Plant Cell Tiss Organ Cult (2014) 119:479–488
DOI 10.1007/s11240-014-0548-7

ORIGINAL PAPER

A new and efficient micropropagation method and its breeding applications in *Asparagus* genera

E. Carmona-Martín · J. J. Regalado · I. M. G. Padilla · N. Westendorp · C. L. Encina

Received: 28 January 2014 / Accepted: 20 June 2014 / Published online: 23 July 2014

Abstract Cultivated asparagus (*Asparagus officinalis* L.) is an economically important plant worldwide. "Morado de Huetor" is a Spanish autochthonous landrace characterized by their longevity, organoleptic characteristics, differential biocompound content and high heterozygosity, resulting in heterogeneous plantations with limited productivity. Consequently, this landrace suffers high risk of extinction due the lack of productivity. The preservation of the genetic pool of asparagus requires the development of a reliable micropropagation method. A new, rapid and efficient method of micropropagation for asparagus using rhizome bud explants has been developed. The rate of disinfection reached 90 %, and the system for shoot development and rooting on Asparagus Rhizome Bud Medium took place in one step. Recovery of the full plantlets ranged between 65 and 90 %. The plantlets were ready to be transplanted by 8 weeks, with a successful acclimatization of 80 % in average. The micropropagated plants were normal in phenotype, and the genetic stability was verified using molecular markers expressed sequence tags–microsatellites or simple sequence repeats and Flow Cytometry and certified as true-to-type. Applying this method, an in vitro breeder collection of "Morado de Huetor" landrace, *A. officinalis*, wild asparagus relatives and hybrid progenies has been established.

Keywords Flow cytometry · Germplasm preservation · Molecular markers · Rhizome explants · Rooting · *Asparagus officinalis* · *Asparagus maritimus* · "Morado de Huetor" · Landrace

Abbreviations

AFLP	Amplified fragment length polymorphism
ANC	Ancymidol
ARBM	Asparagus rhizome bud medium
DAM	Desjardins Asparagus medium
EDDHA	Ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid)
EST	Expressed sequence tags
FCM	Flow cytometry
KIN	Kinetin
MS	Murashige and Skoog medium (1962)
NAA	1-Naphthalene-acetic acid
RAPD	Random amplified polymorphic DNA
SSRs	Microsatellites or simple sequence repeats
2iP	2-Isopentenil adenine
BA	6-Benziladenine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
bp	Base pairs

Introduction

Cultivated asparagus (*Asparagus officinalis* L.) is a monocotyledonous diploid (2n = 20) plant belonging to the Asparagaceae family. *A. officinalis* L. is an important economical species cultivated around the world. Modern commercial varieties of asparagus derived from the Dutch population "Gewone Hollandse" (Geoffriau et al. 1992;

E. Carmona-Martín · J. J. Regalado · I. M. G. Padilla (✉) · N. Westendorp · C. L. Encina
Instituto de Horticultura Subtropical y Mediterránea "La Mayora", CSIC-UMA, 29750 Algarrobo-Costa, Málaga, Spain
e-mail: ecarmona@eelm.csic.es

I. M. G. Padilla
Centro IFAPA-Churriana, 29140 Churriana, Málaga, Spain

Knaflewsky 1996). Due to the common origin of present day commercial cultivars, their genetic base is quite narrow, as it has been demonstrated with molecular markers (Caruso et al. 2008; Moreno et al. 2006, 2010a).

"Morado de Huetor" is a Spanish asparagus landrace originated by natural crossing between *A. officinalis* x *Asparagus maritimus* (Moreno et al. 2008a). This hybrid landrace presents different ploidy levels (2n = 2x, 3x, 4x, 5x, 6x, 8x) and high genetic variability so it is a good candidate for increasing the genetic variability of commercial varieties by hybridization. Moreno et al. (2010b) demonstrated the feasibility of generating triploid hybrids with new agronomic characteristics by crossing "Morado de Huetor" specimens with commercial cultivars, mobilizing new genetic resources.

Since 1987, the cultivated area of this landrace has been drastically reduced (Moreno et al. 2008b) due to the competence of the commercial diploid varieties with higher productivity. For this reason, this landrace is at risk for extinction, which would result in the loss of valuable genetic resources, making necessary the development of an in vitro breeder collection of "Morado de Huetor" to preserve elite genotypes, which requires the development of a reliable method of micropropagation.

Clonal micropropagation of asparagus to obtain allegedly true-to-type copies of elite genotypes of *A. officinalis* has been performed using meristem culture (Murashige et al. 1972; Hasegawa et al. 1973) or nodal axillary sections obtained from the asparagus spear (Yang and Clore 1973, 1974; Chin 1982; Desjardins et al. 1987; Yukimasa et al. 1990; Desjardins 1992). Yukimasa et al. (1990) and Desjardins (1992) micropropagation methods are similar, being both based on the use of proliferating "crowns" induced in vitro from initial spear explants. To date, all micropropagation methods using aerial explants of asparagus are slow, inefficient due to low rooting rate and not valid for all asparagus genotypes.

In asparagus when micropropagation protocols through callus induction and regeneration via organogenesis or embryogenesis, the micropropagated plantlets showed morphological variations (Kohmura et al. 1996), ploidy variation and somaclonal variation (Araki et al. 1992; Odake et al. 1993; Kunitake et al. 1998; Raimondi et al. 2001; Pontaroli and Camadro 2005), so through these procedures, the production of "true-to-type" plantlets of asparagus is not guaranteed.

