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ORIGINAL ARTICLE

# Production of "super-males" of asparagus by anther culture and its detection with SSR-ESTs

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**Abstract** Anther culture is used to develop asparagus "super-male" (di-haploids) in asparagus, which can be used to develop "all-male" varieties, by crossing them with suitable females; their progenies will be formed only by males which is advantageous for producers. This report describe a new anther culture protocol adapted to "Morado de Huétor", a Spanish tetraploid landrace, and studied the different factors involved in callus proliferation success from anther explants such as the microspore development stage, or the type of stress used to induce the symmetric division of the microspores, to obtain a high success rate (90 %). For plantlets regenerates from anther culture (PRACs) regeneration we develop a proliferation media supplemented with a combination of pCPA and BA able to induce callus proliferation and plantlet regeneration in the same step in a 50 % of calli, simplifying the procedure. The high percentage of heterozygous male recovery, originated from somatic cells, is an important problem in the anther culture, and to elucidate the origin of PRACs we have combined different tools: ploidy analysis, characterization with the linked sex-marker Asp1-T7 and with EST-SRRs. We can establish that 50 % of PRACs obtained in this work were regenerated from diploid microspores of "Morado de Huétor", regenerating diploid, di-diploid and tetra-diploid plantlets. The di-diploids males *(MMmm)* would generate a ratio male:female of 5:1 (83.3 %) and the tetra-diploid males (*MMMMmmmm*) a ratio male:female of 69:1 (98.6 %), so the tetra-diploid males could be considered "super-males" and be used to develop "all-male" varieties of "Morado de Huétor".

**Keywords** Haploid · Micropropagation · "Morado de Huétor" · Organogenesis · Sex-linked marker · Somaclonal variation

## Introduction

*Asparagus officinalis* L. is an important crop plant that is grown in temperate climate regions worldwide. This crop is a dioecious species in which the sex is determined by a dominant gene, *M* (Flory 1932), located on the homomorphic chromosome pair L5 (Löptien 1979). In diploid asparagus ($2n = 2x = 20$), the female genotypes are homozygous recessive (*mm*) and the male genotypes are heterozygous (*Mm*), resulting in a ratio 1:1 (male:female) in traditional cultivars.

Male plants show advantages over female plants. The male plants will never produce seeds, avoiding the growth of these seeds into weeds. In addition, their yield, longevity and tolerance to diseases are higher than in female plants (Hartmann 1985; Ellison 1986; López-Anido and Cointry 2008). Male plants sometimes bear bisexual flowers, and these male genotypes are named andromonoecious. Self-pollination of those flowers can produce "super-males" (*MM*). Crosses between "super-males" and female genotypes will raise a progeny consisting exclusively in male plants (Sneep 1953). Hence, the "super-males" can be used to develop "all-male" cultivars (Ellison and Kinelski 1985;

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Ellison et al. 1990). These "super-males" suppose less than 2 % of the wild populations (Galli et al. 1998), and not always appear into the appropriate genetic background. For this reason the introgression of this trait into an appropriate genetic background a long time is required (Riccardi et al. 2011; Regalado et al. 2014).

The development of di-haploids (DH) males (*MM*) is a faster alternative to obtain "super-male" asparagus clones from males with the appropriate genetic background (Doré 1974, 1990). Additionally, the "super-male" DH clones are homozygous at all loci, and the "all-male" varieties derived from these "super-males" are more uniform (hybrids F1) than the "all-male" varieties obtained from "super-males" generated by the self-pollination of andromonoecious plants (Riccardi et al. 2011). There are two methods to obtain "super-male" DH clones already used with success: the culture of isolated microspores (Zhang et al. 1994; Delaitre et al. 2001) and the anther culture (Doré 1974; Qiao and Falavigna 1990; Feng and Wolyn 1991). The culture of isolated microspores requires important technical requirements as the microspore isolation from the anther and the inoculation into liquid medium, limiting its application (Seguí-Simarro 2010). On the other hand, some drawbacks of anther culture methods are: (1) the duration of the plantlet regeneration process; (2) the low percentage of plant regenerated from embryos and (3) the high percentage of heterozygous male recovery, originated from somatic cells existing in the anther and filament tissues (Falavigna et al. 1983; Riccardi et al. 2011).

It is necessary to consider that the success of methods for DH development is highly influenced by different factors. Firstly, these processes are highly genotype dependent, which has been already described in different model species (Ferrie et al. 1995; Touraev et al. 2001; Malik et al. 2007) as well as in *A. officinalis* (Qiao and Falavigna 1990; Wolyn and Feng 1993). Secondly, the developmental stage of the microspores, as a general rule, the use of microspores in very early and in very late stages resulting unsuccessful, but the appropriate developmental stage varies for each species (Seguí-Simarro 2010). In many species, including *A. officinalis* (Feng and Wolyn 1991), the appropriate developmental stage is late microspore, when microspores are ready to divide asymmetrically (Seguí-Simarro 2010). Finally microspores must be submitted to a set of different types of physicochemical factors (Shariatpanahi et al. 2006) to induce a disturbing stress in order to trigger the embryogenic growth pathway of the microspores. This embryogenic response begins with a symmetric division of the microspore, as opposed to the asymmetric division that defines the first pollen mitosis (Zaki and Dickinson 1991; Simmonds and Keller 1999; Smykal 2000).

"Morado de Huétor" is a tetraploid ($2n = 4x = 40$) Spanish landrace (Moreno et al. 2006) originated from hybridization between *A. officinalis* and *Asparagus maritimus* (Moreno et al. 2008a). This landrace showed a high genetic variability, mainly due to both their landrace character and hybrid origin between two different species, contrasting with the narrow genetic basis of the current commercial varieties (Brettin and Sink 1992; Geoffriau et al. 1992; Lallemand et al. 1994; Khandka et al. 1996; Moreno et al. 2006). So that, "Morado de Huétor" could be a good breeding tool for broadening the genetic pool of commercial varieties (Moreno et al. 2010). However, this genetic variability causes that in the traditional growing areas of "Morado de Huétor" this landrace is being displaced by commercial hybrids varieties, which are more productive and homogenous, risking the survival of this landrace (Moreno et al. 2008b). Another disadvantage of "Morado de Huétor" against the 100 % of males that presents the "all-male" varieties, is that the population preserved the sex ratio 1:1 (male:female), suggesting that the male genotypes are *Mmmm* and female genotypes are *mmmm* (Moreno et al. 2008b).

The objective of this work was the development of "super-male" genotypes from an elite male of "Morado de Huétor" in order to further development of "all-male" varieties retaining the differential organoleptic traits of this landrace. To develop these "super-males", we have opted for an anther culture approach. Different factors with influence in the process of DH development, such as the developmental stage of the microspores or the type of stress used to trigger the process were studied. As well, the anther culture to reduce its duration or to increase the low percentage of plant regeneration was optimized. Finally we used flow cytometry for ploidy level assessment of the regenerated plantlets, and molecular tools, such as a sex-linked molecular marker (Asp1-T7) and a battery of EST-SSRs, for sex assessment and to determinate the origin of the plants regenerated from anthers.

