

# Micropropagation of wild species of the genus *Asparagus* L. and their interspecific hybrids with cultivated *A. officinalis* L., and verification of genetic stability using EST-SSRs

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**Abstract** An efficient micropropagation method for asparagus species was developed in this study. The method allows the fast cloning of the elite genotypes from different asparagus species and the interspecific hybrids obtained from these species. Rhizome bud explants were disinfected using  $3 \text{ g l}^{-1}$  of benomyl and  $20 \text{ g l}^{-1}$  of sodium hypochlorite. Then, they were cultured on Asparagus Rhizome Bud Medium 3 (ARBM-3) consisting in modified Murashige and Skoog medium with salts with EDDHA-Fe ( $85.7 \text{ mg l}^{-1}$ ) instead of EDTA-Fe and vitamins, supplemented with  $0.3 \text{ mg l}^{-1}$  NAA,  $0.1 \text{ mg l}^{-1}$  KIN,  $2 \text{ mg l}^{-1}$  ancymidol and 6 % sucrose. Results showed that the method developed produced high disinfection rates (70–95 %). More than 70 % of the explants developed shoots and the rooting rate on ARBM-3 medium was 30–45 %. The rooting rate increased to 60–85 % when the unrooted shoots were subjected to an additional cycle of rooting, reaching 100 % after two cycles of rooting. The multiplication was achieved through mechanical division of rooted shoot clusters growing in ARBM-3. The acclimatization rate of the micropropagated plantlets was higher

than 90 %. The micropropagated plantlets were screened for somaclonal variation using 12 expressed sequence tag derived simple sequence repeat markers. The results confirmed the character “true to type” of the plantlets, indicating that the method developed in this study can be used to successfully micropropagate asparagus species.

**Keywords** Rhizome buds · Rooting · Molecular markers · Somaclonal variation · *Asparagus brachyphyllus* · *Asparagus pseudoscaberr*

## Introduction

The genus *Asparagus* includes around 200 species, classified into three subgenera: *Asparagus*, *Protasparagus* and *Myrsiphyllum* (Clifford and Conran 1987). The geographic distribution of these species comprises the arid and semi-arid regions of Europe, Asia, Africa and Australia (Kubota et al. 2012). All the species in the subgenus *Asparagus* are dioecious, while those of the other two subgenera are hermaphroditic. The three subgenera have a basic chromosome number of  $x = 10$ , and species with different ploidy levels are found in the genus *Asparagus*. Polyploidization seems to be common in the genus *Asparagus*, and can be considered an important mechanism in the evolution of the genus (Castro et al. 2013).

Many *Asparagus* species are used for different purposes such as human food (*A. officinalis* L., *A. acutifolius* L., *A. albus* L., *A. aphyllus* L. and *A. maritimus* M.), medical (*A. adscendens* Kunth, *A. racemosus* Willd., and *A. verticillatus* L.) and ornamental plants (*A. asparagoides* L., *A. densiflorus* Kunth, *A. plumosus* Baker and *A. virgatus* Baker). *A. officinalis* L. ( $2n = 2x = 20$ ) is the most economically important *Asparagus* species and the only one

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cultivated worldwide as vegetable crop. Unfortunately, *A. officinalis* has a narrow genetic base because most of commercial cultivars have been developed from a single population of the 18th century, “Violet Dutch” (Knaflewski 1996). Therefore, the introgression of agronomically important traits from other *Asparagus* species would be valuable for breeding programs in order to increase the genetic pool of cultivated *A. officinalis*.

Previous studies (Kunitake et al. 1996; Ito et al. 2008; Kubota et al. 2012) showed that the number of wild species compatible with *A. officinalis* is limited to the species of the subgenera *Asparagus*. So, it is impossible to obtain hybrids between species of the subgenus *Protasparagus* and *Myrsiphyllum* with *A. officinalis*. Even within the subgenus *Asparagus*, the existence of different ploidy levels implicates an additional barrier to obtain interspecific hybrids. The hybridization of plants of *A. officinalis* and wild asparagus species with the same ploidy level could be a solution to overcome this specific barrier (Favavigna et al. 2008; Riccardi et al. 2011; Castro et al. 2013). The Spanish cultivated tetraploid landrace “Morado de Huétor” presents low frequency of different ploidy levels, and has been proposed as “bridge” to transfer genes from wild to cultivated asparagus (Moreno et al. 2006; Castro et al. 2013).