In this paper we report a new method of asparagus micropropagation using rhizome bud explants to preserve the genetic pool of "Morado de Huetor" landrace, we use Flow cytometry (FCM) to evaluate the ploidy level on the asparagus plants recovered after in vitro treatment. FCM is a well established method (Ochatt et al. 2011), successfully used to evaluate the genetic stability at ploidy level in *A. officinalis* (Limanton-Grevet et al. 2000; Shiga et al. 2009) and other species such as *Citrus* (Cao et al. 2011), *Prunus* (Vujovic et al. 2012), *Medicago* (Ochatt et al. 2013), *Musa* (Escobedo-GraciaMedrano et al. 2014).

For testing the genetic stability we applied microsatellites (EST-SSRs). Previous works assessing the genetic stability of Asparagus micropropagated plantlets were carried out, using different molecular markers such as RAPD (Raimondi et al. 2001) or AFLP (Pontaroli and Camadro 2005). However currently, microsatellites, or SSRs (Gao et al. 2009, Liu et al. 2011, Cao et al. 2011), are

Table 1 List of selected elite genotypes of cv. "Morado de Huetor" asparagus landrace ordered by the outstanding agronomical traits that were used for selection

	Agronomical trait of selection	Selected specimens
A	Thick spear caliper >14 mm	A1, A2, A3, 93B, 114, HT-199, 151, HT-090, HT-091, HT-111, HT-145, HT-515, HT-152, HT-153
B	Closed spear heads (acuminate or acute)	A2, 9, 9B, 93B, HT-192, HT-193, HT-075, HT-199, 151, 168, 169, 173, 179, 187, 208C, HT-119, HT-145, HT-515, HT-152, HT-153, HT-160
C	Wild morphological shape	197B, 197 C, HT-128, HT-129, HT-127, HT-150, Paerones, *A. maritimus*, HT-168, Triguero (0,1 5), HT-029, HT-063, HT-064, 181, HT-109, HT-115, HT-118
D	Productivity >1,100 g/year	HT-030, HT-178, HT-179, HT-180, Peñuelas (7,8,9), 136G, HT-075, HT-118, 197 (B,C,H,G,F), HT-119, HT-160, HT-161
E	Plant vigor (tall shape and/or high no. of shoots in summer) >2.5 m tall and/or >250 shoots/plant	HT-168, HT-029, HT-089, HT-111, HT-114, HT-118, 197B, 197 C, HT-128, HT-129, HT-127, HT-119, HT-145, HT-515, HT-150, HT-152, HT-153, HT-160, HT-161, Paerones, Soto
F	Persistence of green color during autumn season	HT-089, HT-117
G	Intense purple/bronze color	HT-030, HT-032, HT-033, HT-026, HT-027, 151, 181, HT-103, HT-107, HT-110, HT-145
H	Intense green color	9, HT-023, HT-109, HT-160
I	Low fiber content <2 %	36B, HT-075, HT-199
J	Hermaphrodite specimens (andromonoic plants)	HT-100, HT-150
K	Triploid plants	HT-134
S	High level of bioactive compounds >420 mg kg^{-1}	HT-117

considered the consensus markers for studies of somaclonal variation (Bairu et al. 2011).

This innovative method is versatile enough to be applied for micropropagation of other species of subgenera *Asparagus.*

Materials and methods

Plant material

All of the experiments were performed with the genotype HT-089 of "Morado de Huetor", using rhizome buds as explants, obtained by extracting pieces of underground rhizomes from fields and/or potted plants of asparagus growing in a glasshouse.

Also, the micropropagation method was applied to multiple genotypes (Table 1) belonging to "Morado de Huetor" landrace and to commercial cultivars of *A. officinalis* (UC-157, Atlas, Grande, Baitoru and Cipres), and a wild relative, *A. maritimus.*

Dissection and disinfection of explants

The initial pieces of rhizome were thoroughly washed with tap water, and roots and necrotic material around the rhizome bud clusters were cut away and discarded. After the first isolation, the rhizome bud clusters (2 cm^2) were carefully washed again with a soap solution and tap water and then treated with fungicide (benomyl 0.3 %) for 15 min under stirring (120 rpm). After washing the explants in sterile, distilled water, the rhizome bud clusters were disinfected in a 2 % sodium hypochlorite solution for 20 min under vacuum conditions and washed with sterile distilled water. Immediately, the rhizome bud clusters were divided with a scalpel into individual rhizome buds. Each rhizome bud was dissected using a blade, and a number of external bracts that covered the shoot meristem were discarded until the explants reached a size of 0.2–0.5 cm. A piece of parenchymatic tissue, dissected into an inverted, truncated pyramid, was always left at the base of the bud. After dissection, the rhizome bud explants were maintained in an antioxidant solution containing 150 mg l^{-1} of citric acid plus 100 mg $l^{-1}$of ascorbic acid. All of the dissected rhizome buds were treated again with fungicide (benomyl 0.3 %) for 15 min under stirring and, rinsed in sterile distilled water, disinfected in a 2 % sodium hypochlorite solution for 15 min under vacuum conditions and washed again three times with sterile, distilled water in aseptic conditions before they were established in vitro. The rhizome bud explants, which were approximately 0.2–0.5 cm in length, were used as explants for all micropropagation assays. One rhizome bud was incubated per test tube for in vitro establishment.

Culture conditions

The pH of all culture media was adjusted to 5.7 before autoclaving. For culture initiation and rooting experiments, 25 ml of medium was aliquoted into 150 mm × 25 mm test tubes that were covered with polypropylene tops (Bellco Corp.) and autoclaved for 15 min at 121 °C and 1.05 kg cm^{-2}. All cultures were incubated at 25 ± 1 °C during a 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.

