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ORIGINAL PAPER

Study of the somaclonal variation produced by different methods of polyploidization in *Asparagus officinalis* L.

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Abstract Polyploid plants have been induced in different *Asparagus officinalis* L. breeding programs in order to obtain plants with improved agronomical traits, such as large spear diameter or segregation ratios with a higher number of males. The polyploidization methods can produce somaclonal variation in the polyploid plants obtained and, therefore, unwanted changes in the agronomical traits of the initial elite plants. We used two different polyploidization methods to induce polyploid plants from diploid genotypes of commercial varieties and tetraploid genotypes of the Spanish landrace "Morado de Huétor". The first method was the culture of rhizome buds in the medium ARBM-3 (Asparagus Rhizome Bud Medium), supplemented with different concentrations of colchicine (0.1–0.75 g l^{-1}) for 10 and 20 days. The best polyploidization rate obtained was 25 % (0.5 g l^{-1} colchicine for 10 days). The second method was the regeneration of polyploid plants from callus culture, resulting in a polyploidization rate of 40 and 12.5 % for the diploid genotype CM077 and the tetraploid genotype HT156, respectively. Additionally, we have developed a new protocol to separate the mixoploids generated into their different genetic components, obtaining plants with a unique ploidy level. EST-SSRs markers were employed to analyze the genetic stability of polyploidy plants. Somaclonal variation was not detected for polyploidy plants obtained through the culture of rhizome bud explants. Therefore, these polyploid plants should maintain the agronomical traits of the initial elite plants. However, somaclonal variation was detected in the polyploid plants regenerated from callus culture.

Keywords Colchicine · Rhizome buds · Endoreduplication · EST-SSRs · Organogenesis · Separation of mixoploids

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Introduction

Polyploidization is a frequent process within the genus *Asparagus* (Castro et al. 2013). The basic chromosomal number of the genus is x = 10, but there are different ploidy levels: diploid (2n = 2x = 20) such as *A. officinalis* and *A. albus*, tetraploid (2n = 4x = 40) appearing in *A. acutifolius* and *A. prostatus*, hexaploid in the case of *A. maritimus*, *A. pseudoscaber* and *A. brachyphyllus*, even dodecaploid (2n = 12x = 120) in the recently catalogued *A. macrorrhizus* (Pedrol et al. 2013). Diversity also can be found within the same population. That is the case of the Spanish landrace "Morado de Huétor", which is mostly tetraploid (4x). However, triploid (3x), pentaploid (5x), hexaploid (6x) and octoploid plants (8x) were also found within this landrace (Moreno et al. 2006). The broad diversity in the ploidy level suggests that the polyploidization process could play an important role in the evolution within the genus *Asparagus* (Castro et al. 2013), and can be explained by the formation of unreduced gametes. The formation of unreduced gametes is caused by errors in the second meiotic division restitution (SDR) and was detected in different species of the genus *Asparagus* (Camadro 1992, 1994).

Polyploidy induction is a tool used in different *Asparagus officinalis* L. breeding programs (Braak and Zeilinga 1957;

Mac Key 1987; Skiebe et al. 1991). The polyploid plants (tetraploids) obtained in these breeding programs showed some differences from the initial plants (diploids), such as a darker color or a bigger size of all plant organs (Braak and Zeilinga 1957; Kunitake et al. 1998). The increase of the size is very interesting in the case of the diameter of the spears because it is an important agronomical trait, specific to each cultivar. The natural octoploid genotypes of "Morado de Huétor" showed better agronomical traits (vigor, spear diameter, productivity and tolerance to pathogens) than the other genotypes maintained in the in vivo collection of this landrace, established by Moreno et al. (2008a). The tetraploid landrace "Morado de Huétor" is characterized by small diameter spears and low productivity. Hence, in order to improve these agronomical traits, octoploid plants of "Morado de Huétor" were used to develop the hybrid variety HT801 (CESURCA et al. 2012). The cultivar HT801 is more productive and shows spears with bigger diameters, indicating that the polyploidization can be a solution for breeding in "Morado de Huétor" landrace.

Sex in *Asparagus* is determined by a dominant gene, M (Flory 1932). The female genotypes are homozygous recessive (mm) and the male genotypes are heterozygous (Mm), leading to the male: female ratio 1:1 in traditional diploid cultivars. Therefore, an autotetraploid male will be MMmm and the hybrid varieties in which that male was used as parent will present a male: female ratio 5:1 (Braak and Zeilinga 1957; Skiebe et al. 1991). Producing a higher number of males is important because male plants show advantages over female plants. The male plants do not produce seeds, avoiding the further growth of the asparagus seeds into weeds; also male plants are more long-lived and yield higher than females (Ellison 1986; López-Anido and Cointry 2008). In addition, tetraploid plants offer breeding advantages because they can make it easier the hybridization between *Asparagus officinalis* and tetraploid closely related wild species such as *A. prostratus* or *A. acutifolius* (Falavigna et al. 2008), and even can be crossed with diploid plants, obtaining triploid hybrid varieties of *Asparagus officinalis* (Skiebe et al. 1991; Kunitake et al. 1998).

Colchicine is the antimitotic most frequently used to induce polyploidization in many plant species (Dhooghe et al. 2011). It has been previously employed, among other species, in *Phlox subulata* (Dhooghe et al. 2009), *Gerbera jamesonii* (Gantait et al. 2011), *Passiflora edulis* (Rêgo et al. 2011), *Arachis Paraguarensis* (Aina et al. 2012) and *Pfaffia glomerata* (Gomes et al. 2014). In asparagus, different authors have subjected pregerminated seeds to different concentrations of colchicine (Braak and Zeilinga 1957; Mac Key 1987; Skiebe et al. 1991; Kunitake et al. 1998). The polyploidization rates obtained with this method are very low (<8 %). Moreover, the use of seeds supposes an additional drawback because the development of polyploids from plants with unknown agronomical traits is not interesting in breeding. The only way to know the precise agronomical characteristics of the adult plant of asparagus in advance is using seeds from F_1 hybrids, which have been previously evaluated. However, the development of F_1 hybrids is relatively recent (Corriols et al. 1990) and yet there are no F_1 hybrids available from landraces such as the Spanish "Morado de Huétor". Recently, Carmona-Martin et al. (2014a) have developed a new protocol of polyploidization to produce polyploids from adult elite plants with known agronomical characteristics. The method consisted in the treatment of asparagus rhizome bud explants with colchicine before incubating them in ARBM-3 medium. The polyploid plants resulting from this method should maintain the elite agronomical traits of the initial adult plants. One problem of this method is the generation of chimeras or mixoploid genotypes which are genetically unstable. The use of colchicine usually produces mixoploid and it has been reported other species such as in *Phlox subulata* (Dhooghe et al. 2009), *Arachis paraguarienssis* (Aina et al. 2012) and *Pfaffia glomerata* (Gomes et al. 2014).

An alternative to the use of antimitotics to generate polyploidy in asparagus is plant regeneration from callus culture. If the plants are regenerated by organogenesis (Raimondi et al. 2001; Pontaroli and Camadro 2005) or if the callus is used to obtain somatic embryos (Odake et al. 1993), the generation of polyploid plants is common because there are spontaneous endoreduplications in the cells during the callus proliferation (Odake et al. 1993; Kunitake et al. 1998; Raimondi et al. 2001; Pontaroli and Camadro 2005). This polyploidization method has been used in other species such as *Solanum lycopersicum* (Koornneef et al. 1989), *Pyrus pyrifolia* (Kadota and Niimi 2002) and *Cyphomandra betacea* (Currais et al. 2013).