The dioecious character of all the species of the subgenus *Asparagus* prevents the maintenance through sexual reproduction of elite genotypes of the different species. Therefore, the use of micropropagation methods adapted to the different species would be necessary to propagate the materials. Different micropropagation procedures have been published for different species of the genus *Asparagus* such as: *A. officinalis* (Desjardins et al. 1987; Kunitake and Mii 1998; Raimondi et al. 2001), *A. maritimus* (Štajner et al. 2002), *A. densiflorus* (Benmoussa et al. 1996), *A. plumosus* (Ghosh and Sen 1994a), *A. verticillatus* (Ghosh and Sen 1996), *A. robustus* (Nayak and Sen 1998), *A. cooperi* (Ghosh and Sen 1994b) and *A. racemosus* (Bopana and Saxena 2008). However, a general protocol has not been reported that is valid for all the species of *Asparagus* and interspecific hybrids between them. The only method that has been used for the micropropagation of multiple species is the protocol developed by Carmona-Martín et al. (2014). This procedure was developed for micropropagation of the Spanish landrace “Morado de Huétor” ( $2n = 4x = 40$ ). Nevertheless, it has been also employed for micropropagation of the commercial cultivars of *A. officinalis* ( $2n = 2x = 20$ ) and the wild species *A. maritimus* ( $2n = 6x = 60$ ). The objective of the current work was to optimize the micropropagation method developed by Carmona-Martín et al. (2014) for the micropropagation of two additional species of the genus *Asparagus*, *A. pseudoscaber* and *A. brachyphyllus*, and their interspecific

hybrids with cultivated asparagus, and to demonstrate the genetic stability of the micropropagated plants obtained through this procedure.

## Materials and methods

### Plant material

Plants from five wild species of the genus *Asparagus* (Table 1), two hexaploid interspecific hybrids obtained from “Morado de Huétor” and the wild species *A. pseudoscaber* (HTPS1) and *A. brachyphyllus* (HTAB 2) (Castro et al. 2013) were used for the micropropagation assays.

### Extraction and disinfection of rhizome buds

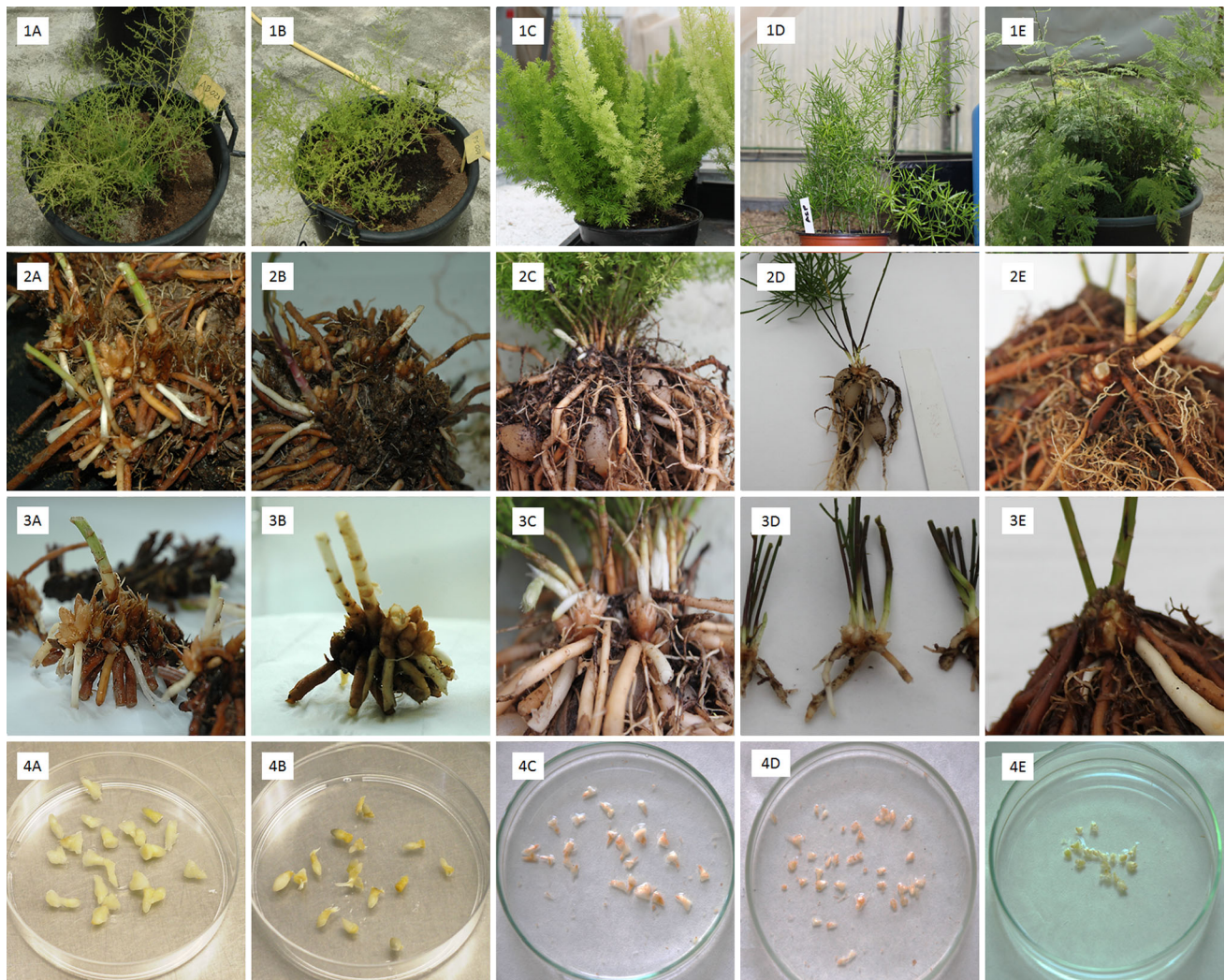
During the months of March and April, rhizome bud explants were collected from asparagus mother plants growing in pots in a glasshouse. The explants were extracted and submitted to a first disinfection following the protocol described by Encina et al. (2008) and Carmona-Martín et al. (2014) (Fig. 1).