Micropropagation method

The effect of sucrose, plant growth regulators, iron and a plant growth retardant were evaluated by comparing to the control medium DAM (Desjardins, 1992), consisting on MS salts (Murashige and Skoog, 1962) supplementing with the following (mg l^{-1}): thiamine-HCl (100), pyridoxine-HCl (50), nicotinic acid (50), glycine (200), i-inositol (100) and 0.8 % of Agar A-1296 (Sigma),0.1 mg l^{-1} KIN, 0.1 mg l^{-1} NAA, 1.3 mg l^{-1} Ancymidol (ANC) and 30 g l^{-1} sucrose. We studied the effect of different sucrose levels (20, 30, 40, 50, 60, 70, 80 g l^{-1}), ANC levels (1.3, 2.0, 3.0, 5.0 mg l^{-1}) and different levels of KIN (0.1, 0.5, 0.7, 1.0 mg l^{-1}) combined with NAA (0.1, 0.3, 0.5, 0.6 mg l^{-1}) on the rhizome buds sprouting and development.

The main parameters considered to evaluate the method were rooting in first place, bud sprouting and acclimatization. The selected medium (MS plus 0.5 mg l^{-1} NAA, 0.7 mg l^{-1} KIN, 2 mg l^{-1}ANC and 60 g l^{-1} sucrose, was compared to DAM medium.

In a subsequent experiment the effect of a different iron source on the buds growth and development, comparing EDDHA-Fe, at 85.7 mg l^{-1}, versus EDTA-Fe in the selected medium was studied. After this modification the selected medium [that we called asparagus rhizome bud medium (ARBM)] was used for rhizome bud sprouting, development and rooting. All experiments were carried out using a minimum of thirty explants per treatment and repeated three times.

When necessary, to eliminate bacterial proliferation appearing at the beginning of the cultures, the media were supplemented with 200 mg l^{-1} of filter-sterilized antibiotic cefotaxime.

This micropropagation method is a one-step method, after disinfection and dissection the rhizome bud explant was incubated 8 weeks in ARBM medium and then transplanted for acclimatization. For in vitro multiplication, we proceeded to mechanically separate the clusters of rooted buds that were obtained by developing the initial

rhizome bud explants into single-rooted plantlets and subculturing them in fresh ARBM medium. The multiplication rate was calculated as the average of the number of plantlets obtained from every primary explant after 8 weeks incubation in ARBM medium.

For subculturing, it was occasionally necessary to prune the roots and shoots of the individual plantlets due to the large size that was reached by these roots and shoots to avoid contamination and necrosis resulting from mistakes during the difficult transfer of large plantlets to fresh medium. After pruning, the plantlets were recovered without problems developing new roots and shoots. The survival percentage, rooting, shoot and root number and length were recorded after 6–8 weeks.

Acclimatization of asparagus plantlets

Plantlets with well-developed roots were thoroughly washed in tap water and transplanted to 4 cm × 4 cm polyethylene alveolus trays containing a mixture of autoclaved peat:perlite (1:1). Potted plantlets were maintained for 1 month in a polyethylene tunnel with 80 % relative humidity. The temperature inside the tunnel ranged from 19 to 30 °C, and the mean temperature was 25 °C. The plants were transferred to 12 cm diameter pots containing the same substrate and maintained at 50–60 % relative humidity for another 3 months. During the experiment, the plants were lightly watered and periodically fertilized. The plants grew under 80 % shade. Data on the survival rate was recorded at monthly intervals for 4 months.

Statistical analysis

Normally distributed variables were analyzed by analysis of variance (one-way ANOVA), and where significant differences were found, the values were compared according to Student-Newman-Keuls (SPSS/PC + program). Variables expressed in percentages were analyzed by maximum likelihood analysis of variance and treatment means were compared by contrast test (SAS program, Littell et al. 2002).

Verification of the genetic stability of clonal lines: flow cytometry and Microsatellites EST-SSR

The ploidy level of two micropropagated genotypes (HT-089, HT-156) and their mother plants maintained at the glasshouse was determined by estimating the relative DNA content using FCM (Ploidy Analyser PA-I; Partec GmbH, Münster, Germany). Three replicas of each mother plant and eight replicas of each micropropagated genotypes (HT-089, HT-156) were analyzed. A total of eleven samples per genotype were evaluated.

For analysis, 0.5 cm pieces of young shoot tips of asparagus were chopped with a razor blade for 30–60 s to release nuclei (Galbraight et al. 1983) 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, the nuclei were stained with fluorescent dye (commercial Partec CyStain UV precise P, high-resolution DNA staining kit 05-5002, staining buffer, approximately 1.6 ml). Lastly, the samples were analyzed after 30 s of incubation. *A. officinalis* cv. "Baitoru" ($2n = 2\times = 20$) was always used as an external standard. The nuclear DNA ploidy levels of the samples were determined using channel values that corresponded to the average G_0/G_1 peaks of the sample and standard plants. The peak relative to the standard nuclei was set to channel 100. Three independent repetitions were performed, with over 10,000 nuclei being analyzed in each.

The DNA used in the study of the genetic stability of the micropropagated plantlets was extracted from young spears that were sampled from eight different micropropagated plants that were developed in vitro from two genotypes HT-089 and HT-156 belonging to "Morado de Huetor" landrace, and three replicas of each mother plant maintained in the glasshouse. A total of eleven samples of each genotype were analyzed. The maternal DNA material was used to establish the control pattern for EST-SSR analysis. Extraction was performed using a standard CTAB protocol following the protocols and methods indicated by Torres et al. (1993).

Twelve SSRs (TC1, AAT1, AG3, TC3, AG6, AG7, TC5, AG8, TC7, AG10, TC9 and AG12) were sed to verify the genetic stability of the micropropagated plantlets (Caruso et al. 2008). Forward primers were synthesized with fluorescent dyes 6FAM or HEX (Applied Biosystems) at the 5'ends. Amplification of the markers was performed as in Caruso et al. (2008). The PCR reaction included the two specific primers (0.025 mM), 2.5–10 ng ml^{-1} of DNA, 0.2 mM dNTPs, 1· Promega PCR buffer, 2 mM magnesium chloride and 0.1 units/ml Promega Taq DNA polymerase.