The methods of polyploidization above mentioned, e.g. the method developed by Carmona-Martín et al. (2014a) and the methods based on callus regeneration, involved the use of in vitro techniques. Consequently, the polyploid plants obtained can show somaclonal variation (Bairu et al. 2011) and the modification of some agronomical traits of the original plants. Currently, the EST-SSRs are considered the molecular markers best-suited to study the somaclonal variation produced in micropropagated plants (Bairu et al. 2011). The EST-SSRs have been used to evaluate somaclonal variation in the genus asparagus (Carmona-Martin et al. 2014b) and other species such as sorghum (Zhang et al. 2010), rice (Gao et al. 2009), wheat (Khlestkina et al. 2010) and sugarcane (Singh et al. 2008).

The main objective of this work is to study of the somaclonal variation produced by different methods of polyploidization in *Asparagus officinalis* using a set of EST-SSRs. In addition, we have optimized the two methods of polyploidization employed in this study, the

treatment with colchicine of asparagus rhizome bud explants and the regeneration of plants from callus culture. We have used diploid commercial genotypes of *Asparagus officinalis* and tetraploid genotypes of the Spanish landrace "Morado de Huétor" in both protocols.

Materials and methods

Plant material

The plant material used in this work and their characteristics (genotype, ploidy level, origin, explant type and use) are shown in Table 1.

Staining and observation of microspores

HT156 flower buds were collected and selected by size. The buds around 2 mm size were fixed in a 3 ethanol (95 %): 1 glacial acetic acid solution for 24 h and stored at 4 °C until use. After fixation, the flower buds were stained with acetic-carmine (Snow 1963) and observed under a light microscope at 1250× to search for failures in the microspore formation.

Dissection and disinfection of rhizome bud explants

Explants of the tetraploid landrace "Morado de Huétor" were obtained from potted plants of asparagus growing in a glasshouse. Then they were cleaned, dissected and disinfected following the protocol described by Carmona-Martin et al. (2014b). Briefly, dissected rhizome buds were treated with fungicide (benomyl 3 g l^{-1}) for 15 min under shaking. After washing the explants in sterile distilled water, they were disinfected in a 20 g l^{-1} sodium hypochlorite solution for 15 min under vacuum conditions and washed again three times with sterile distilled water in aseptic conditions. Rhizome bud explants approximately 3–5 mm length were used as explants for colchicine treatments.

Rhizome buds polyploidization

The rhizome bud explants were incubated in shoot regeneration medium ARBM-3, consisting in MS (Murashige and Skoog 1962) salts modified with EDDHA-Fe 85.7 mg l^{-1} instead of EDTA-Fe and vitamins, supplemented with 0.3 mg l^{-1} ANA, 0.1 mg l^{-1} KIN, 2 mg l^{-1} Ancymidol and 6 % sucrose with pH 5.74, and solidified with 0.8 % agar (Table 2). In order to induce the polyploidization of these rhizome bud explants, the medium was supplemented with different concentrations of filter sterilized colchicine (0.1, 0.25, 0.5 and 0.75 g l^{-1}). In previous studies, we observed that the use of colchicine in the culture medium induces proliferation of different bacteria (Carmona-Martín et al. 2014a). To decrease this secondary effect we supplemented the medium with the antibiotic cefotaxime (200 mg l^{-1} of filter sterilized). Aliquots of 25 ml of ARBM-3 medium was added into 150 mm × 25 mm test tubes covered with polypropylene tops (Bellco Corp.), and autoclaved for 20 min at 121 °C and 1.05 kg cm^{-2}. Forty rhizome buds were cultured in each of the colchicine concentrations tested; one explant was cultured per test tube. All buds were incubated at 25 ± 1 °C under 16 h day photoperiod under cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania) with 45 μmol m^{-2} s^{-1} (400–700 nm) Photosynthetic Active Radiation.

For the assay consisting in the incubation in ARBM-3 medium supplemented with filter-sterilized colchicine for 10 days, 20 rhizome bud explants treated with each concentration of colchicine were transferred to test tubes with ARBM-3 medium without colchicine and incubated in

Table 1 Characteristics of plant materials

Genotype	Ploidy level	Nature	Explant	Origin	Use
HT156	4x	Elite genotype "Morado de Huétor"	Flowers	Greenhouse "IHSM-La Mayora"	Microspore study
			Rhizome buds	Collection "in vivo" UCO[a]	Rhizome buds polyploidization
			Callus	Collection "in vitro" "IHSM-La Mayora"[b]	Polyploid plants regeneration
CM077	2x	Elite commercial genotype	Callus	Collection "in vitro" "IHSM La Mayora"[b]	Polyploid plants regeneration
CM095	2x	Elite commercial genotype	Rhizome buds	Collection in vivo UCO[a]	Rhizome buds polyploidization
Landrace "Morado de Huétor"	4x	Unknown genotypes	Rhizome buds	Greenhouse "IHSM La Mayora"[b]	Optimization of polyploidization method of rhizome buds

HT: Genotype of the tetraploid landrace "Morado de Huétor"; CM: Genotype of commercial variety

[a] Moreno et al. (2008a)

[b] Carmona-Martin et al. (2014b)

Table 2 Composition of the culture media

Culture medium	Hormone					Sucrose (g l^{-1})	Agar (g l^{-1})	Container used	Utility
	ANA (mg l^{-1})	KIN (mg l^{-1})	ANC (mg l^{-1})	BA (mg l^{-1})	2.4-D (mg l^{-1})				
ARBM-3[a]	0.3	0.1	2	–	–	60	8	Tubes 25 × 150 mm	Rooted buds
ARBM-0[a]	0.1	0.1	–	–	–	30	8	Tubes 25 × 150 mm	Plant growth and multiplication
APC[b]	–	–	–	–	2	30	6	Petri dishes 90 × 25 mm	Proliferation of callus
ARS[b]	–	–	–	0.3	–	20	6	Petri dishes 90 × 25 mm	Shoot regeneration

[a] Culture media consisting in full strength MS salts and vitamins (Murashige and Skoog 1962), modified with EDDHA-Fe 85.7 mg l^{-1} instead of EDTA-Fe

[b] Culture mediums consisting in full strength MS salts and vitamins (Murashige and Skoog 1962)

these tubes for 4 weeks. After these 4 weeks, we collected data of contamination, survival and rooting. Identical protocol was applied to carry out the assay incubating rhizome buds in ARBM-3 medium supplemented with filter-sterilized colchicine for 20 days. 20 rhizome buds per colchicine doses were also used. The contamination rate for each treatment was calculated as the number of contaminated buds divided by the total number of buds. The survival rate was defined as the number of buds becoming plants after the treatment with colchicine regardless of the contaminated buds. The rooting rate was calculated as the number of rooted plants divided by the total number of plants obtained. The success rate in the polyploidy induction was calculated as the number of polyploid plants divided by the total number of plants obtained. The polyploidization rate was calculated as the number of rooted polyploid plants divided by the initial number of buds for treatment.

