Plant Cell Tiss Organ Cult (2015) 121:249–254
DOI 10.1007/s11240-014-0680-4

RESEARCH NOTE

In vitro induction of autooctoploid asparagus genotypes

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Received: 22 August 2014 / Accepted: 28 November 2014 / Published online: 5 December 2014

Abstract Octoploid genotypes of *Asparagus officinalis* L. cv. 'Morado de Huetor', a tetraploid Spanish landrace, were successfully induced by treating in vitro rhizome buds explants with colchicine. Pulses during 24 h with different concentrations of colchicine are able to induce polyploid plants, colchicine applied at 0.1 % induce a 7 % of octoploid genotypes with a stable ploidy level and a 3.5 % of mixoploid genotypes. The maximum rate of mixoploid genotypes (26 %) was obtained with 0.3 % colchicine. The plant survival and rooting rates of explants decrease for increasing doses of colchicine. All octoploid genotypes were micropropagated, rooted, transplanted and successfully acclimatized (100 %) in a glasshouse. The four octoploid plants recovered show significantly better agronomical parameters such as spear diameter, canopy area and shoot length than the original tetraploid plants and histological studies confirm the size increase of octoploid cells respect the original tetraploid cells.

Keywords Rhizome bud · Colchicine · Ploidy · Chromosome · Flow cytometry · Chlorophyll

Asparagus officinalis L. (2n = 2x = 20), which is cultivated worldwide, is the most economically important *Asparagus* species. The modern commercial varieties only offer a limited genetic variability due to their common origin (Knaflewsky 1996).

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With a basic chromosome number (x = 10), most of the species of *Asparagus* genera are diploid (Moreno et al. 2008) but also show different ploidy levels, such as *A. maritimus* (6x, 4x), *A. tenuifolius* (6x), *A. prostratus* (4x), *A. acutifolius* (4x) and some tetraploid (4x) landraces such as 'Violetto d'Albenga' and 'Morado de Huetor' (MH).

Cultivated in the South of Spain, MH is an autochthonous Spanish landrace, originating from the natural crossing between *A. officinalis* and *A. maritimus*. This landrace shows high genetic variability due to its hybrid origin, including different levels of ploidy (2n = 2x, 3x, 4x, 5x, 6x, 8x), but most of their genotypes are tetraploid (Moreno et al. 2006, 2008). The main problem of MH landrace is their small spear diameter, resulting in lower productivity and market success with respect to other commercial varieties. A minimal number of natural octoploid genotypes have been detected during field screening. These octoploid genotypes show superior agronomical traits (vigor, thicker spears, higher productivity), and are extremely interesting for breeding and farming.

Colchicine is the most commonly used antimitotic to induce polyploidy in plants (Petersen et al. 2003; Eeckhaut et al. 2004).The regeneration of polyploid genotypes from selected plants applying colchicine in vitro has been successful in different plant species (Shao et al. 2003; Praça et al. 2009).

Polyploidy induces a wide range of physiological and morphological changes, such as an increase in organ size or chlorophyll content, in different species (Gao et al. 1996; Zhang et al. 2008). In *A. officinalis*, Braak and Zeilinga (1957) also detected morphological differences between diploid and tetraploid genotypes. Autotetraploid asparagus show darker color and larger flowers, pollen, and spears, than the original diploid plants.

Table 1 Effects of different concentrations of colchicine on survival and rooting of explants and ploidy of regenerated plantlets

Concentrations of colchicine (% w/v)	Survival (%)	Rooting (%)	No. of plants analyzed for FCM	Octoploids no. (%)	Mixoploids no. (%)
0	97 a	90 a	–	–	–
0.01	45.5 bc	43 b	41	0 (0)	0 (0)
0.05	68 b	39 b	61	0 (0)	0 (0)
0.1	63.3 b	45 b	57	4 (7)	2 (3.5)
0.2	33.3 bc	14 bc	30	0 (0)	0 (0)
0.3	25.5 c	20 bc	23	0 (0)	6 (26)
0.5	20 c	7 c	10	0 (0)	1 (10)
0.7	29 c	9 c	26	0 (0)	1 (3.8)
1	17 d	0 d	15	0 (0)	0 (0)
2	17 d	0 d	15	0 (0)	3 (20)

Different low case letter in the same column means statistical difference according to the contrast test (SAS)

Flow cytometry (FCM) is the choice method for polyploidy determination, screening for cytotypes, detection of chimeras and identification of hybrids in crop species (Ochatt et al. 2011).