## Materials and methods

### Plant material

Anthers collected from the tetraploid male HT156 belonging to the Spanish landrace "Morado de Huétor" were used in this study. This genotype was selected among males of a collection of "Morado de Huétor" (Carmona-Martin et al. 2014) due to its outstanding agronomical traits (as big spear diameter, branching height of shoots, bronze hue of spears and spear tips well-formed and closed). A plant stock of this genotype has been maintained growing

in a greenhouse of the IHSM "La Mayora" as source of explants.

### Study of microspore development

Flower buds of the genotype HT156 were harvested in early 2010s spring to study the different developmental state of its microspores. The flower buds were fixed in a 3:1 solution of 95 % ethanol and glacial acetic acid during 24 h and maintained at 4 °C until been used. The flowers buds were classified by size in six groups: S1 (<1.5 mm), S2 (1.5–2.0 mm), S3 (2.1–2.5 mm), S4 (2.6–3.0 mm), S5 (3.1–3.5 mm) and S6 (>3.5 mm). Ten flowers buds of each group were stained with acetic-carmine (Snow 1963) and observed with an optical microscope at ×1250 magnification. In order to know the prevalent developmental stage of the microspores inside the flower buds and according to its size, one hundred microspores by each anther were counted. The proportion of microspores in each developmental stage obtained for each flower size was analyzed statistically using analysis of variance (ANOVA) with the program SPSS (version 19.0; SPPS Inc., Chicago, IL. USA). Pairwise comparisons among groups were performed by HSD Tukey test.

### Harvesting, disinfection and dissection of flower buds explants

During 2011 spring, one hundred of each flower buds size S2, S3, S4 and S5 from HT156 were collected. The flower buds were collected at 8:00 am, with an alcohol sterilized forceps. The collected buds were disinfected following different steps: firstly, buds were washed with soapy water; secondly, they were treatmented with 1 % of sodium hypochlorite during 15 min under vacuum conditions; and finally, buds were subjected to three washes with sterile water under aseptic conditions to eliminate the sodium hypochlorite. In order to obtain the individual anthers, the dissection of sterilized flowers was performed in aseptic conditions, under a binocular magnifier and with the help of sterilized tweezers.

### Induction of callus proliferation in anther explants

Four different assays were carry out with the objective of establish a protocol adapted for the induction of callus formation in anthers obtained from the tetraploid genotype HT156. In the first assay the results of two culture, A1 (Qiao and Falavigna 1990) and the MAE (Feng and Wolyn 1991) mediums, proposed in the bibliography to induce callus from anthers of diploid *A. officinalis* genotypes, were compared. The composition of these mediums is shown in the Table 1. A total of 480 anthers, obtained from twenty flower buds corresponding to sizes S2, S3, S4 and S5, were used in this assay. After disinfection and dissection, 60 anthers of each size were established in each medium. The anthers were cultured in Petri dishes with 25 ml of both mediums, twelve anthers per Petri dish, and incubated during 8 weeks at 25 ± 1 °C under 16 h day photoperiod under cold white fluorescent tubes (F40 tubes Gro-lux, Sylvania) with 45 μmol m-2 s-1 (400–700 nm) Photosynthetic Active Radiation.

With the aim of analyzing the effect of cold shock in the success rate of callus proliferation in anther explants, a second assay was performed. This experiment was a repetition of the first one but subjecting the anthers before dissection to a cold pretreatment. This pretreatment consisted in incubation at 4 °C in dark conditions during 7 days.

The effect of the incubation temperature over callus proliferation in anther explants was studied in a third assay. Three different incubation temperatures (25 ± 1, 32 ± 1 and 37 ± 1 °C) were tested. Sixty anthers of each size (S2, S3, S4 and S5) were subjected to a cold pretreatment and cultured in Petri dishes containing MAE medium and incubated in dark conditions at 25, 32 and 37 °C during 4 weeks. After these weeks, anthers were incubated at 25 ± 1 °C in standard light conditions, through four additional weeks. The same assay with an incubation temperature of 32 ± 1 °C and A1 instead of MAE medium was repeated as control.

Finally, the effect of the osmotic stress in callus proliferation on anther explants was examined. In order to change the osmotic level in MAE medium, the concentration of sucrose was increased. Two different concentrations of sucrose, 10 % (MAE10) and 14 % (MAE14) (Table 1), were tested. After the cold pretreatment, 60 anthers per each flower bud size (S2, S3, S4 and S5) were cultured for each sucrose concentration. These anthers were incubated at 32 ± 1 °C in dark conditions during 4 weeks, and then incubated during four additional weeks at 25 ± 1 °C in standard light conditions.

The success in the induction of callus from anther explants was measured after 8 weeks of incubation in all the assays. Data were analyzed statistically by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution, with the program SPSS (version 19.0; SPPS Inc., Chicago, IL. USA). Pairwise comparisons among groups were performed by Fisher's least significant difference (LSD) test.

### Callus proliferation and shoot regeneration

The calli obtained through anther culture were transferred to different proliferation media (PM) for mass increasing. The proliferation media consisted in MS (Murashige and

**Table 1** Composition of the culture medium used in anther explants for callus proliferation

| Media[a] | Growth regulators (mg $l^{-1}$) | | | Casein (mg $l^{-1}$) | Glutamine (mg $l^{-1}$) | Sucrose (g $l^{-1}$) | Glucose (g $l^{-1}$) | Agar (g $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | NAA | 2,4-D | BA | | | | | |
| A1 | 0.1 | 0.5 | 0.5 | – | – | 20 | 20 | 6 |
| MAE | 2 | – | 1 | 500 | 800 | 60 | – | 6 |
| MAE10 | 2 | – | 1 | 500 | 800 | 100 | – | 6 |
| MAE14 | 2 | – | 1 | 500 | 800 | 140 | – | 6 |

[a] All media containing MS salts and vitamins (Murashige and Skoog 1962) and adjusted to pH 5.7

Skoog 1962), salts and vitamins, supplemented with different combinations of plant growth regulators (Table 2) and 6 % sucrose, adjusted to pH 5.7, and solidified with 0.6 % agar. Calli were transferred to 25 ml of fresh medium in Petri dishes and incubated in dark conditions at 25 ± 1 °C. Twenty-five calli of each type were cultivated in each proliferation media and classified according to their size as bigger than 2 mm or smaller than 2 mm. The calli were subcultured to Petri dishes containing fresh medium every 4 weeks, six times in total; in these subcultures calli were divided in fragments to improve their proliferation. During these subcultures the shoots regenerated were removed and transferred to new media to proceed with their rooting and multiplication.

The data of proliferation and regeneration, defined as the number of calli showing regeneration respect to the number of callus with proliferation, obtained for each PM were analyzed statistically by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution, with the program SPSS (version 19.0; SPPS Inc., Chicago, IL. USA). Pairwise comparisons among groups were performed by Fisher's least significant difference (LSD) test.

## Rooting, multiplication, and acclimatization of plantlets

Rooted and unrooted shoots were regenerated from callus. The rooted shoots were transferred to test tubes with 25 ml of Asparagus Rhizome Bud Medium 0 (ARBM-0) (Table 3) and incubated at 25 ± 1 °C under 16 h day photoperiod and cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania) with 45 µmol $m^{-2}$ $s^{-1}$ (400–700 nm) Photosynthetic Active Radiation. The plantlets were subcultured every 4 weeks to fresh medium for further growth and multiplication following the protocol described by Regalado et al. (2015a).