To obtain polyploid plants from both elite genotypes HT156 and CM095 (Table 1), we dissected and disinfected rhizome bud explants of these genotypes and incubated them for 10 days in test tubes with ARBM-3 medium supplemented with colchicine (0.5 g l^{-1}). After the incubation, we applied the standard method developed for micropropagation of "Morado de Huétor" rhizome buds.

Rhizome buds rooting and multiplication

Rhizome buds rooted in ARBM-3 were transferred to 25 ml test tubes with medium ARBM-0, consisting in MS (Murashige and Skoog 1962) salts modified with EDDHA-Fe 85.7 mg l^{-1} instead of EDTA-Fe and vitamins, supplemented with 0.1 mg l^{-1} ANA, 0.1 mg l^{-1} KIN, and 3 % sucrose with pH 5.74, and solidified with 0.8 % agar (Table 2). They were incubated at standard conditions and transferred every 4 weeks to fresh medium for growth and multiplication of developed plants. The multiplication consists in the mechanical division of the plants into individual plantlets with shoots and roots. The number of plants obtained in each division will depend on the size of the different plants and the number of shoots and roots that these plants generate.

To increase the rooting rates, unrooted shoots growing in ARBM-3 were subjected to a cyclic process of rooting, alternating subcultures in media ARBM-0 and ARBM-3. We usually applied two cycles of rooting, and the rooting data were collected after each cycle. Once rooted, the shoots were incubated in ARBM-0 and continued with the normal process of multiplication.

Callus proliferation and shoots regeneration

Formation of small callus was observed during the in vitro maintenance of the collection of *Asparagus sp*. in our laboratory (Carmona-Martin et al. 2014b). Calli from the genotypes HT156 (4x) and CM077 (2x) (Table 1) were dissected and incubated for 16 weeks at 25 ± 1 °C in dark conditions in Petri dishes (90 × 25 mm) with 25 ml of APC medium (asparagus proliferation callus), consisted in MS salts and vitamins, supplemented with 2 mg l^{-1} 2,4-D, 3 % sucrose with pH 5.74, and solidified with 0.6 % agar (Table 2). Calli were subcultured to fresh medium every 4 weeks.

For shoot regeneration, callus pieces approximately 15 mm of diameter were transferred to Petri dishes (90 × 25 mm) containing 25 ml of ARS medium (asparagus regeneration shoots), consisting of MS salts and vitamins supplemented with 0.3 mg l^{-1} BA, 2 % sucrose with pH 5.74, and solidified with 0.6 % agar (Table 2). Five calli were cultured in each Petri dish and incubated at the same environmental conditions indicated above for rhizome bud explants. Calli were subcultured in fresh medium every 4 weeks and the shoots regenerated were transferred to test tubes with 25 ml of ARBM-0 for elongation.

Shoots rooting and multiplication

Some shoots directly rooted in ARS medium and were multiplied following the standard method developed from rhizome bud explants. For unrooted shoots, we applied the cyclic process of rooting for shoots developed from rhizome bud explants above mentioned. After two rooting cycles, the unrooted shoots were discarded.

Ploidy analysis

The ploidy level of the plants was determined by estimating the relative DNA content using flow cytometry (Ploidy Analyser PA-I; Partec GmbH, Münster, Germany). For analysis, 0.5 cm^2 of young in vitro shoots was chopped with a razor blade for 30–60 s to release nuclei in a Petri dish containing 0.4 ml of nuclei isolation buffer (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, extraction buffer). The homogenate was filtered through a 50 µm nylon mesh (Partec 50-lm CellTrics disposable filter), and subsequently nuclei were stained with fluorescent dye (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, staining buffer, about 1.6 ml). Finally, the samples were analyzed after 30 s of incubation. *Asparagus officinalis* cv. UC157 F1 ($2n = 2x = 20$) was used as an external standard.

Mechanical separation of plant chimeras

After detecting ploidy differences among the different single shoots/plantlets existing in a mixoploid plant, we proceeded to separate the parts with different ploidy levels from the chimeras obtained in the process of polyploidization. The mixoploid plants detected through flow cytometry were dissected in different parts, leaving a single shoot with a root in each explant. When the explants developed, we repeated the ploidy analyses of these explants and when the results confirmed that some of the asparagus plants evaluated were polyploid, the process of separation was considered successful.

Spear diameter study of asparagus developed in vitro

In this study, three types of plants from each one of the three selected genotypes (CM077, CM095 and HT156) were used. Type 1: Control plants, obtained from our in vitro collection; these plants have never been subjected to polyploidization processes. Type 2: Polyploid plants, plants that were obtained from successful processes of polyploidization. Type 3: Non-polyploid plants, plants resulting from an unsuccessful procedure of polyploidization; these plants maintain the original ploidy level of each genotype after the induction process.

To synchronize the growth of these plantlets, they were subcultured at the same time in tubes with fresh ARBM-0 medium and incubated at the standard conditions of temperature and light for 4 weeks. After these 4 weeks, we recorded the diameter of the new spears developed in vitro from plants of the three plant types of each genotype under study (CM077, CM095 and HT156). The measures of the spear diameter were estimated with the image analysis program Image J. (Rasband 1997–2013), using photographs of the in vitro spears.

Acclimatization of asparagus plantlets

The plantlets of the genotypes CM077, CM095 and HT156 were acclimatized following the method reported by Carmona-Martin et al. (2014b). Plantlets of the three types above described (Type1, 2 and 3) were acclimatizated, and the acclimatization rates of the different types of plants were compared to evaluate the viability of the polyploid plants.

Study of genetic stability of polyploid plants using EST-SSRs

For the genetic stability study, we also employed the same three plant types (Type 1, 2 and 3) of each genotype previously mentioned. In the case of HT156, we differentiated the plants obtained from rhizome bud explants from those obtained from shoot regeneration.

Total genomic DNA of the plants under study was extracted from 1 g of in vitro young spears tips, following a modified CTAB extraction protocol described by Torres et al. (1993). The quality and concentration of extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA).

Twenty-four plants (eight of Type 1, eight of Type 2 and eight of Type 3) from each genotype (CM077, CM095 and HT156) were analyzed using EST-SSR markers in order to search for somaclonal variation generated during the process of polyploidization and micropropagation. For HT156, we analyzed plants obtained from rhizome buds and plants regenerated from callus. A set of twelve EST-SSR markers (AAT1, AG3, AG6, AG7, AG8, AG10, AG12, TC1, TC3, TC5, TC7 and TC9) previously developed by Caruso et al. (2008) was employed. Forward primers were synthesized with fluorescent dyes 6FAM or HEX (Applied Biosystems) at the 5′ ends. Amplification of these markers was performed as in Caruso et al. (2008). The PCR products were separated using an automated capillary sequencer (ABI 3130 Genetic Analyzer; Applied Biosystems/HITACHI, Madrid, Spain) in the Unit of Genomics of the Central Research Support Service at the University of Córdoba. The size of the amplified bands was calculated based on an

internal DNA standard (400HDROX) with GeneScan software (version 3.x) and the results were interpreted using the Genotyper program (version 3.7) all from Applied Biosystems.