The initial tests with rhizome bud explants of *A. pseudoscaber* and *A. brachyphyllus* showed a high presence of microorganisms in both species. Therefore, the disinfection protocol for these explants was optimized in order to eliminate the contaminants. Two different chemicals were used for disinfection: the fungicide benomyl ( $3 \text{ g l}^{-1}$ ) and a sodium hypochlorite solution (2 % w/v). The concentrations used were the same as those reported in previous works developed with rhizome buds of *Asparagus officinalis* (Encina et al. 2008; Carmona-Martín et al. 2014). The rhizome bud explants were first disinfected by immersing and shaking in a solution of benomyl ( $3 \text{ g l}^{-1}$ ). The explants were next rinsed with distilled water to eliminate the fungicide. In the second step, the explants were immersed in a solution of sodium hypochlorite (2 % w/v) under vacuum conditions. Finally, the explants were washed three times with sterile distilled water in aseptic conditions in order to eliminate the sodium hypochlorite. A combination of different times of disinfection (15 and 20 min) with each disinfectant was applied to select the best combination (Table 2). Twenty buds of both species *A. pseudoscaber* and *A. brachyphyllus* were disinfected using each treatment, the buds were collected from six plants of each species and randomly distributed between different sterilization treatments.

Establishing in vitro rhizome bud explants of *A. densiflorus* cvs. Myersii and Sprengeri and *A. plumosus*, we never detected the high levels of microorganisms found in *A. pseudoscaber* and *A. brachyphyllus* explants. Due to the

**Table 1** Origin and ploidy levels of mother plants of the genus *Asparagus* used in the micropropagation method

Species	Subgenus	Ploidy level	Origin	Donor
<i>Asparagus pseudoscaber</i> (PS)	Asparagus	6x	Czech Republic	Genebank UCO (Spain)
<i>Asparagus brachyphyllus</i> (AB)	Asparagus	6x	Russia	Genebank UCO (Spain)
<i>Asparagus densiflorus</i> cv. Myersii (AME)	Protasparagus	6x	South Africa	Nurseries “El Alamillo” (Malaga, Spain)
<i>Asparagus densiflorus</i> cv. Sprengeri (ASP)	Protasparagus	6x	South Africa	Nurseries “El Alamillo” (Malaga, Spain)
<i>Asparagus plumosus</i> (APL)	Protasparagus	2x	South Africa	Nurseries “El Alamillo” (Malaga, Spain)



**Fig. 1** Procedure for extraction of rhizome bud explants from *Asparagus brachyphyllus* (A), *Asparagus pseudoscaber* (B), *Asparagus densiflorus* cv. Myersii (C), *A. densiflorus* cv. Sprengeri (D) and

*Asparagus plumosus* (E). (1) Mother plants, (2) Morphology of plant rhizomes, (3) Details of rhizome bud clusters, (4) Dissected rhizome bud explants

low level of contamination found in the explants of *A. densiflorus* and *A. plumosus*, we used the disinfection treatment A (Table 2), which is the less aggressive. Sixty buds collected from six plants of each species were disinfected.

Finally, twenty rhizome bud explants obtained from the interspecific hybrids HTPS1 and HTAB 2 were disinfected using the treatment that was the best for the wild-species parent (*A. pseudoscaber* and *A. brachyphyllus*) of each hybrid.

**Table 2** Disinfection rates obtained for rhizome bud explants of *A. pseudoscaber*, *A. brachyphyllus*, *A. densiflorus* cvs. Myersii and Sprengeri, *A. plumosus* and the interspecific hybrids HTPS1 and HTAB 2 using different treatments

Species/hybrid	Disinfection treatment	Treatment duration (min)		Disinfection rate (%)
		Benomyl (3 g l <sup>-1</sup> )	Sodium hypochlorite (20 g l <sup>-1</sup> )	
<i>A. pseudoscaber</i>	A	15	15	56 ± 12 b
	B	15	20	59 ± 12 b
	C	20	15	86 ± 9 a
	D	20	20	88 ± 8 a
<i>A. brachyphyllus</i>	A	15	15	57 ± 11 b
	B	15	20	57 ± 11 b
	C	20	15	86 ± 8 a
	D	20	20	90 ± 6 a
<i>A. densiflorus</i> cv. Myersii	A	15	15	83 ± 5 a
<i>A. densiflorus</i> cv. Sprengeri	A	15	15	82 ± 5 a
<i>A. plumosus</i>	A	15	15	75 ± 6 a
HTPS1	D	20	20	75 ± 10 a
HTAB 2	D	20	20	70 ± 10 a

a, b Letters indicate groups that were significantly different by LSD at  $\alpha = 0.05$