After a first subculture in ARBM-0 for elongation, the unrooted shoots were subjected to a cyclic process of rooting, alternating subcultures in ARBM-0 and ARBM-3 (Table 3) as described by Regalado et al. (2015a). Once rooted, the normal procedure of growth and multiplication was followed as described above. After two cycles of rooting the rooting rate was established and unrooted shoots were discarded. Finally, the plantlets regenerated from anther culture (PRAC) were acclimatized using the method described by Encina et al. (2008).

**Table 2** Composition of auxin and cytokinins of proliferation media (PM)

| Cytokinins (mg $l^{-1}$) | Auxin | | | |
|---|---|---|---|---|
| | pCPA (mg $l^{-1}$) | | 2,4-D (mg $l^{-1}$) | |
| | 1 | 2 | 1 | 2 |
| – | PM1 | PM2 | PM3 | PM4 |
| BA1 | PM5 | PM6 | PM7 | PM8 |
| Kinetin1 | PM9 | PM10 | PM11 | PM12 |

## Ploidy analysis

The ploidy level of plantlets regenerated from anther culture (PRAC) 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 1.6 ml of fluorescent dye (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, staining buffer). Finally, the samples were analyzed after 30 s of incubation. *A. officinalis* cv. UC157 F1 (2n = 2x = 20) was used as an external standard. A maximun of 16 plantlets regenerated from callus developed from a single anther were analyzed. The parts with different ploidy level of the plantlets cataloged as mixoploid were separated following the protocol described by Regalado et al. (2015b).

**Table 3** Composition of culture media used in the rooting and further multiplication of regenerated plantlets

| Medium[a] | Growth regulators (mg $l^{-1}$) | | | Sucrose (g $l^{-1}$) | Agar (g $l^{-1}$) | Goal |
|---|---|---|---|---|---|---|
| | NAA | KIN | Ancymidol | | | |
| ARBM-3 | 0.3 | 0.1 | 2 | 60 | 8 | Shoots rooting |
| ARBM-0 | 0.1 | 0.1 | – | 30 | 8 | Plantlet development |

[a] All media contain MS salts with EDDHA-Fe (85.7 mg $l^{-1}$) instead of EDTA-Fe and vitamins (Murashige and Skoog 1962)

**Table 4** Percentage of microspores in different developmental stages in side anthers of buds flowers with different sizes, belonging to genotype HT156

| Flower size (mm) | Development stage | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Pollen mother cells | Dyads | Tetrads | Early microspore | Middle microspore | Late microspore | Bicellular pollen | Mature pollen |
| S1 (<1.5) | 94.4 ± 21.0 a | 5.6 ± 21.0 b | | | | | | |
| S2 (1.5–2.0) | 31.6 ± 44.3 b | 28.8 ± 31.8 a | 39.6 ± 39.7 a | | | | | |
| S3 (2.1–2.5) | | | 9.6 ± 27.5 b | 30.4 ± 32.6 a | 42.5 ± 31.7 a | 17.5 ± 30.3 b | | |
| S4 (2.6–3.0) | | | | | 29.1 ± 33.8 b | 54.9 ± 28.2 a | 16.0 ± 22.5 b | |
| S5 (3.1–3.5) | | | | | | 8.5 ± 14.3 b | 65.9 ± 25.5 a | 25.6 ± 28.7 b |
| S6 (>3.6) | | | | | | | 5.2 ± 14.2 c | 94.8 ± 14.2 a |

a, b, c, Letters indicate groups that were significantly different by HSD Tukey test at $\alpha = 0.05$

### Sex determination of plantlets regenerated from anther culture (PRAC)

Eight regenerated plantlets from each anther, were selected for sex determination. The sex-linked marker Asp1-T7 developed by Jamsari et al. (2004) was used for that goal, following the procedure described by Regalado et al. (2014).

### Characterization with EST-SSRs of the plantlets regenerated from anther culture

In order to determine the origin of the calli from which the plantlets were regenerated, the EST-SSRs AAT1, AG10, TC1 and TC5 (Caruso et al. 2008) were used. These EST-SSRs were chose because they amplified at least three alleles in plants of tetraploid genotype HT156 (Regalado et al. 2015b), source of the anthers used as explants in this work. The same plantlets characterized with the sex-linked marker Asp1-T7 were characterized with these EST-SSRs. PCR conditions used to carry out this characterization were described by Carmona-Martin et al. (2014).

## Results

### Study of microspores development

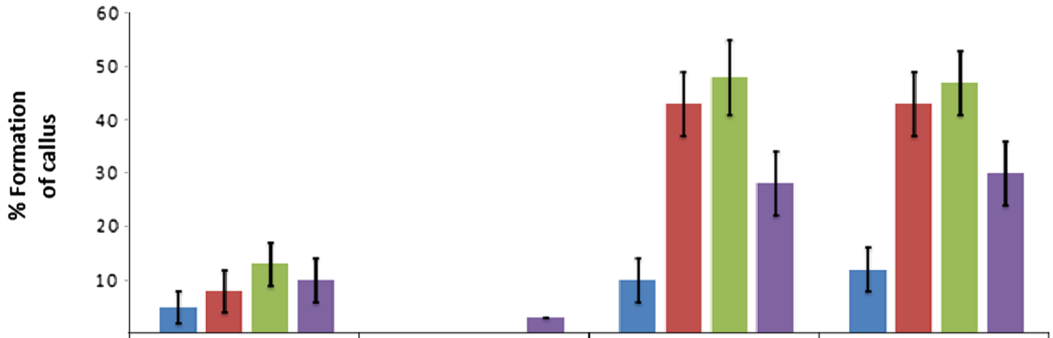
The percentages of microspores in different developmental stages obtained from different sizes flower buds belonging to genotype HT156 are shown in Table 4. These percentages were always accompanied by a high standard deviation, because of microspores development has a high variation inside the flower buds with the same size, even for different anthers belonging to the same flower bud. Apart from the high deviation obtained, correlation between the most abundant microspores stage and the flower bud sizes was established.

The development stage of the microspores more suitable to carry out anthers culture was the late microspore stage, this developmental stage was most abundant in S4 flower buds (54.9 ± 28.2 %), being also present in flower buds types S3 (17.5 ± 30.3 %) and S5 (8.5 ± 14.3 %). Based on these results, the flower buds with these sizes were used to obtain anther explants. S2 stage was also included as control because it shows a development stage of microspores previous to the suitable stage. S1 and S6 flowers were discarded because the most common developmental stages were pollen mother cells (94.4 ± 21.0 %) and mature pollen (94.8 ± 14.2 %), respectively.