Statistical analysis

All data were analyzed using SPSS software package (version 19.0; SPPS INC., Chicago, IL, USA). The rates of contamination, survival, rooting, polyploidization and number of polyploid plants obtained in the different assays developed with rhizome bud explants were analyzed by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution. Pairwise comparisons among groups were performed by Fisher's least significant difference (LSD) test. To facilitate the independent analysis of the effect produced by colchicine concentration and the duration of each treatment, we pooled the results obtained in each assay by the colchicine concentration used and the duration of each treatment. The same analysis was used to analyze the acclimatization rate of the different plants. Finally, the data of the diameter of the in vitro spears were analyzed by one-way ANOVA, using a HSD-Turkey test in the post-hoc analysis for comparisons among groups.

Results

Microspore formation in cv. "Morado de Huétor"

Figure 1 illustrates a microspore preparation from the genotype HT156, which is a tetraploid male of "Morado de Huétor". These microspores were in the tetrad phase during the maturation period, and normal tetrads, with four 2n microspores were observed (Fig. 1a). However, triads were also observed (Fig. 1b). Triads give rise to two 2n microspores and one 4n microspore, which give rise to an unreduced gamete.

Rhizome buds polyploidization

Data on contamination, survival, rooting (after two cycles of rooting) and success in polyploidy induction rates obtained in different assays of polyploidization with rhizome bud explants are shown in Fig. 2. Supplementing the medium ARBM-3 with colchicine increased the contamination rate of the rhizome bud explants incubated in this medium. There were no significant differences in the contamination rates between the different colchicine treatments pooled by the duration of the treatment. However when the analysis was carried out with the colchicine treatments pooled by the concentration of colchicine significant differences appeared between the contamination rates of the different concentrations, 17 ± 6 % with the lowest concentration (0.1 g l^{-1}) and 37 ± 8 % with the highest concentration (0.75 g l^{-1}). These results indicate that the contamination rate was independent of the duration of the treatment with colchicina but increased with the concentration of colchicine used. The treatment with colchicine also decreased the survival rate of the rhizome bud explants in around 25 % in all the assays performed, regardless of the colchicine concentration applied and the assay duration.

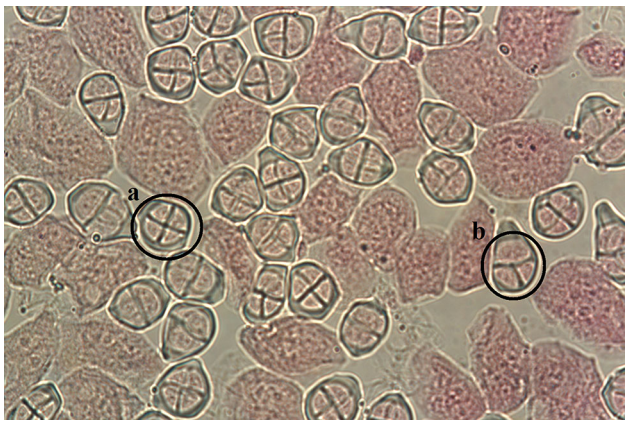
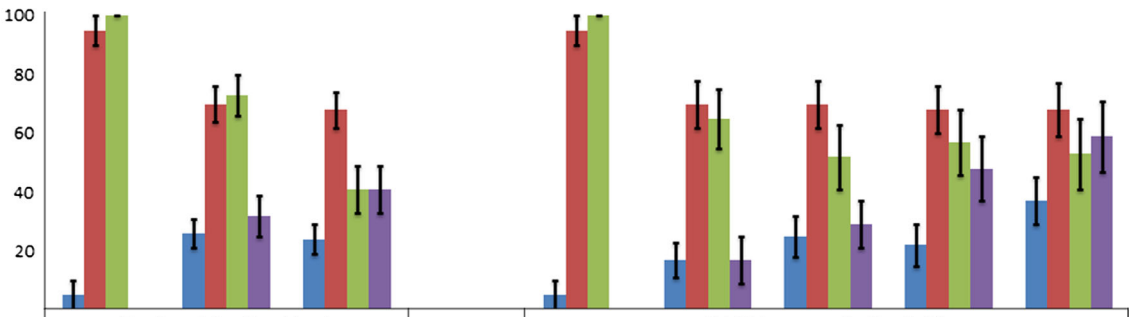


Fig. 1 Microspores from genotype HT156 in tetrad stage (×1250). **a** Normal tetrads with 2n microspores. **b** Triad with a 4n microspore and two 2n microspores

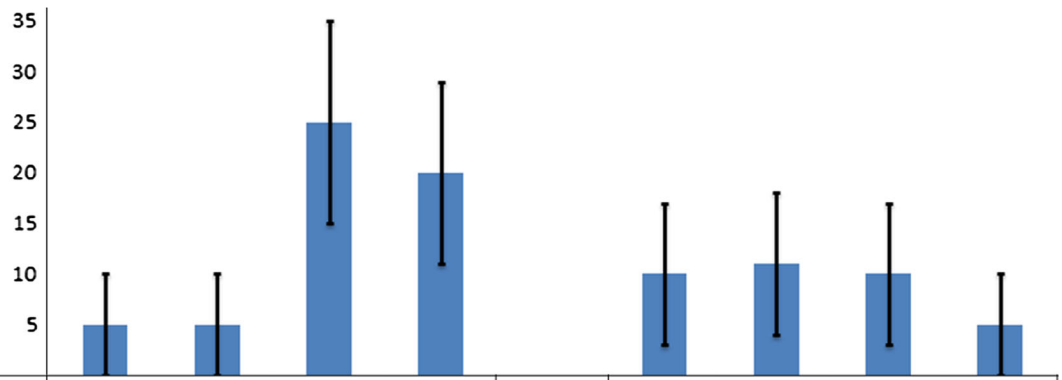
According to our results, the strongest effect of colchicine was on the rooting rate of the rhizome buds (Fig. 2). After two cycles of rooting, the differences between the rooting rates of the control and the different treatments were high and directly proportional to the incubation time. Thus, the 20 days treatment showed a percentage of rooting 30 % lower than the 10 days treatment. No significant differences were detected on rooting between the different doses of colchicine assayed. The rate success in polyploidy induction was higher for the 20 days treatment than for the 10 days treatment, though there were no significant differences between them. However, there were significant differences in this rate among the assays using different concentrations of colchicine. The rate of success in the polyploidy induction increased proportionally with the concentration of colchicine.

The polyploidization rates of the rhizome buds using different assays are shown in Fig. 3. The best results were obtained using 0.5 g l^{-1} of colchicine for 10 days. In contrast, as is shown in Fig. 2, the 20 days treatments showed higher success rate on polyploidy induction than the 10 days treatments, while the negative effect of colchicine on rooting was stronger for the 20 days treatments (Fig. 3). Thus, the number of polyploid rooted plants obtained (polyploidization rate) was higher for the 10 days treatments using 0.5 and 0.75 g l^{-1} of colchicine.