Cycling conditions consisted of 95 °C for 5 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min; and one cycle of 72 °C for 15 min. PCR reactions were performed in a Perkin Elmer Cetus DNA Thermal Cycler (9600). 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 (400 HD-ROX) with GeneScan software (version

3.x) and the results were interpreted using the Genotyper program (version 3.7) all from Applied Biosystems.

Establishment of an in vitro collection of Morado de Huetor

Plants of "Morado de Huetor" were collected in the cultivation area of this landrace in the banks of the Genil river valley (Granada, Spain) following the advice of producers and technicians working in this area.

The selection of the elite genotypes was carried out after outstanding agronomical traits, such as spear diameter, precocity, color, active biocompounds content, morphology of spear, productivity, pest and disease tolerance, sex, ploidy level, vigor and plant size (Table 1).

Plants were labeled, and moved to the laboratory of Plant Tissue Culture and Biotechnology of the IHSM La Mayora (Malaga, Spain), cleaned, dissected, disinfected and established in vitro following the above indicated method. All the plants of the in vitro collection were maintained in culture jars containing 50 ml of ARBM-0 medium. This medium consist on MS with 85.7 mg l^{-1} EDDHA-Fe, supplemented with 0.1 mg l^{-1} KIN, 0.1 mg l^{-1} NAA, 30 g l^{-1} sucrose and without ANC.

The ploidy level of the full germplasm collection of "Morado de Huetor" was established using FCM methods.

Results and discussion

Dissection and disinfection of rhizome bud explants

The selection of good quality rhizome buds (healthy, turgent and bigger than 5 mm) and their correct dissection played a key role in the success of the disinfection and micropropagation process. The disinfection rates obtained for multiple (>80) genotypes belonging to "Morado de Huetor" landrace, five different varieties of *A. officinalis* and four wild relative species ranged between 80 and 95 % in average (Table 2), depending on the location of the donor plant (field, glasshouse). Specifically, the rhizome buds of HT-089, showed an excellent disinfection rate (94 %). We have not found any data about similar procedures of disinfection of rhizome bud explants in asparagus, but similar rhizome explants used in other species, such as *Zingiber* sp (Idris et al. 2010), showed much lower disinfection rates (30 %,) than most of the asparagus genotypes cleaned following our method. We successfully controlled (>90 %) the occasional proliferation of bacteria in the early stages of the micropropagation process by supplementing the first culture medium with cefotaxime.

Table 2 Rooting rate and plant recovery after micropropagation of different asparagus genotypes on ARBM medium after 4 weeks of acclimatization

Genotype	Disinfection rate (%)	Rooting (%)	Acclimatization (%)
"Morado de Huetor"	94b	74a	95b
A. officinalis "UC-157"	80a	70a	80a
Asparagus maritimus	80a	80a	70a

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

Table 3 Rhizome bud explant growth, rooting and acclimatization percentages of "Morado de Huetor" obtained supplementing DAM medium with different concentrations of SUC, after 8 weeks of incubation

Sucrose (g l^{-1})	Bud sprouting (%)	Rooting (%)	Acclimatization (%)
20	100a	20c	60b
30	100a	15c	70b
40	93.3a	38.5b	70b
50	97a	34.6b	80a
60	100a	52a	85a
70	80b	50a	80a
80	70b	60a	60b

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

Micropropagation of "Morado de Huetor"

Our initial assays using DAM medium (Desjardins 1992) with rhizome buds explants result in 96–100 % of bud sprouting and 28–30 % of rooting after 8 weeks incubation. To improve this percentage we modify some of the components. Our goal was to develop a new method of micropropagation which avoids the phase of de novo "crown" induction. We use individual underground rhizome buds as explants, with the goal of drastically shortening the duration of the micropropagation method and making the process independent of the asparagus genotype.

An increase of the rooting level (52 %) was detected when the medium was supplemented with 60 g l^{-1} of sucrose (Table 3). Higher values of sucrose results in similar rooting rates (50–60 %) but decrease the bud sprouting and the acclimatization levels.

Desjardins et al. (1987) reported that increasing the concentration of sucrose in media containing ANC increases rooting, remarking the complementary effects between sucrose and ANC. Under our conditions, after testing different levels of sucrose and ANC, our results

Table 4 Rhizome bud explant rooting percentages that were obtained for cv. "Morado de Huetor" after supplementing basal DAM medium with different combinations of NAA and KIN for 8 weeks

Rooting (%)				
NAA/KIN (mg l^{-1})	0.1	0.5	0.7	1
0.1	28.6c	17.2c	20.7c	3.9c
0.3	58.6a	57.7a	35b	36.8b
0.5	58.6a	31b	70a	57a
0.6	44.8b	40b	35.7b	45.8b

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

Table 5 Effects of different culture media on shoot development, rooting, quality of roots and acclimatization incubating rhizome bud explants of "Morado de Huetor"

Medium	Shoot no.	Rooting (%)	Good quality plants (%)[a]	Acclimatization (%)
DAM	1.5 b	41b	28b	70a
ARBM	4.4 a	74a	64a	80a

Different low case letter in the same column means statistical difference according to the Student–Newman–Keuls' test ($p < 0.5$) (shoot number) and contrast test (rooting, good quality plants and acclimatization)

[a] Plants with a balanced number of thick and thin roots (30/70) adequate for acclimatization

indicate that the use of high doses of ANC (2 mg l^{-1}) and sucrose (60 g l^{-1}) were beneficial for the in vitro development of rhizome bud explants of "Morado de Huetor".