### Induction of callus proliferation in anther explants

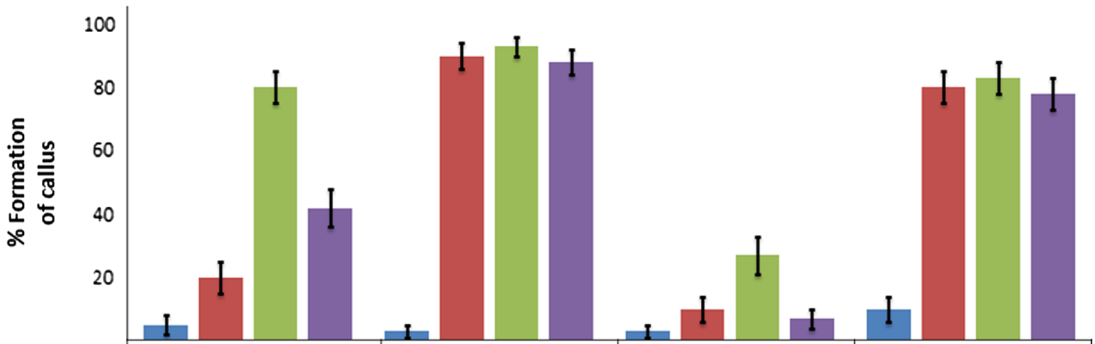
The percentage of callus proliferation in the different assays involving anther culture is shown in Fig. 1. Better rate of callus proliferation without pretreatment was obtained in A1 medium (13 ± 4 % anthers S4), while none or few was obtained using MAE. On the other hand, the cold pretreatment increased proliferation in both media (Fig. 1a). This increasing was remarkable higher in callus

**(A)** Effect of culture media and pretreatment



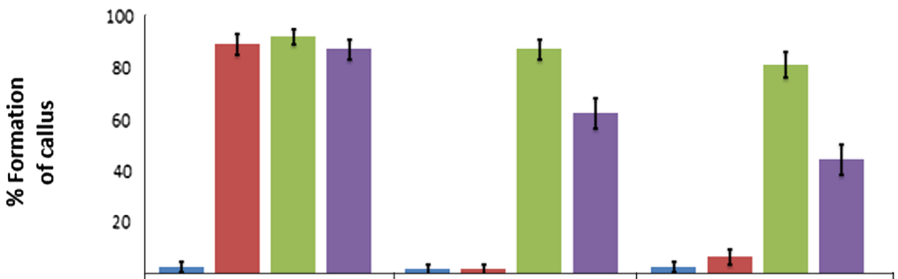

| | A1 | Mae | A1 with pretreatment | Mae with pretreatment |
|---|---|---|---|---|
| Anthers S2 | 5 ± 3 de | 0 ± 0 f | 10 ± 4 c | 12 ± 4 c |
| Anthers S3 | 8 ± 4 cd | 0 ± 0 f | 43 ± 6 a | 43 ± 6 a |
| Anthers S4 | 13 ± 4 c | 0 ± 0 f | 48 ± 7 a | 47 ± 6 a |
| Anthers S5 | 10 ± 4 c | 3 ± 0 ef | 28 ± 6 b | 30 ± 6 b |

**(B)** Effect of the temperature of incubation




| | Mae 25°C | Mae 32°C | Mae 37°C | A1 32°C |
|---|---|---|---|---|
| Anthers S2 | 5 ± 3 fg | 3 ± 2 g | 3 ± 2 g | 10 ± 4 f |
| Anthers S3 | 20 ± 5 e | 90 ± 4 ab | 10 ± 4 f | 80 ± 5 bc |
| Anthers S4 | 80 ± 5 bc | 93 ± 3 a | 27 ± 6 e | 83 ± 5 bc |
| Anthers S5 | 42 ± 6 d | 88 ± 4 ab | 7 ± 3 fg | 78 ± 5 c |

**(C)** Effect of the osmotic stress




| | Mae 6 | Mae 10 | Mae 14 |
|---|---|---|---|
| Anthers S2 | 3 ± 2 e | 2 ± 2 e | 3 ± 2 e |
| Anthers S3 | 90 ± 4 ab | 2 ± 2 e | 7 ± 3 e |
| Anthers S4 | 93 ± 3 a | 88 ± 4 ab | 82 ± 5 b |
| Anthers S5 | 88 ± 4 ab | 63 ± 6 c | 45 ± 6 d |

◀**Fig. 1** Rate of callus proliferation obtained in the different assays carried out with anther explants: **a** Effect of culture medium and cold pretreatment. **b** Effect of the temperature of incubation. **c** Effect of the osmotic stress: anther cultured in Mae medium supplemented with 6, 10 and 14 % of sucrose. *Different letters* indicate groups that were significantly different by LSD at $\alpha = 0.05$

growing in MAE media The differences between anthers obtained from flower buds of different sizes were very important in this assay. Regardless of the culture medium used, the sizes S3 and S4, that presented a higher percentage of late microspore showed a higher rate of callus formation, being 43 ± 6 and 48 ± 7 % for S3 and S4, respectively. S5, that presented a low percentage of late microspore, had an intermediate rate (30 ± 6 %). As expected, the stage without late microspores (S2) had a minimum rate of proliferation (10 ± 4 %).

The rates of callus proliferation increased in the third assay, in which the effect of the incubation temperature in callus proliferation from anther explants was studied. A significant increase in the success rate (80 ± 5 % in S4) was obtained when the incubation was made at 25 °C in dark conditions during 4 weeks (Fig. 1b) comparing with the rate obtained in light conditions (48 ± 7 % in S4, Fig. 1a). The rate obtained for S3 in this assay (20 ± 5 %) was lower than the expected; this inconsistency could be due to a mistake during the culture or to a problem of plant material. The incubation at 32 °C in dark during 4 weeks resulted in a rate close to 90 % for all flower buds sizes with presence of late microspore (S3, S4 and S5) (Fig. 1b), these results indicated that the stress produced by a temperature of 32 °C enables the callus proliferation inside anther explants. On the other hand when the incubation temperature was 37 °C a decreasing in callus proliferation was observed (Fig. 1a, b). Finally the results obtained in the incubation at 32 °C in dark conditions during 4 weeks but using the A1 medium instead MAE medium, used as control in this assay, confirmed the results obtained in MAE medium. All flower buds sizes with presence of late microspore (S3, S4 and S5) showed very high rates of callus proliferation in these conditions (Fig. 1b), ranging a 80 %.

Finally, a comparison of different osmotic stresses was made. The increase of the osmotic stress supposed a significantly decrease in the rate of callus proliferation inside the anther explants for the flower buds size S3 (Fig. 1c), being smaller for S5 state. S4 flower buds did not appear to be affected by osmotic changes and high levels of proliferation were observed in both concentrations tested. These results allowed us to discard the use of osmotic stress to induce callus proliferation inside anther explants.