	Treatment duration (days)			Colchicine concentration (g l^{-1})				
	0	10	20	0	0.1	0.25	0.5	0.75
% Contamination	5 ± 5 b	26 ± 5 a	24 ± 5 a	5 ± 5 c	17 ± 6 bc	25 ± 7 ab	22 ± 7 ab	37 ± 8 a
% Survival	95 ± 5 a	70 ± 6 b	68 ± 6 b	95 ± 5 a	70 ± 8 b	70 ± 8 b	68 ± 8 b	68 ± 9 b
% Rooting	100 ± 0 a	73 ± 7 b	41 ± 8 c	100 ± 0 a	65 ± 10 b	52 ± 10 b	57 ± 11 b	53 ± 12 b
% Success polyploidy induction	0 ± 0 b	32 ± 7 a	41 ± 8 a	0 ± 0 c	17 ± 8 b	29 ± 10 b	48 ± 11 a	59 ± 12 a

Fig. 2 Rate of contamination, survival, rooting (after two cycles of rooting) and success in polyploidy induction obtained from rhizome bud explants applying different colchicine concentrations (g l^{-1}) and durations of treatment (days)



Treatment duration (days)	10				20			
Colchicine concentration (g l^{-1})	0.1	0.25	0.5	0.75	0.1	0.25	0.5	0.75
% Octoploid plants rooted (Polyploidization rate)	5 ± 5 bc	5 ± 5 bc	25 ± 10 a	20 ± 9 a	10 ± 7 ab	11 ± 7 ab	10 ± 7 ab	5 ± 5 bc

Fig. 3 Polyploidization rate or percentage of rooted polyploid plants obtained for the different polyploidization treatments with colchicine in rhizome bud explants

We selected the incubation of the rhizome buds for 10 days in ARBM-3 medium supplemented with 0.5 g l^{-1} of colchicine due to produce the best results. Polyploidization assays with these conditions were conducted with the genotypes CM095 and HT156. The polyploid plants of CM095 and HT156 obtained in these assays were used in further analysis.

Regeneration of polyploid shoots

Around 40 % of the shoots regenerated on ARS medium rooted. The other shoots were subjected to a cyclic process of rooting, and after two cycles of rooting the rooting rate rise to 90 %. The unrooted shoots were discarded.

Forty plantlets of the diploid genotype CM077 and forty plantlets of the tetraploid genotype HT156 were randomly selected to analyze their ploidy level. Sixteen out of forty CM077 plants analyzed were tetraploids, meaning that 40 % of the shoots regenerated from callus were under a process of endoreduplication resulting in a polyploidization rate of 40 %. In the case of HT156, five of forty plants analyzed were octoploids, which represent a polyploidization rate of 12.5 % for this tetraploid genotype. Therefore, according to our results, the polyploidization rate was lower for the tetraploid genotype HT156 than for the diploid genotype CM077.

Mechanical separation of plant chimeras

Asparagus officinalis cv. UC157 F1 (2n = 2x = 20) was used as an external standard in all ploidy level determinations, establishing in the flow cytometry histogram a value of 50 as corresponding to the peak of the G1 somatic nuclei and a value of 100 as corresponding to the peak of the G2 somatic nuclei (Fig. 4a). In a first ploidy analysis, 33 of 220 plantlets were catalogued as mixoploids. The flow cytometry histogram of the mixoploids showed two peaks of similar height in 100 and 200 (Fig. 4b). The first peak represents G1 somatic nuclei of the tetraploid fragment (2n = 4x = 40) and the second one represents G1 somatic nuclei of the octoploid fragment (2n = 8x = 80) plus G2 somatic nuclei of the tetraploid fragment (4n = 8x = 80). A third small peak was observed in 400, which represents G2 somatic nuclei of the octoploid fragment. Thirty-one of the mixoploids resulted from the assays with rhizome buds and the other two plantlets were regenerated from callus. In 29 of these plantlets (88.9 %), we were able to separate the polyploid shoots (Fig. 4d) from the shoots that maintained the original ploidic level (Fig. 4c) by mechanical separation of the different shoots. The flow cytometry histogram of the octoploid shoots showed two peaks (Fig. 4d). The principal peak situated in a value of 200 represents G1 octoploid somatic nuclei (2n = 8x = 80) and the secondary peak located in a value of 400 represents G2 octoploid somatic nuclei (4n = 16x = 160). The flow cytometry histogram of the tetraploid shoots also presented two peaks (Fig. 4c). The principal peak was located near a value of 100 and represents G1 tetraploid somatic nuclei (2n = 4x = 40) and the secondary peak was located near a value of 200 and represents G2 tetraploid somatic nuclei (4n = 8x = 80). Subsequent ploidy analysis showed that the ploidy level of the different shoots mechanically separated from the chimeras was stable and was maintained in the adult plants.

Spear diameter study of asparagus developed in vitro

The effect of polyploidization on the diameter of in vitro spears is shown in Fig. 5. No significant differences were

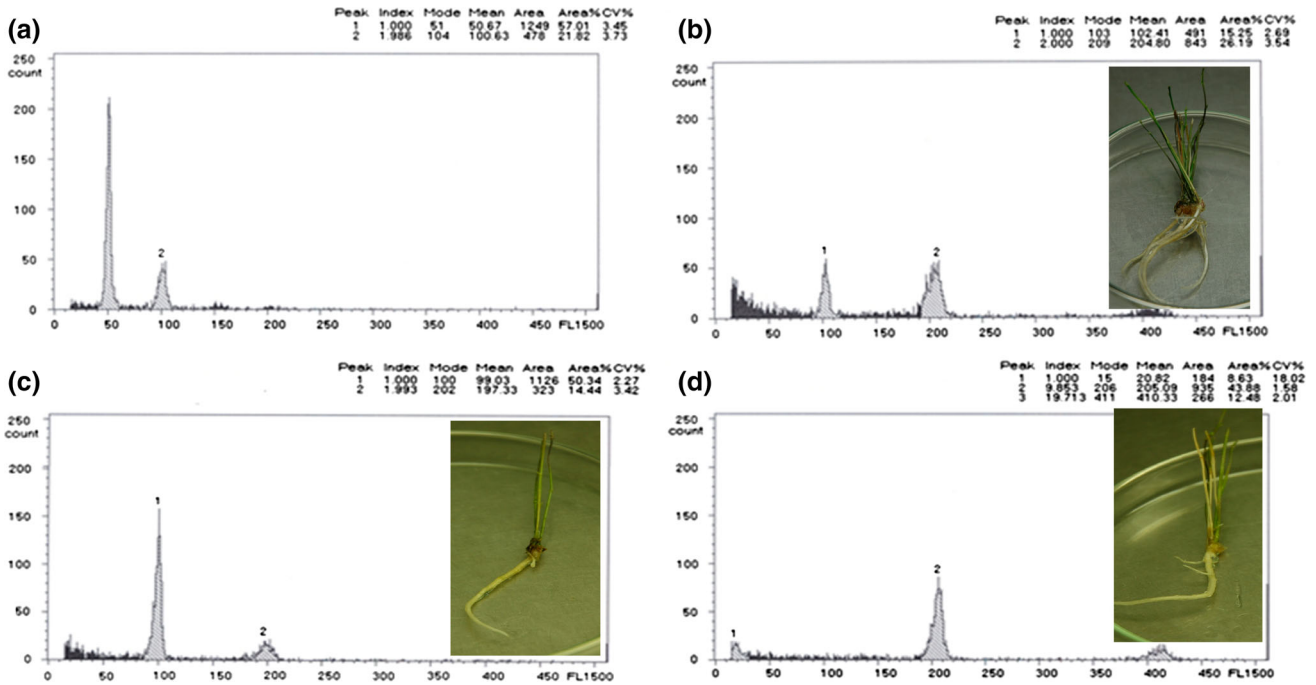


Fig. 4 Flow cytometry traces for four Asparagus plants. **a** *Asparagus officinalis* cv. UC157 F1 (2n = 2x = 20). **b** Mixoploid plantlet regenerated from rhizome buds of "Morado de Huétor" landrace treated with colchicine (2n = 4x = 40 – 2n = 8x = 80). **c** and **d** Separation of different components of mixoploid plants isolating the tetraploid component (2n = 4x = 40) (**c**) and the octoploid component (2n = 8x = 80) (**d**)