To date, polyploidy induction in *Asparagus* has only been assayed over diploid seeds (Braak and Zeilinga 1957; Skiebe et al. 1991). The induction of polyploids using vegetative tissues instead of seeds allows the agronomical traits of the mother plant to be maintained or improved. Furthermore, there are no reports about the regeneration of autooctoploid genotypes of *Asparagus* from tetraploid genotypes.

The present work aims to establish a methodology for in vitro induction and recovery of octoploid plants in MH using rhizome bud explants of elite genotypes and evaluate the effects of colchicine over agronomical traits of interest.

Colchicine was applied as a pulse treatment (24, 48 h) over MH rhizome bud explants (3–5 mm), incubated in flasks containing 3 ml of colchicine solutions (0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 1 y 2 % w/v), under shaking conditions (80 rpm). In each experiment, 30 rhizome bud explants per colchicine treatment were used, and all experiments were repeated three times. Later, the explants were incubated in Asparagus Rhizome Bud Medium (ARBM) supplemented with 200 mg l^{-1} of filter sterilized Cefotaxime, for 4 weeks and then transferred to ARBM without antibiotic following the methods of Carmona-Martín et al. (2014).

All cultures were incubated at 25 ± 1 °C under a 16 h day photoperiod under cool white fluorescent tubes (Gro-lux, Sylvania) with 45 µmol m^{-2} s^{-1} (400–700 nm) Photosynthetic Active Radiation.

After 8 weeks, incubation data on survival and rooting were recorded. The mother plants were preserved in vitro for further comparisons. The polyploid regenerated plants were acclimatized following the method of Carmona-Martín et al. (2014).

After 8 weeks of incubation, the ploidy level of survival plantlets was determined by FCM (Ploidy Analyser-PA-I, Partec GmbH, Münster, Germany) following the procedures of Moreno et al. (2008), *A. officinalis* cv. "Baitoru" (2n = 2x = 20) was always included in the sample as an external standard. Three independent repetitions were performed, with over 10,000 nuclei being analyzed in each repetition. These analyses were repeated 3 months later to confirm the stability of the polyploids and repeated again after acclimatization of the autooctoploid plants.

Karyotyping was performed following the Singh (2002) protocol over root tips (8–10 mm long), obtained from autooctoploid (8x) and two control (2x and 4x) genotypes regenerated after the treatments with colchicine.

The content of chlorophyll a, b and the total was calculated applying the formulas and protocols developed by Goodwin (1976); the results were expressed in mg of chlorophyll per g of fresh weight. Five samples of leaves of micropropagated autooctoploid and tetraploid genotypes of MH, maintained in the greenhouse for 6 months, were analyzed.

The morphological parameters of regenerated asparagus were studied on 4-month-old plants maintained in big containers at a greenhouse. Data on shoot number, length, and basal diameter were recorded for five shoots belonging to autooctoploid and tetraploid plants and the canopy area was estimated in all the autooctoploid plants generated. Data showed correlations between the average of four autooctoploid plants and their respective four tetraploid mother plants.

To study the size of both type of cells (4x and 8x), fresh cuts of spears were made, stained with toluidine blue and photographed. Using the image analysis program Image-J, the cell area/diameter measurements were estimated over three different micrographs obtained from an autooctoploid plant and the corresponding tetraploid mother plant. Data presented correspond to the average of five measures repeated three times.

Data were previously transformed with $\arcsin\sqrt{x/100}$ to obtain the normality criteria and then analyzed with the