## Callus proliferation and shoot regeneration

The callus proliferation rates obtained with the different proliferation media (PM) assayed are shown in Table 5. Calli were classified by size bigger or smaller than 2 mm (Fig. 2b, c). For calli smaller than 2 mm the rates obtained were very low, ranging from 8 ± 5 to 20 ± 8 %. No statistically significant differences were found in any of the PM tested for this calli size. Nevertheless, significant differences appeared in the proliferation rates for calli greater than 2 mm in the different PMs assayed. The PMs with a higher success rate were those with a composition including different auxin concentrations without cytokinins, PM1, PM2, PM3 and PM4, as well as PM9 combination (1 mg $l^{-1}$ KIN + 1 mg $l^{-1}$ pCPA). The proliferation rates of these PMs were close to 80 %. PM4 showed the highest

**Table 5** Callus proliferation rates obtained with the different proliferation media (PMs) assayed in anther culture

| Cytokinins | Initial size | Auxins | | | |
|---|---|---|---|---|---|
| | | pCPA (1 mg $l^{-1}$) PM1 | pCPA (2 mg $l^{-1}$) PM2 | 2,4-D (1 mg $l^{-1}$) PM3 | 2,4-D (2 mg $l^{-1}$) PM4 |
| – | Smaller than 2 mm | 8 ± 5 e | 12 ± 7 e | 8 ± 5 e | 20 ± 8 e |
| | Bigger than 2 mm | 80 ± 8 ab | 76 ± 9 bc | 76 ± 9 bc | 92 ± 5 a |
| | | PM5[a] | PM6[a] | PM7 | PM8 |
| BA (1 mg $l^{-1}$) | Smaller than 2 mm | 12 ± 7 e | 8 ± 5 e | 8 ± 5 e | 12 ± 7 e |
| | Bigger than 2 mm | 48 ± 10 d | 48 ± 10 d | 60 ± 10 cd | 56 ± 10 d |
| | | PM9 | PM10 | PM11 | PM12 |
| KIN (1 mg $l^{-1}$) | Smaller than 2 mm | 16 ± 7 e | 16 ± 7 e | 12 ± 7 e | 16 ± 7 e |
| | Bigger than 2 mm | 84 ± 7 ab | 60 ± 10 cd | 56 ± 10 d | 64 ± 10 c |

Different letters indicate groups that were significantly different by LSD at $\alpha = 0.05$

[a] Shoot regeneration rate in PM5 (46.6 %) and in PM6 (57.1 %) media

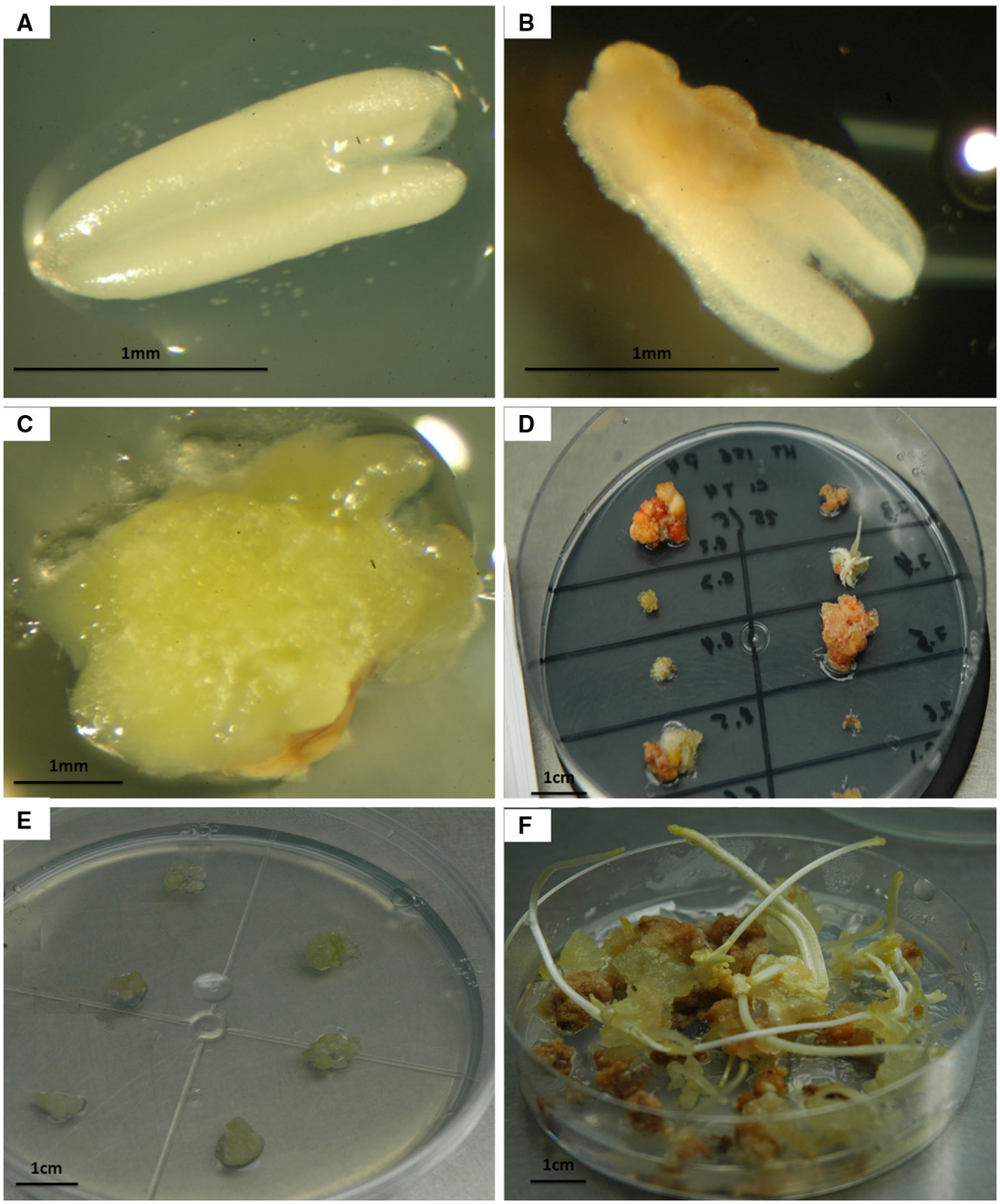



**Fig. 2** Different steps of anther culture. **a** Anther without callus. **b** Anther with proliferating calli smaller than 2 mm. **c** Anther with proliferating calli bigger than 2 mm. **d** Anther subcultures with calli bigger than 2 mm in PM. **e** Subculture of callus fragments to facilitate their proliferation. **f** Shoot regeneration in PM5 medium

proliferation rate (92 ± 5 %). The PMs containing BA plus 2,4-D (PM7 and PM8) or kinetin combined with pCPA or 2,4-D (PM10, PM11 and PM12) produced a proliferation rate close to 60 %. Lastly, PM5 and PM6, that contained BA with pCPA, showed the lowest proliferation rates (48 %). In spite of showed the lowest calli proliferation rates, PM5 and PM6 where the only PM showing regeneration rate of shoots. The plantlets regenerate in a 46.6 and 57.1 % of calli proliferating in PM5 and PM6, respectively (Fig. 2f). Although proliferation rates in PM5 and PM6 media were lower than the one obtained in other PMs, these media allowed the combination of callus

with shoot regeneration in a unique medium, eliminating a second step of regeneration and shortening substantially the time required to develop this procedure.

### Rooting and acclimatization of plantlets

After two cycles of rooting, shoots obtained from 93 % of the shoot-regenarating-calli were successfully rooted. The acclimatization rate of the plantlets regenerated from anthers culture (PRAC) was above 90 %.

### Ploidy analysis

The ploidy analyses results are shown in Table 6. Diploid plantlets (2n = 2x = 20) were detected only in PRAC1. Tetraploid (2n = 4x = 40) and octoploid (2n = 8x = 80) ones were also detected (Fig. 3) among the plantlets regenerated from PRAC1. The plantlets obtained from the remaining PRACs were tetraploid (2n = 4x = 40) or a mix of tetraploid and octoploid (2n = 8x = 80) plantlets. Tetraploid plantlets (2n = 4x = 40) were detected in PRAC2, PRAC3, PRAC4, PRAC5, PRAC7, PRAC10, PRAC12, PRAC13 and PRAC14. Both tetraploid and octoploid (2n = 8x = 80) plantlets were identified in PRAC6, PRAC8, PRAC9 and PRAC11. In addition, an increase in the spear diameter associated with ploidy level can be observed in the plantlets from the same PRAC (Fig. 3).