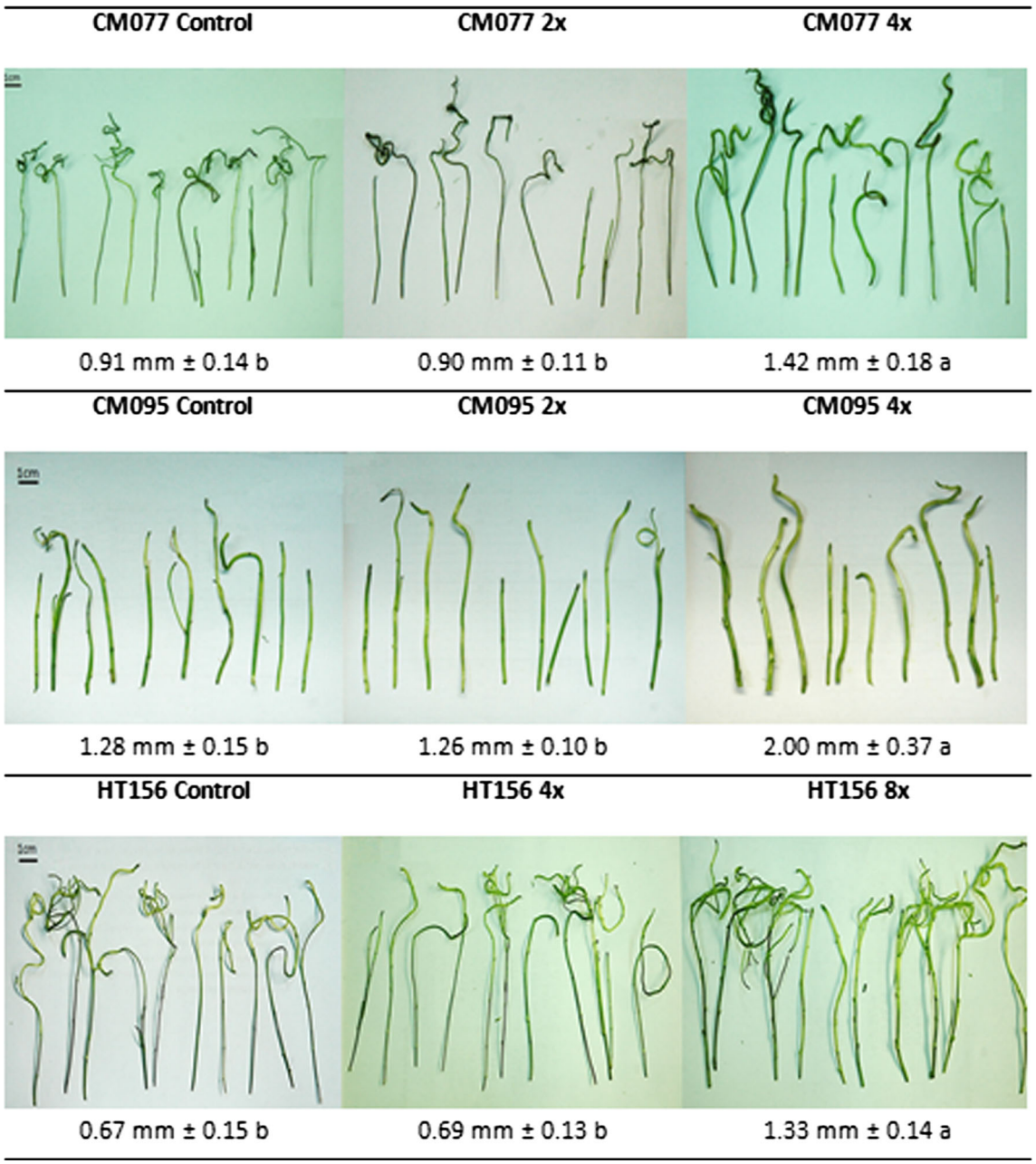


Fig. 5 Analysis of spear diameter of asparagus shoots developed in vitro for polyploid and non-polyploid plantlets obtained from the genotypes CM077, CM095 and HT156. *a*, *b* significant differences by one-way ANOVA, using a HSD-Turkey test in the post-hoc analysis for comparisons among groups

detected between the spear diameter of the control plants (Type1) and the non-polyploid plantlets (Type3) in any of the genotypes analyzed: CM077 (2x), CM095 (2x) and HT156 (4x). However, significant differences were observed when the spear diameter of the polyploidized plantlets (Type2) from the genotypes CM077 (4x), CM095 (4x) and HT156 (8x) were compared with the other two types of plants. The spear diameter increased 156 % in the case of the diploid genotypes CM077 and CM095 and 198 % in the case of the tetraploid genotype HT156.

Acclimatization of asparagus plantlets

The acclimatization rates of the different types of plants obtained in this work are detailed in Table 3. No significant differences were found between the control plants of each

Table 3 Acclimatization rates of three types of plantlets from three asparagus genotypes

CM077		CM095		HT156	
Control (Type 1)	87 ± 9[a]	Control (Type 1)	93 ± 7[a]	Control (Type 1)	87 ± 9[a]
2x (Type 3)	86 ± 9[a]	2x (Type 3)	86 ± 9[a]	4x (Type 3)	86 ± 9[a]
4x (Type 2)	93 ± 7[a]	4x (Type 2)	86 ± 9[a]	8x (Type 2)	100 ± 0[a]