When different concentrations of KIN and NAA were applied in combination, we recorded higher values on rooting percentage respect the control (0.1 mg l^{-1} NAA +0.1 mg l^{-1} KIN). The best rooting rate (70 %) correspond to 0.5 mg l^{-1} NAA + 0.7 mg l^{-1} KIN (Table 4).

After defining the optimal conditions of sucrose level, growth regulators and ANC were established, we tested a different iron chelate (EDDHA-Fe) modifying the DAM medium mineral formulation and we determined that the use of EDDHA-Fe instead of EDTA-Fe (Table 5) improved significantly the rate of bud sprouting (93.7 vs. 73.3 %) and shoot growth compared with EDTA-Fe (5.5 vs. 2.7 cm, respectively), similar results were obtained by Christensen et al. (2008) on micropropagation of *Hibiscus rosa-sinensis* using EDDHA-Fe versus EDTA-Fe.

After preliminary tests involving modifications in the components of the culture medium (auxins: NAA, IAA, IBA; cytokinins: BA, 2iP, KIN, alone or in combination) we concluded that for our material and explants, the best cytokinin was KIN combined with NAA, which was in agreement with previous protocols (Yang and Clore 1973, 1974; Chin 1982; Desjardins et al. 1987; Desjardins 1992).

The combination of 0.5 mg l^{-1} NAA and 0.7 mg l^{-1} KIN when added to the modified mineral formulation of DAM medium, worked synergistically resulting in higher rooting and multiplication percentages of "Morado de Huetor" rhizome bud explants.

The multiplication rate of "Morado de Huetor" following this method was an average of 3.5 plants per explant after 8 weeks incubation.

We applied our micropropagation method to different genotypes of "Morado de Huetor" landrace and other cultivated species such *A. officinalis* (UC-157) and *A. maritimus* we found similar results (Table 2). That could be explained for the interspecific origin of "Morado de Huetor" based on these two species.

Acclimatization

In order to obtain a correct acclimatization of micropropagated asparagus, we need to remark that it is necessary to obtain plantlets with a balanced number of thick and thin roots (a rate approximately 30/70) and in this process the sucrose plays a key role. The high quality plantlets obtained, provide excellent results during the acclimatization step. Comparing the results of percentage of quality plantlets with the adequate root balance for acclimatization, 64 versus 28 % was obtained for ARBM and DAM medium respectively (Table 5). The acclimatization rate was always high (70–95 %) for all asparagus genotypes assayed.

Applying the method of Yukimasa et al. (1990), the minimal amount of time that was required for plantlet development until it was ready for transplant and acclimatization was 8 weeks. However, these authors do not include the period of time that is necessary at the beginning of the process to develop the initial crown from the primary explants, which, when successful, could easily duplicate the explant incubation time for the primary explants (spear nodal sections).

Using our one-step protocol, the time necessary to recover high-quality plantlets ready to be acclimatized was shortened drastically and it is possible to obtain rooted plants of asparagus within 4–6 weeks, but the acclimatization of the resulting micropropagated plants was better when the plantlets were acclimatized after 8 weeks incubation (Fig. 1).

Verification of the genetic stability of clonal lines

Flow cytometry

In contrast with the results obtained using other methods of propagation in vitro that were applied to asparagus and

Fig. 1 Micropropagation method for asparagus through rhizome bud explants. **a** Raw crown of asparagus; **b** Detail of rhizome bud that is ready for dissection after first disinfection; **c** Rhizome bud explants in aseptic conditions that are ready for in vitro establishment; **d** The initiation of rhizome bud explants; **e** In vitro development of rhizome buds; **f** Acclimatization of micropropagated asparagus plantlets

generated anomalies at the ploidy level (Araki et al. 1992; Odake et al. 1993; Kunitake et al. 1998; Raimondi et al. 2001; Pontaroli and Camadro 2005), our results confirm the genetic stability of the micropropagated material after analyzing eleven samples of two genotypes selected for it (Fig. 2). In our material, all coefficients of variation (CV) scored below 5 % of the acceptance criteria for FCM samples (Galbraight et al. 2002). However, even if we never detected major changes in ploidy, it is possible that other minor changes in DNA content could occur, making it necessary to analyze our micropropagated asparagus using molecular markers such as EST-SSR for a detailed analysis of genetic stability and somaclonal variation.

EST-SSR assay

Some studies using molecular markers, such as RAPDs (Raimondi et al. 2001) and AFLPs (Pontaroli and Camadro 2005), have already revealed that somaclonal variation in asparagus appears when micropropagation methods based in callus induction and regeneration were applied.

To our knowledge, this is the first time that EST-SSRs were applied in asparagus to evaluate the possible somaclonal variation that is induced by micropropagation. Under our experimental conditions, we never detected differences when we analyzed several of our mother plants (HT-089 and HT-156) and their micropropagated progenies