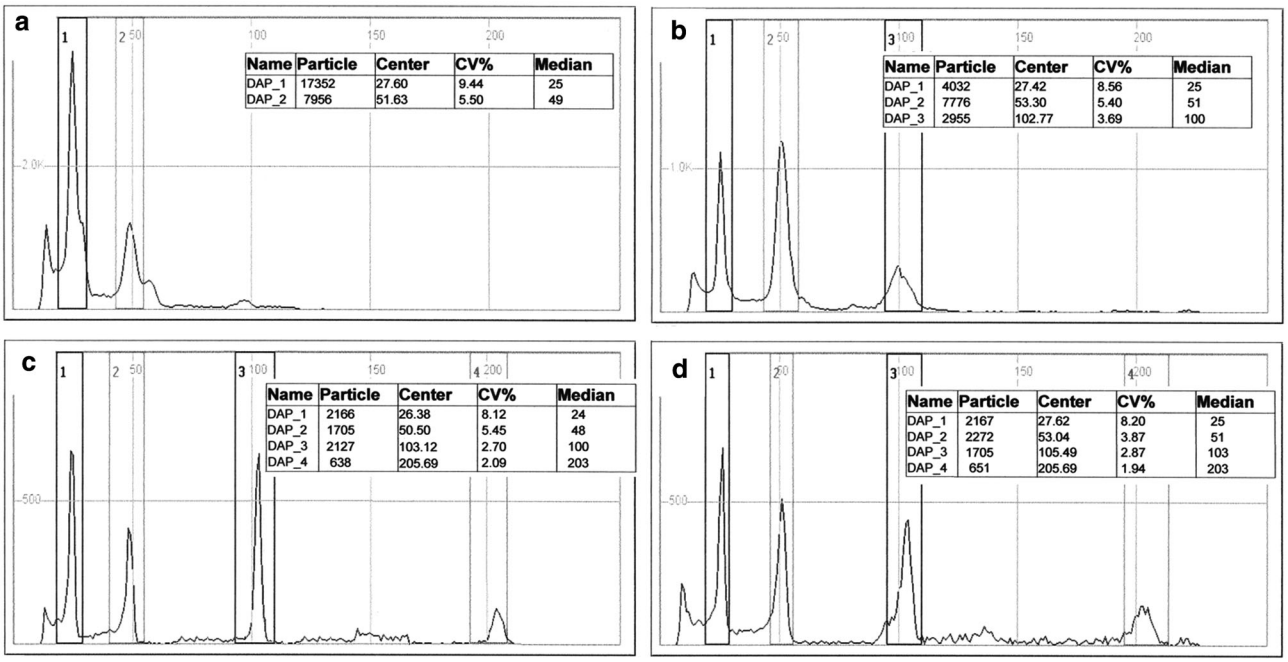

Fig. 1 Histograms corresponding to the analysis of asparagus samples: **a** diploid sample 2n = 2x, **b** tetraploid sample 2n = 4x plus diploid control, **c** autooctoploid sample 2n = 8x plus diploid control, **d** mixoploid sample 2n = 4x − 8x plus diploid control

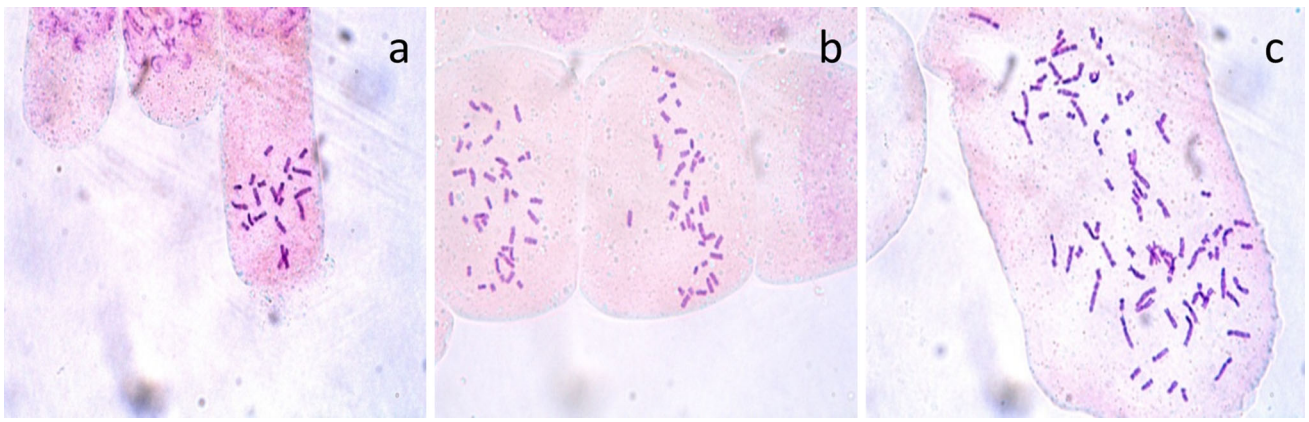

Fig. 2 Root tip chromosomes of *Asparagus officinalis* L. **a** Control diploid cell with 2n = 8x = 80 (cv. Baitoru), **b** tetraploid cell with 2n = 4x = 40 (cv. Morado de Huetor), **c** octoploid cell with 2n = 8x = 80 (cv. Morado de Huetor) obtained after colchicine treatement

SPSS/PC+program. Normally distributed variables were analyzed by analysis of variance (ANOVA). When significant differences were found, the values were compared according to a Student–Newman–Keuls test.

The 24 h colchicine pulses were selected due to the extreme toxicity of 48 h pulses. The 24 h colchicine pulses also negatively affects the growth of rhizome bud explants, showing decreased levels of survival (97–17 %) and rooting (90–0 %) when colchicine doses increase (Table 1).