[a] Without significant differences

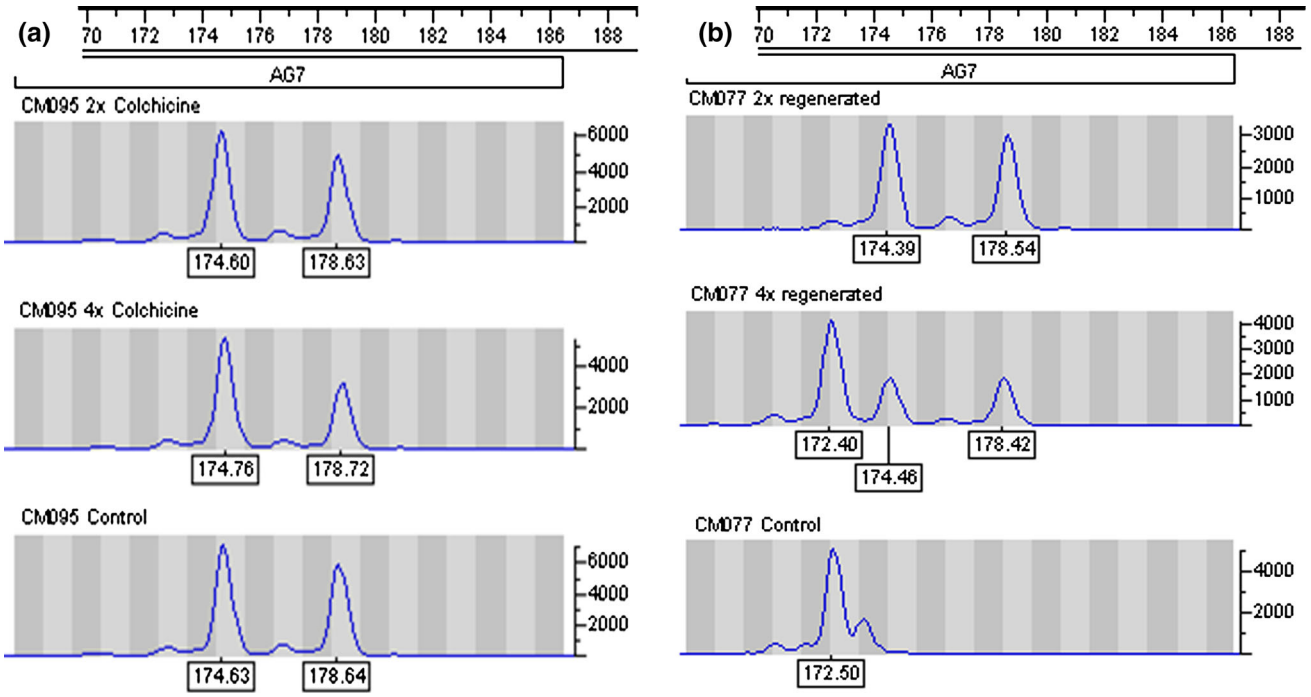


Fig. 6 Chromatograms corresponding to the amplification of EST-SSR AG7 in different plantlets: **a** Diploid plantlet (2x) and tetraploid plantlet (4x) belonging to genotype CM095 micropropagated from rhizome bud explants, and control plantlet of CM095 obtained from the in vitro collection of the IHSM-"La Mayora". **b** Diploid plantlet (2x) and tetraploid plantlet (4x) of genotype CM077 micropropagated through regeneration from callus, and control plantlet of genotype CM077 belonging to the in vitro collection of the IHSM-"La Mayora"

genotype evaluated and the plants obtained from the different methods of polyploidy induction.

Study of genetic stability of polyploid plants using EST-SSRs

As an example, Fig. 6a shows the chromatograms obtained in the amplification of the EST-SSR AG7 in the three types of plants from the diploid genotype CM095. The same two alleles (175 and 179 bp) were detected in the three types of plants analyzed (control plants (2x), polyploid plants (4x) and non-polyploid plants (2x)). These alleles indicate that there is no somaclonal variation for AG7 in the plants from CM095. Figure 6b shows a different example that corresponds to the chromatograms obtained in the amplification of the same marker (AG7) in the three types of plants from the diploid genotype CM077. The polyploid (4x) and non-polyploid plants of this genotype (CM077) were obtained by callus proliferation and shoot regeneration. The CM077 control plants (2x) presented only one allele (172 bp). This result is not preserved in the other plants analyzed. A polyploid plant of CM077 (4x) showed three alleles (172, 174 and 179 bp) and a diploid regenerated plant of CM077 (2x) showed two alleles (174 and 179 bp). These chromatograms indicate that somaclonal variation exists for the EST-SSR AG7 in the regenerated plants whether they are polyploid or not.

The results of the genetic stability study are detailed in Table 4. Somaclonal variation was not detected for any of the twelve EST-SSRs analyzed in the plants obtained from the assays carried out with rhizome bud explants, regardless of the genotype employed and the success or failure of

Table 4 Number of EST-SSR markers detecting somaclonal variation in the different polyploidization treatments

Genotype	Original ploidy level	Treatment of polyploidization	No. of EST-SSRs detecting somaclonal variation
CM077	2x	Plants regenerated from callus	3
CM095	2x	Induction with colchicine in rhizome buds	0
HT156	4x	Plants regenerated from callus	1
HT156	4x	Induction with colchicine in rhizome buds	0

the polyploidization protocol. However, somaclonal variation was observed in the plants regenerated from callus of polyploid and non-polyploid plants. We detected somaclonal variation for three of twelve EST-SSRs (AAT1, AG7 and TC9) in the diploid genotype CM077, while in the tetraploid genotype HT156 the somaclonal variation was only detected for one EST-SSR (AG7).

Discussion

The 4n microspores of the tetraploid genotype HT156 observed in Fig. 1 become unreduced gametes (4x). The formation of unreduced gametes has been previously detected in *Asparagus officinalis* (Camadro 1992, 1994) and *Asparagus densiflorus* (Camadro 1994). The fusion of two unreduced gametes (4n) of "Morado de Huétor" will result in the formation of an octoploid genotype. That was the case of the parent used to develop the octoploid hybrid variety of "Morado de Huétor" HT801 (CESURCA et al. 2012). If the fusion between an unreduced gamete (4x) and a normal gamete (2x) occurs, the result would be a hexaploid genotype (6x), which was also found in the landrace "Morado de Huétor" (Moreno et al. 2006). The existence of triploid and pentaploid genotypes in this landrace (Moreno et al. 2006) could be explained by the fact that plots cultivated with the tetraploid cultivar alternate with plots cultivated with diploid commercial varieties. Pollen from the diploid varieties (2x) can contaminate the tetraploid females (4x) producing triploid plants (3x) if the gamete of "Morado de Huétor" is normal, and pentaploid plants (5x) if the gamete of "Morado de Huétor" is an unreduced gamete. Obtaining triploid varieties of *Asparagus officinalis* by crossing diploid and tetraploid plants induced with colchicine has been described in this species by several authors (Skiebe et al. 1991; Kunitake et al. 1998), and triploid hybrids of *Asparagus officinalis* and "Morado de Huétor" have been already developed (Moreno et al. 2010; Castro et al. 2014).

Recently, (Carmona-Martin et al. 2014a) have developed a method to induce polyploid plants from rhizome bud explants. In this method, rhizome bud explants were subjected to pulses with different concentrations of colchicine for 24 h before incubation in a micropropagation medium (ARBM). A polyploidization rate of 7 % was obtained using 10 g l^{-1} of colchicine. Several authors have supplemented the culture medium with small concentrations of colchicine instead of using pulses of colchicine to induce polydiploidization. As a result, they obtained higher polyploidization rates in different species such as *Salvia miltiorrhiza* (17 %) (Gao et al. 1996), *Phlox subulata* (31.6 %) (Zhang et al. 2008) and *Ranunculus asiaticus* (23.3 %) (Dhooghe et al. 2009). In asparagus, we supplemented the medium ARBM-3 with different concentrations of colchicine (0.1–0.75 g l^{-1}) and incubated rhizome bud explants of "Morado de Huétor" in this medium for 10 and 20 days. To optimize the polyploidization method, we used buds from "Morado de Huétor" plants that are in the field and whose genotype is unknown, because a high number of buds (>200) is necessary for testing different protocols. The best result was obtained using 0.5 g l^{-1} of colchicine for 10 days that led to a polyploidization rate of 25 % (Fig. 3), improving the previous results of Carmona-Martin et al. (2014a). Our results indicated that colchicine increases the innate difficulty of asparagus rooting (Fig. 2). The use of a cyclic process of rooting, consisting in alternating incubation of shoots in the rooting medium ARBM-3 and the growth medium ABRM-0, increases the rooting rate. We have used this protocol to induce polyploid plants from the diploid genotype CM095 and the tetraploid genotype HT156.