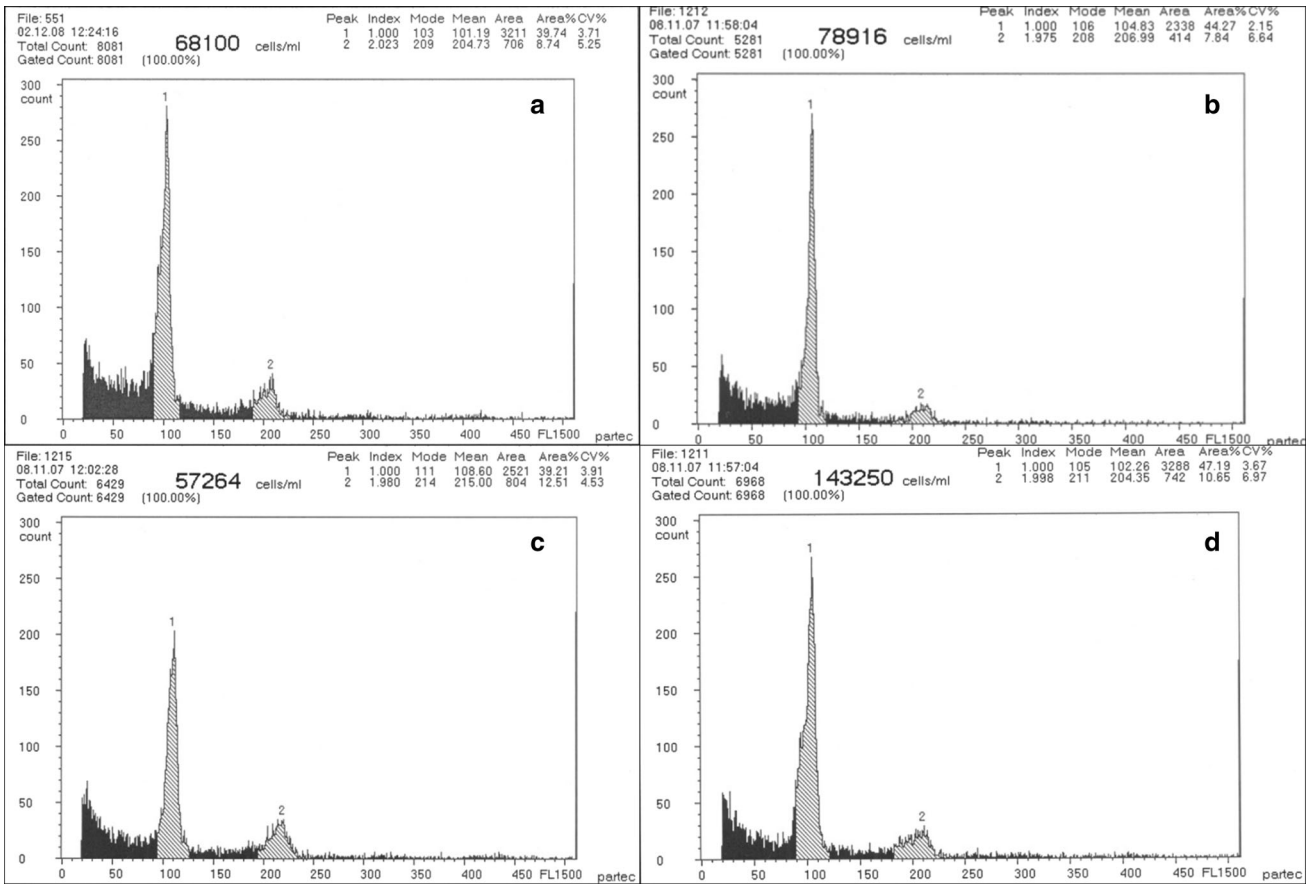

Fig. 2 Diagrams of FCM for original plants and micropropagated genotypes HT-089 and HT-156. **a** Original HT-089, **b** Micropropagated HT-089, **c** Original HT-156, **d** Micropropagated HT-156

using EST-SSR (Fig. 3), which makes our method extremely reliable and efficient for producing true-to-type plantlets of asparagus. These results support the genetic stability that was shown by our above-indicated FCM analysis.

In vitro breeder collection

Using the micropropagation method based in rhizome bud explants it has been possible to establish and maintain a wide collection of asparagus for breeding purposes. At the present time, the collection includes around 80 different genotypes from "Morado de Huetor", several genotypes of *A. officinalis* such as UC-157, Atlas, Grande, Baitoru and Cipres, some wild species such as *A. maritimus, A. brachyphyllus, A. pseudoscaber and A. densiflorus* and also the collection is now growing quite fast due the continuous entry of new genotypes, as result of our breeding works and from different in vitro assays of polyploidization, callus regeneration and anther cultures. The establishment and maintenance of the collection confirms the versatility of our micropropagation method for multiple genotypes of Asparagus, improving the previous methods (Desjardins 1992; Yukimasa et al. 1990) only applied to a limited number of genotypes and without data about their further success in micropropagating other asparagus genotypes.

To date our micropropagation method has been applied successfully to all the species tested belonging to Subgenera Asparagus.

Conclusions

A new method of micropropagation of asparagus based on the establishment and development in vitro of rhizome bud explants. These explants were disinfected with fungicide and Sodium hypochlorite under vacuum conditions. It has been developed a new medium (ARBM), consisting on full-strength MS salts (Murashige and Skoog 1962), modified with EDDHA-Fe (85.7 mg l^{-1}) and supplemented with the following (mg l^{-1}): thiamine-HCl (100), pyridoxine-HCl (50), nicotinic acid (50), glycine (200) and i-inositol (100), and 60 g l^{-1} sucrose, 0.8 % of Agar A-1296 (Sigma), 0.5 mg l^{-1} NAA, 0.7 mg l^{-1} KIN and