Table 1 indicates that 0.1 % w/v colchicine was the optimal doses for autooctoploid induction (7 %). At the end of the first subculture after colchicine treatment (0.1 % w/v) and before ploidy analysis, the survival rate was approximately 63 % and the rooting rate reached 45 %. After the ploidy analysis, the 7 % of autooctoploid genotypes generated were labeled, multiplied and after several subcultures in ARBM containing Ancymidol, rooted, with a 100 % of success following the method of Carmona-Martín et al. (2014).

The treatment of 0.3 % w/v generates a 26 % of mixoploids genotypes, which is similar to rates obtained in *Alocasia* (4.5 % tetraploids and 8.4 % mixoploids) applying colchicine pulses (Thao et al. 2003). This author found that for all the supraoptimal concentrations of colchicine

the rate of chimeras increases, again, similar results were recorded for MH.

Multiplication of selected material was achieved by simple mechanical separation of rooted buds clusters developed in vitro, obtaining simple-rooted plantlets and subculturing them in fresh ARBM medium.

Table 2 Differences in chlorophyll content between tetraploid and autooctoploid genotypes of *Asparagus* cv. Morado de Huetor

Chlorophyll content	Octoploids	Tetraploids
Chlorophyll a + b (mg g^{-1})	56.3 a	50.9 b
Chlorophyll a (mg g^{-1})	36.2 a	36.7 a
Chlorophyll b (mg g^{-1})	29.9 a	22.4 b
Ratio Chlorophyll a:b	1.2 b	1.6 a

Different letters in the same row represent significant differences analyzed with One Way ANOVA ($p < 0.05$)

Table 3 Morphological characteristics in micropropagated tetraploid and autooctoploid asparagus plants

Morphological traits	Octoploids	Tetraploids
Shoot number	25.3 a	25.0 a
Spear diameter (mm)	6.2 a	3.0 b
Shoot length (cm)	230 a	170 b
Canopy area (cm^2)	0.95 a	0.64 b

Different low case letter in the same row means statistical difference according to the *Student–Newman–Keuls* test ($p < 0.5$)

Figure 1 shows the diagrams obtained for diploid (2n = 2x = 20), tetraploid (2n = 4x = 40), octoploid (2n = 8x = 80) and mixoploid (2n = 4x − 8x = 40–80) genotypes corresponding to FCM analysis of plantlets regenerated after the colchicine treatments. The control diploid peak appears at channel 25 and the tetraploid and the octoploid peaks appear at channel 50 and 100, respectively; the peaks for mixoploids appear at channel 50 and 100.

Four autooctoploid and 13 mixoploid genotypes were obtained after the colchicine treatments. The ploidy stability of the autooctoploid genotypes was confirmed after an incubation of 3 months in vitro.

Figure 2 shows the chromosome number of diploid, tetraploid and autooctoploid plantlets confirming the ploidy levels determined with FCM. The diploid control shows 20 chromosomes (Fig. 2a), the tetraploid sample shows 40 chromosomes (Fig. 2b) and the autooctoploid sample shows 80 chromosomes (Fig. 2c).

Chlorophyll a, b and total chlorophyll content in leaves of tetraploid and octoploid genotypes of asparagus were quantified; clear differences were observed between them (Table 2). The total content of chlorophyll was on average significantly higher in octoploid plants (56.3 mg chlorophyll/g of fresh weight (FW) respect the total content in tetraploid asparagus (50.9 mg/g of FW).

In our asparagus polyploids the ratio of chlorophyll a:b was 25 % greater in 4x than in 8x leaves, which is in