Currently, flow cytometry is replacing the traditional techniques used to analyze the ploidy level of plants. Thus, the ploidy level of the plants can be determined by estimating the relative DNA content, which is faster and easier than traditional cytogenetic techniques (Ochatt et al. 2011). Flow cytometry was routinely used in recent asparagus publications (Ozaki et al. 2004; Moreno et al. 2008b; Carmona-Martin et al. 2014a). We have used flow cytometry to evaluate the polyploid plantlets obtained in the polyploidization processes. A total of 33 plantlets were catalogued as mixoploids. The generation of chimerical plants has been reported in studies of polyploidization carried out in different species (Dhooghe et al. 2009; Aina et al. 2012; Gomes et al. 2014) and has been also detected in previous works of polyploidization in asparagus (Carmona-Martin et al. 2014a).

We have developed a method of mechanical separation that allows separating the different parts of mixoploids. As far as we know, there is only one published protocol describing the separation of the components of a mixoploid plant (Roy et al. 2001). These authors used a complicate technique including a step of callus proliferation from the mixoploid shoots and the regeneration of new shoots with different ploidy. Using the method developed in our study, we successfully separated 88.9 % of the plantlets tested (Fig. 4). Protocols of mechanical separation have been also used to separate the components of other types of chimeras (not mixoploids) in other species such as *Dianthus* sp. (Johnson 1980), *Rubus* sp. (Mc Pheeters et al. Pheeters and Skirvin 1983), *Nicotiana* sp. (Marcotrigiano 1986) and *Pyrus* sp. (Abu-Qaoud et al. 1990).

The other method used in this work to induce polyploidization was the regeneration of polyploid plants from callus culture. It was used with the genotypes CM077 (diploid) and HT156 (tetraploid), reaching a polyploidization rate of 40 and 12.5 % for the diploid and tetraploid genotypes, respectively. The endoreduplication in different cells during the callus incubation of *Asparagus officinalis* (2x) has been described in previous works (Odake et al. 1993; Kunitake et al. 1998; Raimondi et al. 2001; Pontaroli and Camadro 2005) as the cause of the polyploidization. The polyploidization rate (40 %) obtained for CM077 is similar to the rate (37.8 %) obtained by Pontaroli and Camadro (2005) in a different diploid asparagus genotype. Endoreduplications are less frequent in tetraploid genotypes such as HT156 because the genetic stability of the tetraploid genotypes is higher (Kunitake et al. 1998). The higher stability of HT156 resulted in a lower polyploidization rate (12.5 %), which also was smaller than the rate obtained for rhizome bud explants in this work (25 %).

As indicated in the Introduction, one of the most interesting agronomical traits of polyploid plants is the bigger diameter of spears respect to the original plants (Braak and Zeilinga 1957; Kunitake et al. 1998; Carmona-Martin et al. 2014a). To confirm this agronomical effect, we have studied the spear diameter using in vitro spears obtained from different polyploid genotypes (Fig. 5). The polyploid plantlets showed spears with a diameter increase of 156 % between the tetraploid plantlets and the diploid plantlets, and of 198 % between the octoploid plantlets and the tetraploid plantlets. The non-polyploid plantlets obtained after the polyploidization process did not show differences in diameter compared to the control of each genotype. We can confirm that the increase of spear diameter is due to polyploidization and not because of the process of micropropagation. Carmona-Martin et al. (2014a) indicated that the increase in the spear diameters is caused by the larger size of the polyploid cells.

No differences were detected between the acclimatization rate of polyploid plants and the control plants. In all cases the rate was around 90 %. These acclimatization rates indicate that the polyploid plants are viable and the polyploidization does not induce any problem for acclimatization.

Due to the higher productivity and greater longevity of male plants, another advantage of the polyploidization procedures is the modification of the sex ratio towards a higher percentage of male plants. Thus, the tetraploid males obtained in this work from the diploid genotype CM077 are MMmm, and the hybrid varieties derived from this parent will result in a male: female ratio 5:1 (83 % males) (Braak and Zeilinga 1957; Skiebe et al. 1991). The tetraploid males of the Spanish landrace "Morado de Huétor", such as HT156, are Mmmm (Moreno et al. 2008a) and the octoploid males obtained from HT156 will be MMmmmmmm. Therefore, the hybrid varieties derived from it would present a male: female ratio 11:3 (78 % males).

As we indicated in the introduction, the two polyploidization methods employed in this work are based on micropropagation techniques. Therefore, they could induce somaclonal variation in the plants obtained (Bairu et al. 2011), that may cause unwanted changes in the agronomical traits of the initial genotypes. We used twelve EST-SSRs to detect any somaclonal variation. A similar number of microsatellites has been used to study the genetic stability of different micropropagated species in previous studies: 10 SSRs in *Populus tremuloides* (Rahman and Rajora 2001), 7 SSRs in *Pinus pinaster* (Marum et al. 2009), 14 EST-PCRs in *Vaccinium angustifolium* (Debnath 2011) and 8 SSRs in *Chrysanthemum morifolium* (Wang et al. 2014). In the polyploid plants generated from rhizome bud explants treated with colchicine, no somaclonal variation was detected for any EST-SSR, while all the plants obtained by shoot regeneration from callus culture showed somaclonal variation for at least one EST-SSR. Somaclonal variation in plants regenerated from callus culture had already been detected in previous works using different molecular markers such as RAPDs (Raimondi et al. 2001) and AFLPs (Pontaroli and Camadro 2005). Kunitake et al. (1998) also reported morphological changes in these plants. The differences between the two methods of polyploidization on the induction of somaclonal variation were linked to several factors such as the initial explant, the presence of a disorganized growth phase or the type and concentration of the auxin used. The use of a primary explant with preexisting meristems normally involves a higher genetic stability in the micropropagated plants (Sharma et al. 2007). These meristems are present in rhizome bud explants, while the occurrence of a disorganized growth phase, such as callus growth, is considered an important factor on induction of somaclonal variation (Rani and Raina 2000). Finally, our shoot regeneration method from callus includes the use of 2,4-D (2 mg l^{-1}). This auxin is able to induce somaclonal variation in different crops such as strawberry (Nehra et al. 1992),

soybean (Gesteira et al. 2002) and cotton (Jin et al. 2008). The auxin NAA, used in the micropropagation of rhizome buds, induces less somaclonal variation than 2,4-D, when both auxins are used at the same concentration (Ahmed et al. 2004) and, in this work, it is used in a concentration 10 times lower (2 mg l^{-1} for 2,4-D versus 0.3 mg l^{-1} for NAA).

We can conclude that the asparagus polyploid plants obtained through culture of rhizome buds in medium ARBM-3 supplemented with colchicine are plants with high genetic stability in which no somaclonal variations have been detected. Therefore, they should maintain the agronomic traits of the mother elite plant from which the rhizome bud explants were originated. However, we detected somaclonal variations in the asparagus polyploid plants obtained from the callus culture, so these polyploid plants present less genetic stability than the polyploid plants obtained through culture of rhizome buds. All the plants obtained in this work were included in the in vitro collection of the IHSM. These plants can be used in future breeding programs to obtain new varieties of asparagus with better agronomical traits like spears with improved caliber or better rates of male: female than traditional varieties, especially in the case of the Spanish landrace "Morado de Huétor".

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