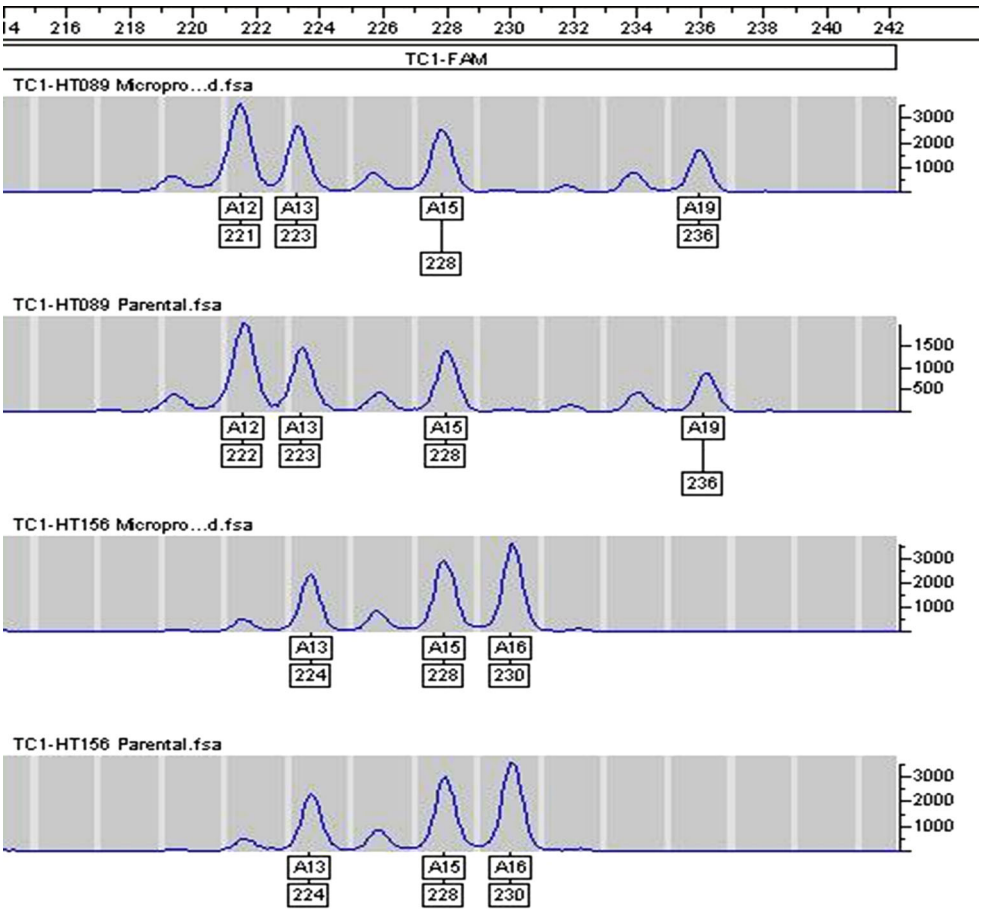

Fig. 3 Comparative analysis of TCI EST-SSR between the mother plants HT-089 and HT-156 and their respective micropropagated progenies

2 mg l^{-1} ANC. The ARBM medium was also supplemented with 200 mg l^{-1} of filter-sterilized cefotaxime, which served as an antibiotic, in the first step of micropropagation when necessary. This method guaranties the genetic stability of the micropropagated genotypes.

Our micropropagation protocol from rhizome bud explants is extremely versatile and can be applied for micropropagation of different species of Asparagus genera. At the present time, is routinely used for the management of the "Morado de Huetor" landrace collection and to introduce in vitro new accessions, such as wild related species of asparagus, new induced hybrids and polyploid genotypes of "Morado de Huetor", and allows us to preserve this endangered landrace of asparagus, a valuable genetic resource extremely interesting for breeding purposes to widening the genetic pool of cultivated asparagus.

References

Araki H, Shimazaki H, Hirata Y, Oridate T, Harada T, Yakuwa T (1992) Chromosome number variation of callus cells and regenerated plants in *Asparagus officinalis* L. Plant Tis Cult Lett 9:169–175

Bairu MW, Aremu AO, Van Staden J (2011) Somaclonal variation in plants: causes and detection methods. Plant Growth Regul 63:147–173

Cao H, Biswas MK, Lü Y, Amar MH, Tong Z, Xu Q, Xu H, Guo W, Deng X (2011) Doubled haploid callus lines of Valencia sweet orange recovered from anther culture. Plant Cell Tiss Organ Cult 104:415–423

Caruso M, Federici CT, Roose ML (2008) EST-SSR markers for asparagus genetic diversity evaluation and cultivar identification. Mol Breeding 21:195–204. doi:10.1007/s11032-007-9120-z

Chin CK (1982) Promotion of shoot and root formation in asparagus in vitro by ancymidol. HortScience 17:590–591

Christensen B, Sriskandarajh S, Serek M, Müller R (2008) In vitro culture of *Hibiscus rosa-sinensis* L.: influence of iron, calcium and BAP on establishment and multiplication. Plant Cell Tiss Organ Cult 93:151–161

Desjardins Y (1992) Micropropagation of Asparagus (*Asparagus officinalis* L.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. Vol. 19: high-tech and micropropagation III. Springer, Berlin-Heidelberg, pp 26–41

Desjardins Y, Tiessen H, Harney PM (1987) The effect of sucrose and ancymidol on the in vitro rooting of nodal sections of asparagus. Hort Science 22:131–133

Escobedo-GraciaMedrano RM, Maldonado-Borges JI, Burgos-Tan MJ, Valadez-González N, Ku-Cauich JR (2014) Using flow cytometry and cytological analyses to assess the genetic stability of somatic embryo-derived plantlets from embryogenic *Musa acuminata* Colla (AA) *P.* malaccensis cell suspension cultures. Plant Cell Tiss Organ Cult 116:175–185

Galbraight DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051

Galbraight DW, Lambert G, Macas J, Dolezel J (2002) Analysis of nuclear DNA content and ploidy in higher plants. In Robinson J, Darzynkiewicz Z, Dean P, Hibbs A, Orfao A, Rabinovitch P, Wheeless L (ed) *Current Protocols in Cytometry*. John Wiley and Sons: New York, pp 7.6.1–7.6.22. doi: 10.1002/0471142956.cy0706s02

Gao DY, Vallejo V, He B, Gai YC, Sun LH (2009) Detection of DNA changes in somaclonal mutants of rice using SSR markers and transposon display. Plant Cell Tiss Organ Cult 98:187–196