**Table 3** Composition of culture media used

Medium <sup>a</sup>	Growth regulators (mg l <sup>-1</sup> )			Sucrose (g l <sup>-1</sup> )	Agar (g l <sup>-1</sup> )	Goal
	NAA	KIN	Ancymidol			
ARBM-3	0.3	0.1	2	60	8	Rhizome bud rooting
ARBM-0	0.1	0.1	–	30	8	Plantlet development

<sup>a</sup> All media contain MS salts with EDDHA-Fe (85.7 mg l<sup>-1</sup>) instead of EDTA-Fe and vitamins (Murashige and Skoog 1962)

### Rhizome bud explants culture

The rhizome bud explants, independently of the origin and the disinfection treatment used, were cultured for shoot and root regeneration on Asparagus Rhizome Bud Medium (ARBM-3) (Table 3). Aliquots of 25 ml of ARBM-3 medium were poured 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<sup>-2</sup>. One explant per tube was cultured and all buds were incubated in a culture room at 25 ± 1 °C under 16 h photoperiod under cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania) with 45 μmol m<sup>-2</sup> s<sup>-1</sup> (400–700 nm) Photosynthetic Active Radiation.

After 4 weeks, data including contamination, survival and rooting of rhizome bud explants of *A. brachyphyllus* and *A. pseudoscaber* for each disinfection treatment, and buds explants of *A. densiflorus* cvs. Myersii and Sprengeri, *A. plumosus* and the interspecific hybrids HTPS1 and HTAB 2 were recorded. The contamination rate was calculated as the number of contaminated explants per treatment divided by the total number of bud explants. The

survival rate was defined as the number of buds becoming plantlets regardless of the contaminated buds. The rooting rate was calculated as the number of rooted plantlets divided by the total number of buds developed.

### Rhizome bud explants multiplication and rooting

Rhizome bud explants of *A. brachyphyllus*, *A. pseudoscaber*, *A. densiflorus* cv. Sprengeri, HTPS1 and HTAB 2 rooted in ARBM-3 were transferred to 25 ml test tubes with ARBM-0 medium (Table 3), and incubated at standard conditions. The plantlets were subcultured every 4 weeks to fresh medium for further growth and multiplication. The multiplication consists in the mechanical division of the rooted shoot groups on individual plantlets, which have at least one shoot and one root. The number of plantlets obtained in each division will depend on the size of the different plants and the number of shoots and roots available.

To increase the rooting rates, unrooted rhizome buds in ARBM-3 were subjected to a cyclic process of rooting. This cyclic process of rooting consists in the culture of the

rhizome buds in the ARBM-0 and ARBM-3 media alternatively. One cycle comprise the incubation during 4 weeks in the ARBM-0 media continued by other 4 weeks incubating in the ARBM-3 media. We routinely applied two cycles of rooting. The rooting data were collected after each cycle. Once rooted, the rhizome buds were incubated in ARBM-0 and continued with the normal procedure of growth and multiplication.

#### Acclimatization of asparagus plantlets

Thirty micropropagated plantlets of the species *A. brachyphyllus* and *A. pseudoscaber* and the interspecific hybrids HTPS1 and HTAB 2 were acclimatized using the method described by Encina et al. (2008). After 8 weeks, the acclimatization rate was calculated as the number of plantlets correctly acclimatized divided by the total number of transplanted plantlets.

#### Genetic stability of the micropropagated plants

Eight micropropagated and acclimatized plantlets of both species (*A. brachyphyllus* and *A. pseudoscaber*) were selected to study their genetic stability using EST-SSR markers. Each plantlet derived from an independently sampled rhizome bud sampled from the same mother plant. Total genomic DNA of each selected plant was extracted from 1 g of 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). DNA from the mother plants of both species was used as a control for amplification pattern and fragment size.

All plants, micropropagated plantlets and mother plants, were characterized with 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) using the method described by Carmona-Martin et al. (2014). The amplified fragments in the characterization of micropropagated plants were compared with those obtained in the characterization of the mother plants in order to study the genetic stability of the micropropagated plantlets and the possible somaclonal variation.

#### Statistical analysis

All data were analyzed using SPSS software package (version 19.0; SPPS INC., Chicago, IL, USA). The rates of contamination, survival and rooting obtained in the different assays carried out 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.

## Results

#### Disinfection of rhizome bud explants

Table 2 shows the disinfection rate obtained using the different treatments assayed with rhizome bud explants of *A. brachyphyllus*, *A. pseudoscaber*, *A. densiflorus* cvs. Myersii and Sprengeri, *A. plumosus* and the interspecific hybrids HTPS1 and HTAB 2.