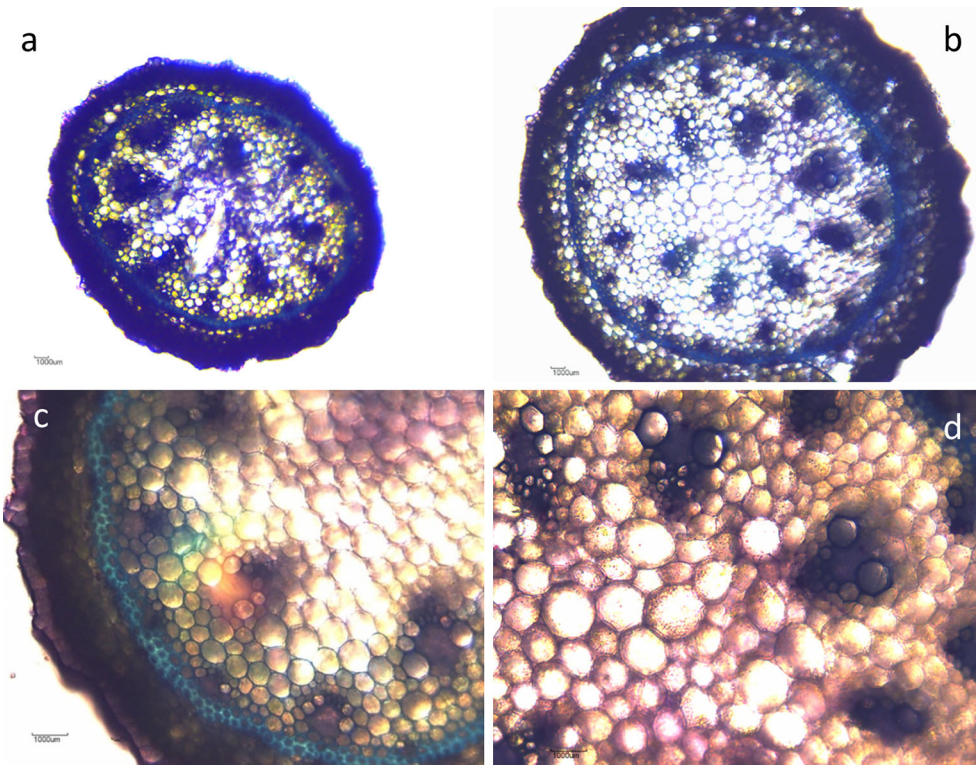

Fig. 3 Histological comparison between tetraploid and autooctoploid spear sections of asparagus cv. Morado de Huetor. **a** Tetraploid sample (×4), **b** octoploid sample (×4), **c** tetraploid sample (×10), **d** octoploid sample (×10). *Bar* 1,000 µm

Fig. 4 Morphological comparison between an autooctoploid plant and its mother tetraploid mother plant

agreement with the results by Romero-Aranda et al. (1997) in *Citrus sinensis.*

Braak and Zeilinga (1957) reported significant differences in some morphological traits (flower size, spear diameter, color) between diploid and tetraploid genotypes of *A. officinalis* induced with colchicine. Table 3 shows statistically significant differences for the spear diameter between octoploid and tetraploid plants. The average values of shoot length and canopy area are also significantly different, the higher values belonging to the autooctoploid genotypes. These morphological traits indicate that the autooctoploid plants are much more vigorous than the tetraploids.

When tetraploid (4x) and autooctoploid (8x) cells are compared (Fig. 3) the autooctoploid cells show a cellular size 2.2 times bigger than the original tetraploid cells. The increase in spear diameter recorded for autooctoploid genotypes is due to the cell size increase since the cell number remains stable. Cavalier-Smith (1985) reported that in polyploids an increase in cell size is proportional to the increase of DNA content in order to balance cell growth through maintenance of a constant ratio between the nuclear volume devoted to transcription and the cytoplasmic volume devoted to protein synthesis.

The results obtained after inducing autooctoploid genotypes confirm the size increase of the genotypes obtained and the generation of plants producing thicker spears (Fig. 4), improving the market competitiveness of this landrace.

Polyploidization studies in asparagus have never been developed over vegetative material. Because the male plants of asparagus the most suitable material for farming, the development of improved male genotypes from vegetative tissues obtained from elite genotypes, will be the best option to maintain the agronomical traits from the parental plant.

For this goal, the rhizome buds have been the explant of choice for polyploidy induction, due to their bipolar pattern of growth and the availability of an efficient method of micropropagation (Carmona-Martín et al. 2014).

The application of the micropropagated method for mass micropropagation of generated polyploids could improve the reported fertility problems of polyploid genotypes (Braak and Zeilinga 1957; Gao et al. 1996).

This method can also be used to increase the genetic variability by selective crossing with different ploidy level genotypes, generating genotypes with diverse ploidy (3x, 5x, 6x) useful for further crosses with genotypes or species with similar ploidy, and could circumvent incompatibilities or fertility problems (Skiebe et al. 1991) detected in intraspecific or interspecific hybrids obtained by crossing parentals with different ploidy.

Finally, the studies of the possibility to overcome the incompatibility barriers and the consequences of the allopolyploidy in interspecific crosses could give interesting new data about polyploid behavior in *Asparagus* genera.

Polyploidy in asparagus also offers another advantage: the standard 1:1 sex ratio male:female increases up to 78.6 % when autooctoploid male pollen was used in breeding.

This methodology has been applied to tetraploid genotypes (MH) with successful regeneration of autooctoploid genotypes with excellent agronomical traits.

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