Geoffriau E, Denoue D, Remeau C (1992) Assessment of genetic variation among asparagus (*Asparagus officinalis* L.) populations and cultivars: agromorphological and isozymic data. Euphytica 61:169–179

Hasegawa PM, Murashige T, Takatori FH (1973) Propagation of asparagus through shoot apex culture II. Light and temperature requirements, transplantability of plants, and cyto-histological characteristics. J Am Soc Hort Sci 98:143–148

Idris TIM, Ujool SAM, Mahdi EM (2010) Disinfection potential of some chemicals and local herbs and proliferation studies on the in vitro culture of ginger (*Zingiber officinale* Rosc). J Sci Tech 11:34–39

Knaflewsky M (1996) Genealogy of asparagus cultivars. In: Nichols M, Swain D (ed). Proceedings VIII Int Asparagus Symp. pp 87–91

Kohmura H, Ito T, Shigemoto N, Imoto M, Yoshikawa H (1996) Comparison of growth, yield, and flowering characteristics between micropropagated asparagus clones derived by somatic embryogenesis and seed-propagated progenies. J Jpn Soc Hort Sci 65:311–319

Kunitake H, Nakashima T, Mori K, Tanaka M (1998) Somaclonal and chromosomal effects of genotype, ploidy and culture duration in *Asparagus officinalis* L. Euphytica 102:309–316

Limanton-Grevet A, Sotta B, Brown S, Jullien M (2000) Analysis of habituated embryogenic lines in *Asparagus officinalis* L.: growth characteristics, hormone content and Ploidy level of calli and regenerated plants. Plant Sci 160:15–26

Littell RC, Stroup WW, Freund R (2002) SAS for linear models, 4th edn. SAS Institute Inc, Cary

Liu F, Huang LL, Li YL, Reinhoud P, Jongsma MA, Wang CY (2011) Shoot organogenesis in leaf explants of *Hydrangea macrophylla* 'Hyd1' and assessing genetic stability of regenerants using ISSR markers. Plant Cell Tiss Organ Cult 104:111–117

Moreno R, Espejo JA, Cabrera A, Millan T, Gil J (2006) Ploidic and molecular analysis of 'Morado de Huetor' *Asparagus* (*Asparagus officinalis* L.) population: a Spanish tetraploid landrace. Genet Resour Crop Ev 53:729–736. doi:10.1007/s10722-004-4717-0

Moreno R, Espejo JA, Cabrera A, Gil J (2008a) Origin of tetraploid cultivated asparagus landraces inferred from nrDNA ITS polymorphisms. Ann Appl Biol 153:233–241. doi:10.1111/j.1744-348.2008.00254.x

Moreno R, Espejo JA, Moreno MT, Gil J (2008b) Collection and conservation of 'Morado de Huetor' Spanish tetraploid asparagus landrace. Genet Resour Crop Ev 55:773–777. doi:10.1007/s10722-008-9358-2

Moreno R, Carmona E, Encina CL, Rubio J, Gil J (2010a) Aplicación de marcadores microsatélites en la mejora del espárrago. Actas Hortic 55:221–222

Moreno R, Espejo JA, Gil J (2010b) Development of triploid hybrids in asparagus breeding employing a tetraploid landrace. Euphytica 173:369–375. doi:10.1007/s10681-009-0103-5

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Murashige T, Shabde MN, Hasegawa PM, Takatori FH, Jones JB (1972) Propagation of asparagus through shoot apex culture. J Am Soc Hort Sci 97:158–161

Ochatt SJ, Patat-Ochatt EM, Moessner A (2011) Ploidy level determination within the context of in vitro breeding. Plant Cell Tiss Organ Cult 104:329–341

Ochatt S, Jacas L, Patat-Ochatt EM, Djenanne S (2013) Flow cytometric analysis and molecular characterization of *Agrobacterium tumefaciens*-mediated transformants of *Medicago truncatula*. Plant Cell Tiss Organ Cult 113:237–244

Odake Y, Udagawa A, Saga H, Mii M (1993) Somatic embryogenesis of tetraploid plants from internodal segments of a diploid cultivar of *Asparagus officinalis* L. grown in liquid culture. Plant Sci 94:173–177

Pontaroli AC, Camadro EL (2005) Somaclonal variation in *Asparagus officinalis* L. plants regenerated by organogenesis from long-term callus cultures. Genet Mol Biol 28:423–430

Raimondi JP, Camadro EL, Masuelli RW (2001) Assessment of somaclonal variation in asparagus by RAPD fingerprinting and cytogenetic analyses. Sci Hortic 90:19–29

Shiga I, Uno Y, Kanechi M, 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. J Japan Soc Hort SCI 78(1):103–108

Torres AM, Weeden NF, Martin A (1993) Linkage among isozyme, RFLP and RAPD markers in *Vicia Faba*. Theor Appl Genet 85:935–945

Vujovic T, Cerovic R, Ruzic D (2012) Ploidy level stability of adventitious shoots of sour cherry 'Čacanski Rubin' and Gisela 5 cherry rootstock. Plant Cell Tiss Organ Cult 111:323–333

Yang HJ, Clore WJ (1973) Rapid propagation of asparagus through lateral bud culture. HortScience 8:141–143

Yang HJ, Clore WJ (1974) Development of complete plantlets from moderately vigorous shoot of stocks plants of asparagus in vitro. HortScience 9:138–139

Yukimasa H, Shigeru T, Rie M, Atsuko K (1990) Patent: Method of multiplying plant belonging to the genus asparagus. Patent No: EP0375218. Assignee: Mitsui Petrochemical Ind (JP). Application No: EP19890312806 19891208