### Sex determination of plantlets regenerated from anther culture (PRAC)

The sex determination of different PRACs through the sex-linked marker Asp1-T7 is shown in Table 6. All plantlets obtained from the same anther belonged to the same sex. Only three PRACs (PRAC1, PRAC2 and PRAC9) were determined as female, the others 11 PRACs were determined as males.

### Characterization with EST-SSRs of plantlets regenerated from anther culture

The size of the fragments amplified in the characterization with four EST-SSRs (AAT1, AG10, TC1 and TC5) of the PRACs is shown in Table 6. In this table the characterization of the male HT156 was also included as reference. The results of this characterization allowed the identification of the origin of the callus from which each PRAC was regenerated (Table 6). This is possible since the plantlets derived from somatic cells should show the same amplification pattern than HT156 and the ones originated from microspores should have as much as two alleles of the three alleles amplified in HT156 from each EST-SSR studied. The combination of this information with the PRACs ploidy level enabled the determination of the PRACs sexual genotype (Table 6).

PRAC14 amplified as much as two alleles in the EST-SSRs studied (AAT1: 214; AG10 160,164; TC1 222,230; TC5 167) (Fig. 4). This indicates that the PRAC14 origin was a diploid microspore male (*Mm*), being that this PRAC has been determined as male. Due to the tetraploid nature of this plantlets, and taking into account that as much as two alleles were found for this genotype in all the microsatellites tested, it could be concluded that it was produced by an endoreduplication of some callus cells during the process of regeneration. So the sexual genotype of these plants would be *MMmm*. The same conclusion could be reached for tetrapoid genotypes of PRAC7, PRAC11 and PRAC12. In the case of the octoploid plantlets of PRAC11 (Table 6), their sexual genotype may be *MMMMmmmm* and would have been produced by a double endoreduplication. Also one or two alleles were found for each marker for PRAC2 (AAT1: 214; AG10: 160,183; TC1: 222,228; TC5: 167) (Fig. 4). However, the origin of the callus regenerating these plantlets was a female microspore, being this PRAC has been determined as female. PRAC1 and PRAC9 have the same origin that PRAC2, a female microspore (Table 6). The sexual genotype of the diploid plantlets of these PRACs would be *mm*, the tetraploid plantlets *mmmm* and the octoploid plantlets *mmmmmmmm.*

On the contrary, PRAC3, PRAC4, PRAC5, PRAC6, PRAC8, PRAC10 and PRAC13 amplified the same alleles that HT156 did in all EST-SSRs studied (Table 6; Fig. 4). These results indicated that these plantlets are clonal to HT156 and were regenerated from a callus which origin was a somatic cell of the anther. The ploidy level and the sexual genotype of this somatic cell would be the same of HT156, tetraploid and *Mmmm,* and therefore the sexual genotype of the tetraploid plantlets would be *Mmmm*. The octoploid plantlets of tPRAC6 and PRAC8 would be *MMmmmmmm* and would have been produced by an endoreduplication. The data obtained for PRAC8 indicated a similar amplification pattern as PRAC3, but in this case only two alleles were obtained for the marker TC5 (167:169) (Table 6; Fig. 4). The absence of 164 bp allele may be due to somaclonal variation produced in the regeneration process. In this case, the origin of the PRAC8 was a somatic cell.

## Discussion

"Lucullus" was the first "all-male" variety of asparagus in the market. The "super-male" used in the development of this variety was originated from the self-pollination of an andromonoecious plant (Boonen 1988). While "Andreas"

**Table 6** Characterization of the plantlets regenerated from anthers belonging to genotype HT156

| Genotype | PM | Ploidy level (Plantlet No.) | Sex | Characterization with EST-SSRs alleles (bp) | | | | Callus origin | Prospected sexual genotype (Ploidy level) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | AAT1 | AG10 | TC1 | TC5 | | |
| HT156 (Mother plant) | – | 4× | Male | 214 | 160 | 222 | 164 | – | *Mmmm* |
| | | | | 216 | 164 | 228 | 167 | | |
| | | | | 217 | 183 | 230 | 169 | | |
| PRAC1 | P5 | 2× (8) | Female | 214 | 160 | 222 | 167 | Microspore | *mm* (2×) |
| | | 4× (7) | | 217 | 183 | 228 | | | *mmmm* (4×) |
| | | 8× (1) | | | | | | | *mmmmmmmm* (8×) |
| PRAC2 | P6 | 4× (16) | Female | 214 | 160 | 222 | 167 | Microspore | *mmmm* |
| | | | | | 183 | 228 | | | |
| PRAC3 | P6 | 4× (16) | Male | 214 | 160 | 222 | 164 | Somatic cell | *Mmmm* |
| | | | | 216 | 164 | 228 | 167 | | |
| | | | | 217 | 183 | 230 | 169 | | |
| PRAC4 | P6 | 4× (16) | Male | 214 | 160 | 222 | 164 | Somatic cell | *Mmmm* |
| | | | | 216 | 164 | 228 | 167 | | |
| | | | | 217 | 183 | 230 | 169 | | |
| PRAC5 | P5 | 4× (16) | Male | 214 | 160 | 222 | 164 | Somatic cell | *Mmmm* |
| | | | | 216 | 164 | 228 | 167 | | |
| | | | | 217 | 183 | 230 | 169 | | |
| PRAC6 | P5 | 4× (14) | Male | 214 | 160 | 222 | 164 | Somatic cell | *Mmmm* (4×) |
| | | 8× (2) | | 216 | 164 | 228 | 167 | | *MMmmmmmm*(8×) |
| | | | | 217 | 183 | 230 | 169 | | |
| PRAC7 | P6 | 4× (16) | Male | 216 | 160 | 222 | 164 | Microspore | *MMmm* |
| | | | | 217 | 164 | 230 | 169 | | |
| PRAC8 | P6 | 4× (4) | Male | 214 | 160 | 222 | –[a] | Somatic cell | *Mmmm* (4×) |
| | | 8× (4) | | 216 | 164 | 228 | 167 | | *MMmmmmmm* (8×) |
| | | | | 217 | 183 | 230 | 169 | | |
| PRAC9 | P5 | 4× (4) | Female | 214 | 160 | 222 | 167 | Microspore | *mmmm* (4×) |
| | | 8× (4) | | | 183 | 230 | 169 | | *mmmmmmmm* (8×) |
| PRAC10 | P5 | 4× (16) | Male | 214 | 160 | 222 | 164 | Somatic cell | *Mmmm* |
| | | | | 216 | 164 | 228 | 167 | | |
| | | | | 217 | 183 | 230 | 169 | | |
| PRAC11 | P6 | 4× (4) | Male | 214 | 160 | 228 | 164 | Microspore | *MMmm* (4×) |
| | | 8× (12) | | 217 | 183 | 230 | 169 | | *MMMMmmmm* (8×) |
| PRAC12 | P5 | 4× (11) | Male | 214 | 160 | 228 | 167 | Microspore | *MMmm* |
| | | | | 217 | 164 | 230 | | | |
| PRAC13 | P6 | 4× (13) | Male | 214 | 160 | 222 | 164 | Somatic cell | *Mmmm* |
| | | | | 216 | 164 | 228 | 167 | | |
| | | | | 217 | 183 | 230 | 169 | | |
| PRAC14 | P5 | 4× (16) | Male | 214 | 160 | 222 | 167 | Microspore | *MMmm* |
| | | | | | 164 | 230 | | | |