In the case of the rhizome bud explants of *A. brachyphyllus* and *A. pseudoscaber*, there were statistically significant differences between the treatments during 20 (86–90 %) and 15 min (56–59 %) with benomyl, independently of the duration (15 or 20 min) of the following treatment with sodium hypochlorite.

The disinfection rates obtained with the treatment A for the rhizome bud explants of *A. densiflorus* cvs. Myersii and Sprengeri and *A. plumosus* were around 80 % (83–82–75 %). The rates obtained with the treatment D for the rhizome buds explants of the interspecific hybrids HTPS1 and HTAB 2 were 75 and 70 %, respectively (Table 3).

#### Micropropagation and rooting of rhizome bud explants

Table 4 shows the survival and rooting rates of rhizome bud explants after disinfection, applying or not different cycles of rooting, for the different species and the interspecific hybrids tested. For rhizome buds explants of *A. pseudoscaber* and *A. brachyphyllus*, the rates have been calculated for each disinfection treatment.

There were no statistically significant differences ( $\alpha = 0.05$ ) between the survival rates obtained for rhizome bud explants of *A. pseudoscaber* (70–90 %) and *A. brachyphyllus* (68–75 %) applying different disinfection treatments. Differences on survival were not observed for HTPS1 (80 %) and HTAB 2 (71 %) either. However, the rhizome buds of the species belonging to subgenus *Protasparagus*, *A. densiflorus* cv. Myersii, *A. densiflorus* cv. Sprengeri and *A. plumosus*, failed to grow in ARBM-3, and only *A. densiflorus* cv. Sprengeri showed a low survival rate (10 %).

The rooting rates of rhizome bud explants of *A. pseudoscaber* and *A. brachyphyllus* did not present statistically significant differences independently of the type of disinfection treatment applied. The rooting rates (30–46 %) directly obtained in ARBM-3 medium, increased to 60–85 % when an additional cycle of rooting was applied, and in some cases, even reached 100 % after applying two cycles of rooting (Fig. 2). HTPS1 and HTAB 2 had rooting

**Table 4** Survival and rooting rates obtained for the different disinfection treatments for rhizome bud explants of *A. pseudoscaber*, *A. brachyphyllus*, *A. densiflorus* cvs. Myersii and Sprengeri, *A. plumosus* and the interspecific hybrids HTPS1 and HTAB 2

Species	Disinfection treatment	Survival rate (%)	Rooting rate in ARBM-3 (%)	Rooting rate with one cycle of rooting (%)	Rooting rate with two cycles of rooting (%)
<i>A. pseudoscaber</i>	A	90 ± 10 a	33 ± 16 b	67 ± 16 b	78 ± 14 b
	B	70 ± 15 ab	43 ± 19 b	71 ± 17 b	86 ± 13 b
	C	83 ± 11 ab	30 ± 15 b	60 ± 16 b	80 ± 13 b
	D	86 ± 9 a	42 ± 14 b	83 ± 11 b	83 ± 11 b
<i>A. brachyphyllus</i>	A	75 ± 13 ab	33 ± 16 b	78 ± 14 b	100 ± 0 a
	B	75 ± 13 ab	44 ± 17 b	67 ± 16 b	89 ± 11 ab
	C	72 ± 11 ab	46 ± 14 b	77 ± 12 b	92 ± 7 ab
	D	68 ± 11 b	38 ± 14 b	85 ± 10 b	100 ± 0 a
<i>A. densiflorus</i> cv. Myersii	A	0 ± 0 d	–	–	–
<i>A. densiflorus</i> cv. Sprengeri	A	10 ± 4 c	100 ± 0 a <sup>1</sup>	100 ± 0 a <sup>1</sup>	100 ± 0 a <sup>1</sup>
<i>A. plumosus</i>	A	0 ± 0 c	–	–	–
HTPS1	D	80 ± 10 ab	42 ± 14 b	75 ± 13 b	100 ± 0 a
HTAB 2	D	71 ± 12 ab	33 ± 16 b	70 ± 17 b	90 ± 10 ab

a, b, c, d Letters indicate groups that were significantly different by LSD at  $\alpha = 0.05$

<sup>1</sup> The rooting rate in *A. densiflorus* cv. Sprengeri is calculated with only five plantlets

rates of 42 and 33 % in ARBM-3, reaching 100 and 90 %, respectively after two cycles of rooting. The five surviving rhizome buds of *A. densiflorus* cv. Sprengeri rooted in the medium ARBM-3 (Table 4).

#### Acclimatization of asparagus plantlets

The acclimatization rate for micropropagated plantlets of *A. brachyphyllus*, *A. pseudoscaber* and the interspecific hybrids HTPS1 and HTAB 2 were similar: 93, 100, 90 and 100 %, respectively (Fig. 2).

