajaniu potrzeb człowieka. In: Malepszy [ed.], *Biotechnologia Roślin*, 3–11. PWN, Warszawa.

MARCELLAN ON, and CAMADRO EL. 1999. Formation and development of embryo and endosperm in intra- and interspecific cross of *Asparagus officinalis* and *A. densiflorus* cv. 'Sprengeri'. *Scientia Horticulture* 81: 1–11.

MARTINSON V. 1972. Polyembryony in *Theobroma cacao* L. *Annals of Botany* 36(5): 947–951

MOREIRA S, GURGEL JTA, and ARRUDA LF. 1947. Poliembrionia em citrus. *Bragantia* 10: 69–106.

MORENO R, ESPEJO JA, CABRERA A, MILLAN T, and GIL J. 2006. Ploidic and molecular analysis of 'Morado de Huetor' asparagus (*Asparagus officinalis* L.) population; a spanish tetraploid landrace. *Genetic Resources of Crop Evolution* 53: 729–736.

MURASHIGE T, and SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.

NOWACZYK P, and NOWACZYK L. 1996. The influence of growth regulators on the frequency of polyembryony in pepper (*Capsicum annuum* L.). *Genetica Polonica* 37A: 204–207.

NOWACZYK P, and NOWACZYK L. 2006. Genetic analysis of tomato (*Lycopersicum esculentum* Mill.) twin forms. *Acta Biologica Cracoviensia Series Botanica* 48/1: 53–58.

OZAKI Y, NARIKIYO K, FUJITA C, and AND OKUBO H. 2004. Ploidy variation of progenies from intra- and inter-ploidy crosses with regard to trisomic production in *Asparagus officinalis* L. *Sexual Plant Reproduction* 17: 157–164.

PEREZ-TORNERO O, and PORRAS I. 2008. Assessment of polyembryony in lemon rescue and in vitro culture of immature embryos. *Plant Cell Tissue and Organ Culture* 93(2): 173–181.

RANDALL TE, and RICK CM. 1945. A cytogenetic study of polyembryony in *Asparagus officinalis* L. *American Journal of Botany* 32: 560–569.

RICCARDI P, CASALI PE, MERCATI F, FALAVIGNA A, and SUNSERI F. 2011. Genetic characterization of asparagus doubled haploids collection and wild relatives. *Scientia Horticulturae* 130: 691–700.

SHIGA I, UNO Y, KANECHI M, and INAGAKI N. 2009. Identification of polyploidy of in vitro anther-derived shoots of *Asparagus officinalis* L. by flow cytometric analysis and measurement of stomatal length. *Journal of Japan Society for Horticultural Science* 78/1: 103–108.

SINGH V, AKADE JH, and NIMBKAR N. 2010. Inheritance of stem fasciation and twin/multi-embryonic seeds and genetic linkage between them in safflower. *Indian Journal of Genetics and Plant Breeding* 70: 281–287.

SPECHT CE, MEISTER A, KELLER ERJ, KORZUN L, and BORNER A. 2001. Polyembryony in species of the genus *Allium*. *Euphytica* 121: 37–44.

STAJNER N, BOHANEC B, and JAVORNIK B. 2002. Genetic variability of economically important *Asparagus* species as revealed by genome size analysis and rDNA ITS polymorphisms. *Plant Science* 162: 931–937.

THEVENIN L. 1968. Les problemes d'amelioration de l'asperge (*Asparagus officinalis* L.). *Annales de l' Amelioration des Plantes* 18: 327–365.

UNO Y, LI Y, KANECHI M, and INAGAKI N. 2002. Haploid production from polyembryonic seeds of *Asparagus*. Proc. X Int. ISHS Symposium on *Asparagus. Acta Horticulturae* 589: 212–224.

VALDES B. 1964. *Asparagus* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM. Valentine DH, Walters SM and

Webb DA [eds.], *Flora Europaea*, 71–73. Cambridge University Press.

VERNON DM, HANNON MJ, LE M, and FORSTHOEFL NR. 2001. An expanded role for the *TWN1* gene in embryogenesis: defects in cotyledon pattern and morphology in the *twn1* mutant of *Arabidopsis* (Brassicaceae). *American Journal of Botany* 88: 570–582.

WEBBER JM. 1940. Polyembryony. *Botanical Review* 6: 575–598

ZENKTELER M, and GUZOWSKA I. 1967. O niektórych zagadnieniach eksperymentalnej embriologii roślin. I. Poliembrionia. *Wiadomości Botaniczne* 11: 181–190.

ZENKTELER M, and NITZSCHE W. 1984. Wide hybridization experiments in cereals. *Theoretical Applied of Genetics* 68: 311–315.

ZENKTELER M, and STRAUB J. 1979. Cytoembryological studies on the process of fertilization and the development of haploid embryos of *Triticum aestivum* L. (2n=47) after crossing with *Hordeum bulbosum* (2n=14). *Zeitschrift fur Pflanzenzüchtung* 82: 32–44.

ZHANG C-J, WANG H-L, MA Y, and KANG Y-Q. 1994. Regeneration of haploid plants from isolated microspores of asparagus (*Asparagus officinalis* L.). *Plant Cell Reports* 13: 637–640.