[a] The fragment of 164 bp of TC5 was not amplified in the genotype PRAC8, possibly due to the somaclonal variation produced in the process of the regeneration of the plantlets of this genotype

was the first true $F_1$ all-male hybrid (Corriols et al. 1990), in which the "super-male" parental was obtained by anther culture. Others authors also used the anther culture to generate diploid "all-male" commercially interesting hybrids (Ellison and Kinelski 1985; Ellison et al. 1990; Falavigna et al. 1999). As we have mentioned in the introduction, these authors described that the genotype used, together the developmental stage of the microspores and the culture conditions of the anthers, is one of the factors to be in consideration to success in anther culture.

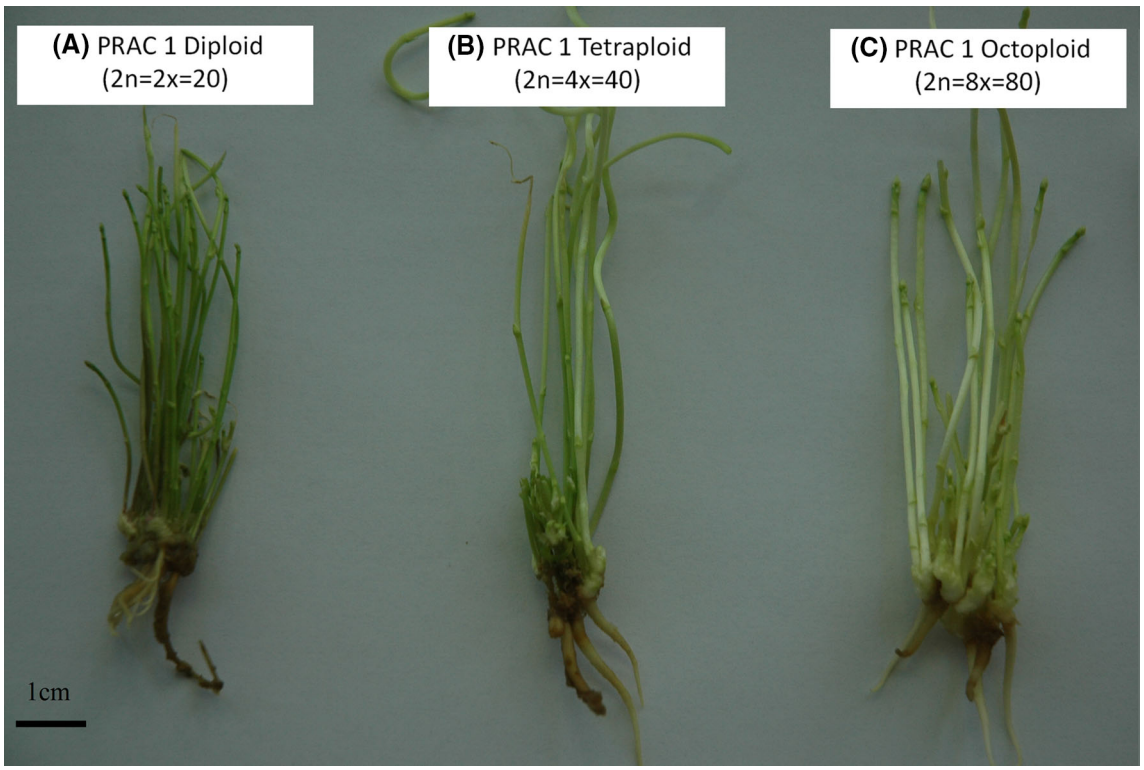



**Fig. 3** Plantlets regenerated from anther culture 1 (PRAC1). **a** Diploid plantlet (2n = 2x = 20). **b** Tetraploid plantlet (2n = 4x = 40). **c** Octoploid plantlet (2n = 8x = 80)

To eliminate the effect of the genotype, anthers from a unique genotype were used in the present study. This allowed the study of the others two factors: developmental stage of the microspores and the culture conditions of the anthers. The genotype selected (HT156) was a tetraploid male selected from the collection of the tetraploid Spanish Landrace "Morado de Huétor".

Several authors reported the late microspore as the best developmental stage of the microspore to succeed on the di-haploid (DH) induction in *A. officinalis* (Qiao and Falavigna 1990; Feng and Wolyn 1991; Zhang et al. 1994; Delaitre et al. 2001). For this reason, different authors have studied the relationship between the flower bud size and the developmental stage of the microspores, to determine the flower bud size in which most of the microspores are in the appropriate developmental stage (Feng and Wolyn 1991; Zhang et al. 1994; Delaitre et al. 2001). These studies were developed using diploid genotypes with non-homogeneous results. Thus, Feng and Wolyn (1991) establish that around 80 % of microspores of the genotype G203 (belonging the variety "Rutgers") between 2.4 and 2.6 mm are late microspores, while Delaitre et al. (2001) establish an appropriate size between 1 and 2 mm in three genotypes of the "Argenteuil" cv. (80 % of late microspore). These results indicated that the appropriate flower bud size to obtain microspores in late microspore is genotype dependent. Therefore, we had to establish the appropriate flower buds size of HT156 before to start with the anther culture. The results obtained in the present study indicated that in this genotype the optimum flower bud size corresponded to flower buds between 2.6 and 3.0 mm length (S4) in which 54.9 ± 28.2 % of the microspores are late microspore (Table 4). Even so to obtain a better study, anthers of the buds flower of S2 (1.5–2.0 mm), S3 (2.1–2.5 mm) and S5 (3.1–3.5 mm), further of anther of the buds flower of S4, were used in the assays of induction of callus in anther.