#### Genetic stability study of micropropagated plantlets using EST-SSR markers

The amplified fragments in the characterization of the mother plants of *A. brachyphyllus* and *A. pseudoscaber* using a set of 12 EST-SSR are shown in Table 5. The same fragments were also amplified in the molecular characterization of eight micropropagated plantlets from each species, further the relative height of the peaks obtained in the chromatograms was consistent between the mother plants and the micropropagated plantlets. Figure 3a and b shows chromatograms obtained after amplification of markers AG8 and TC1 in the mother plant and a micropropagated plant of *A. brachyphyllus* and *A. pseudoscaber*, respectively. According to the chromatogram results, the same fragments were observed in the *A. brachyphyllus* mother plant and their micropropagated plantlets for AG8 (214–216–218–220–226 bp) as well as in the *A. pseudoscaber* mother plant and their micropropagated plantlets

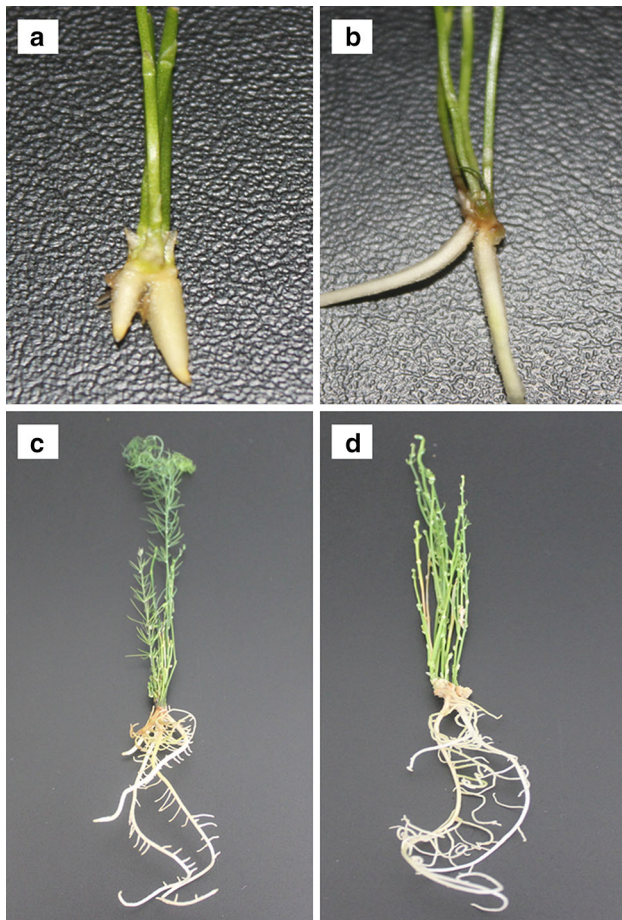
for TC1 (216–218–220–222–226–232 bp). The same peaks and with the same relative height were always found in the mother plants and their micropropagated plantlets for the 12 markers tested, indicating that, at least in these 12 markers, there is no somaclonal variation in the micropropagated plantlets of *A. brachyphyllus* or *A. pseudoscaber*.

#### Discussion

The development of a more broadly applicable micropropagation method for the different species of the genus *Asparagus* could be very useful for the conservation of elite genotypes of asparagus species and the interspecific hybrids obtained in breeding programs.

The disinfection of the rhizome bud explants is the key step of this micropropagation procedure due to the underground origin of the explants. The combination of two consecutive disinfection steps, the fungicide benomyl followed by sodium hypochlorite, allows us to obtain high disinfection rates in our materials. The disinfection step with the fungicide is crucial because of the high presence of fungi in the rhizome. The results obtained in the optimization of the decontamination in this study emphasize the importance of the fungicide. Also, our results showed that the disinfection step with benomyl for 20 min caused little damage to the rhizome buds (Table 4).

The development and rooting in ARBM-3 of rhizome bud explants from different species showed different survival rates between the species belonging to the subgenus *Asparagus* and *Protasparagus*. The species of the subgenus

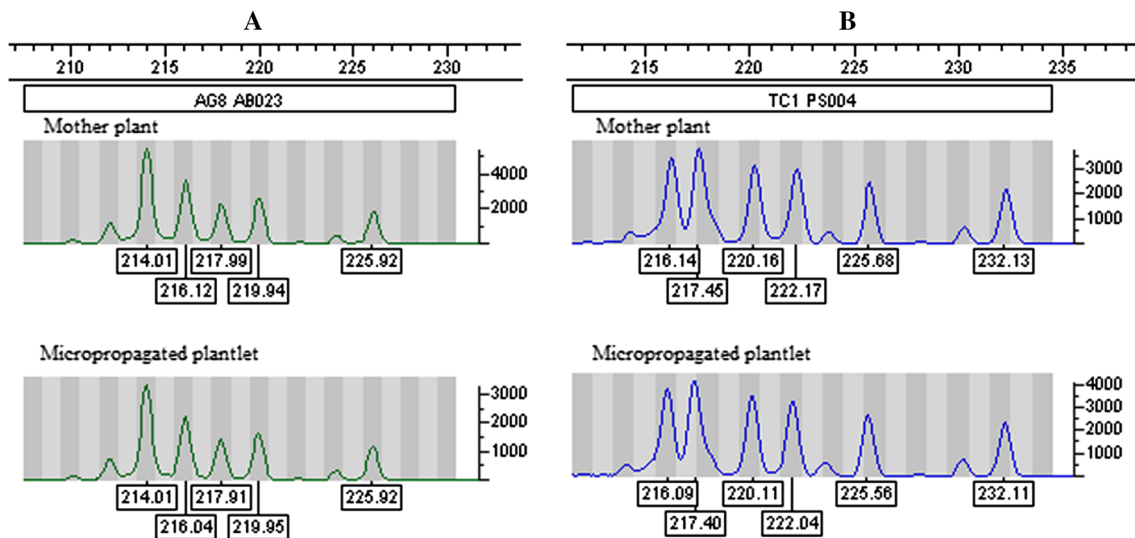


**Fig. 2** Detail of rooting of rhizome bud explants of *A. brachyphyllus* (**1a**) and *A. pseudoscaber* (**1b**). Plantlets of *A. brachyphyllus* (**2a**) and *A. pseudoscaber* (**2b**) ready for acclimatization

*Asparagus* (*A. brachyphyllus* and *A. pseudoscaber*) and their interspecific hybrids with “Morado de Huétor” (HTPS1 and HTAB 2) presented survival rates higher (>70 %) than the species of *Protasparagus* (*A. densiflorus* cvs. Myersii and Sprengeri, and *A. plumosus*). High survival rates for *A. officinalis* and *A. maritimus*, which belong to the subgenus *Asparagus*, were also detected by Carmona-Martín et al. (2014). In this study, the rhizome buds of *Protasparagus* species were not able to grow in ARBM-3 medium, except the 10 % of rhizome bud explants of *A. densiflorus* cv. Sprengeri, which develop to become full plantlets. These results indicate that the method of micropropagation using rhizome bud explants is inappropriate for micropropagation of these species of the subgenus *Protasparagus*. Other methods such as plant regeneration from callus culture (Benmoussa et al. 1996) appear to be more appropriate. The causes of the failure of the micropropagation method including rhizome buds of the subgenus *Protasparagus* as primary explants may reflect the annual growth pattern of the species belonging to the subgenus *Protasparagus*. The *Protasparagus* species employed in this study (*A. densiflorus* cvs. Myersii and Sprengeri and *A. plumosus*) are perennial and their aerial shoots show a slow growth pattern, sprouting just a few shoots per year. In contrast, the *Asparagus* species that we successfully propagated (*A. pseudoscaber* and *A. brachyphyllus*) are deciduous and renovate the aerial shoots every year, producing multiple new shoots every year, with a fast pattern of growth. The slow growth of the shoots of the subgenus *Protasparagus* in nature is mirrored in “in vitro”. The slow growth of the few plantlets of *A. densiflorus* cv.

**Table 5** Fragments (bp) amplified in the molecular characterization of the mother plants of *A. brachyphyllus* (AB023) and *A. pseudoscaber* (PS004) using 12 EST-SSR markers

Mother plant	EST-SSR								
	AG3	AG6	AG7	AG8	AG10	AG12			
AB023	208	193	173	175	214	216	159	230	233
	211				218	220	160	241	243
					226		161	256	
PS004	204	193	173	175	218	232	160	162	228
	218		182	183	240	256	171	177	233
	224								243
	EST-SSR								
	AAT1	TC1	TC3	TC5	TC7	TC9			
AB023	213	216	144	145	161	191	199	144	
	216	232	154	156	169	201	227	146	
	222	248			174	229	231	153	
PS004	213	216	218	138	145	172	173	193	147
	216	220	222	154	155	174	175		
		226	232						



**Fig. 3** **a** Chromatograms obtained in the amplification of the marker AG8 in the mother plant (AB023) and a micropropagated plantlet of *A. brachyphyllus*. **b** Chromatograms obtained in the amplification of

the marker TC1 in the mother plant (PS004) and a micropropagated plantlet of *A. pseudoscaber*

*Sprengeri* obtained (data not shown) could be due to a smaller content of GA in the *Protasparagus* species than in the species belonging to the subgenus *Asparagus*. Furthermore, the rhizome buds of the subgenus *Protasparagus* have a smaller size than the rhizome buds of the subgenus *Asparagus* (Fig. 1).

The rooting rates obtained in the ARBM-3 medium for rhizome bud explants of *A. brachyphyllus*, *A. pseudoscaber*, HTPS1 and HTAB 2 (ranging from 30 to 46 %) were lower than the rooting rates (ranging from 70 to 81 %) obtained in previous works using *A. officinalis*, *A. maritimus* and “Morado de Huétor” (Carmona-Martin et al. 2014). However, the use of a cyclic process of rooting, alternating incubation of unrooted buds in ARBM-3 and ARBM-0 media, increased the rooting rates (>78 %) when two cycles of rooting were applied in our materials. The main differences between the ARBM-0 and ARBM-3 cultures medium are the presence of ancymidol and a higher sucrose concentration (60 g l<sup>-1</sup>) in the latter medium (Table 3). The ancymidol is a growth retardant that, combined with high doses of sucrose, improves rooting and plantlet development in asparagus (Desjardins et al. 1987; Desjardins 1992; Carmona-Martin et al. 2014). In the work cited above, the explants were continuously cultured on the medium with ancymidol and high doses of sucrose. However, our results indicate that the alternation between a medium with ancymidol and high concentration of sucrose (ARBM-3) and a medium without ancymidol and normal concentration of sucrose (ARBM-0) increases the rooting rate of rhizome bud explants of the species of the subgenus *Asparagus* employed in this study. Nevertheless, of the few rhizome buds of *A. densiflorus* cv. *Sprengeri* which

survived, 100 % rooted easily in ARBM-3 medium (Table 4), suggesting that this species could be no recalcitrant for rooting, probably due to a lower endogenous content of GA in this species.

After rooting, the plantlets were cultured on ARBM-0 medium. In approximately 8 weeks, they reached the size and quality suitable for their acclimatization (Fig. 2). The plantlets can also be multiplied by mechanical division in this medium, but the division increases the time necessary to reach the optimal quality for the acclimatization. The acclimatization rates obtained in this work (90–100 %) are higher than the rates obtained in previous research on other species (Carmona-Martin et al. 2014). The improved results in acclimatization could be explained by the continuous use in our laboratory of the acclimation method described by Encina et al. (2008).

Finally, we have compared the fragments amplified using a set of twelve EST-SSRs in the micropropagated plantlets of *A. brachyphyllus* and *A. pseudoscaber* with the fragments obtained in the mother plants of each species in order to look for somaclonal variation and study the genetic stability of these plantlets (Fig. 3; Table 5). Each of the EST-SSRs employed corresponds to a unique locus. So, the number of alleles amplified depends on the ploidy level of the species characterized (Castro et al. 2013). In our case, working with two hexaploid species ( $2n = 6x = 60$ ) we can find up to six different alleles for an EST-SSR (TC1 for PS004, Table 5). We have not found variation in the fragments amplified in the eight micropropagated plantlets analyzed in both species, neither in the relative height of the peaks obtained in the chromatograms. Working with polyploid species, variation in the height of the peaks may



indicate change in the number of repetitions of each allele amplified for each marker. Therefore, we can indicate that there is no somaclonal variation in our experimental conditions for the asparagus species tested, confirming that the micropropagated plantlets have a high genetic stability. These results are in agreement with the results previously obtained using the same method for micropropagated plantlets of the Spanish landrace “Morado de Huétor” (Carmona-Martin et al. 2014). Currently, EST-SSRs are considered the molecular markers of choice for genetic stability studies (Bairu et al. 2011). A similar number of SSRs have been used to detect somaclonal variations in previous studies using different species such as banana (Ray et al. 2006), sugarcane (Singh et al. 2008), rice (Gao et al. 2009), or sorghum (Zhang et al. 2010).

The micropropagation methods reported to date have been developed for only one species of the *Asparagus* subgenus. In addition, all these methods present some problems such as technical difficulties for meristem culture (Murashige et al. 1972). The methods based on axillary bud culture are slow, have low efficiency and their success is highly dependent on the genotype employed (Desjardins et al. 1987; Bopana and Saxena 2008). The methods based on organogenic or embryogenic regeneration produce morphological variations (Kunitake and Mii 1998), changes in the ploidy level (Kunitake and Mii 1998; Raimondi et al. 2001; Pontaroli and Camadro 2005) and somaclonal variations that have been detected using RAPDs (Raimondi et al. 2001) and AFLPs (Pontaroli and Camadro 2005) markers. It is perfectly possible to compare the results of these studies of the genetic stability performed with RAPDs or AFLPs and the results obtained in our work with EST-SSRs. The works developed by Adhikari et al. (2014) and Chavan et al. (2013) are examples of the reliability of this comparison, these authors detected a similar rate of somaclonal variation studying the genetic stability of the same micropropagated plants with different molecular markers (SSRs, RAPDs, AFLPs). So, we can conclude that the method proposed in the current work, based on the use of rhizome buds as primary explant, avoids all the problems linked to the above mentioned methods of micropropagation of asparagus and it is versatile enough to guarantee the fast and efficient recovery of micropropagated plantlets with high genetic stability for different species belonging to the subgenus *Asparagus*. Using the method proposed, hundred micropropagated plants could be produced in eight or nine months starting from an adult plant, of which around 40 rhizome buds were extracted.

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