The success rate obtained in the first assay of callus proliferation in A1 medium (13 ± 4 % for S4) was very similar to percentages obtained in the same culture conditions by Qiao and Falavigna (1990) for their best genotypes (12.8 %). However, this rate is very small comparing with the results obtained in the next assays carried out in this work. The use of a cold pretreatment in the second assay increased the success rate to 50 % for anthers S4, above to 40 and 30 % for anthers S3 and S5, respectively (Fig. 1a). This increasing was remarkable for all anther types containing late microspores, supporting the importance of the developmental stage of the microspores in the success of the anther culture. These results are in agreement with other studies using cold pretreatments to produce the stress necessary to induce the

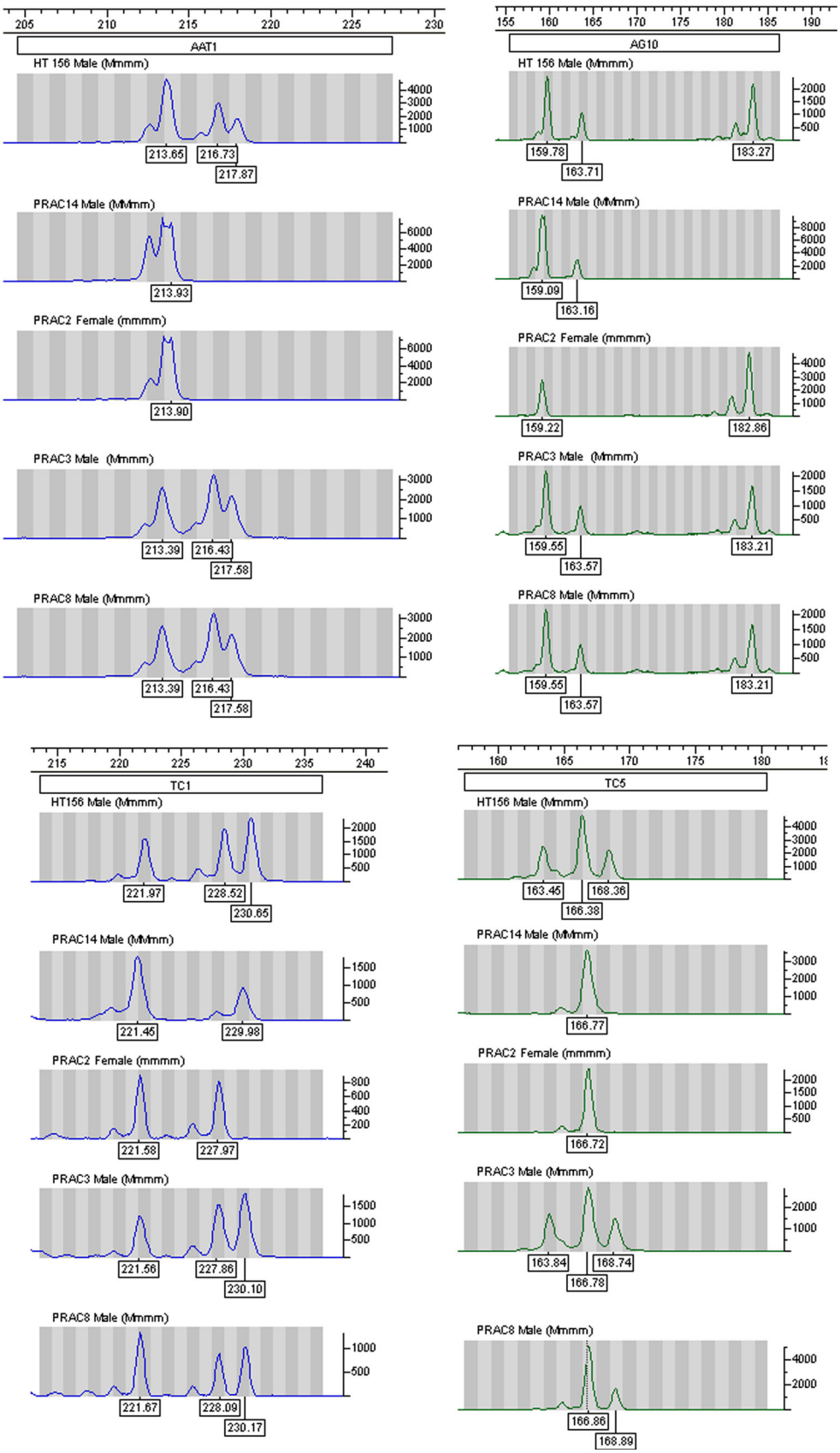



**Fig. 4** Chromatograms obtained in the amplification of the EST-SSRs: AAT1, AG10, TC1 and TC5 in HT156, PRAC14, PRAC2, PRAC3 and PRAC8

symmetric division of the microspore in other species such as tobacco (Heberle-Bors 1983), rice (Cho and Zapata 1988), tangerine (Germana and Chiancone 2003), *Hepatica nobilis* (Nomizu et al. 2004) or wheat (Labbani et al. 2005). In addition, in this second assay two culture media (A1 and MAE) supplemented with a very different composition of growth regulators were used. In spite of this different composition, there were not significant differences between the success rates obtained in both medium. These results suggested the relatively low importance of growth regulators comparing with the stress treatments necessary to reach success on anther culture, as earlier works postulated (Aionesei et al. 2005; Seguí-Simarro 2010). We opted for the MAE medium for the next assays due to the absence of 2,4-D. The auxin 2,4-D is strongly linked to the somaclonal variation production (Nehra et al. 1992; Gesteira et al. 2002; Jin et al. 2008), and the purpose of this work is the development of "super-males" able to maintain the elite agronomical traits of the parental male.

The results obtained changing the incubation temperature, showed that using both MAE and A1 medium, the 32 °C are the optimum temperature to obtain a good callus proliferation rate. The incubation of the anthers in dark conditions during the first 4 weeks after the cold pretreatment resulted in a new increase of success rate in callus proliferation (80 ± 5 % in S4; 42 ± 6 % in S5). Even so, the best success rates were obtained in the third assay carried out (Fig. 1b). The stress produced by incubation at 32 °C induced the symmetric division of microspores in around 90 % of the anthers containing late microspores (S3, S4 and S5). The presence of late microspores in the anthers was sufficient to the success in the callus proliferation, regardless of the proportion of late microspores inside of these anthers. This stress was also used to induce the callus proliferation in anther explants of *A. officinalis* by Wolyn and Feng (1993), obtaining a similar success rate for the diploid genotype G203 (93.2 %) (Feng and Wolyn 1991).

The osmotic stress was the last stress tested to induce the proliferation callus in this work. This stress has been successfully used by other authors in different plant species (Wang et al. 1981; Supena et al. 2006). Nonetheless, our work showed that the osmotic stress supposed a decrease of the success rate, especially in anthers S3 and S5 (Fig. 1c), so we discard the use of osmotic stress to induce callus proliferation inside anther explants of asparagus.

After the different assays carried out, we can conclude that the better culture conditions to induce the callus proliferation in the anthers of HT156 was the incubation at 32 °C in dark during 4 week, after a pretreatment at 4 °C during a week and followed by incubation at 25 °C during other 4 week in standard light conditions.

Different authors have developed protocols for regeneration of plantlets from the callus produced in the anther culture. Some of them used different media for the different steps of the regeneration: callus proliferation, induction, maturation and germination of the somatic embryos obtained from these callus (Feng and Wolyn 1991, 1993; Wolyn and Feng 1993). Others (Qiao and Falavigna 1990) chose protocols with a first medium to induce callus proliferation and shoot organogenesis, and a second medium to root the material regenerated from calli. With the aim of simplify as much as possible the plantlet regeneration different proliferation media (PMs) were tested in order to select a PM in which the callus proliferation occurs together with the shoot regeneration. We have combined different hormones (pCPA, 2,4-D, BA, KIN) in the composition of the PMs. The combination of these hormones has been used previously in different publications to induce the callus proliferation of asparagus (Benmoussa et al. 1996, 1997) and even the PM4 is identical to medium used by Feng and Wolyn (1993). Nevertheless, our results (Fig. 2; Table 5) indicated that only the combination of pCPA and BA (PM5 and PM6) induce the shoot regeneration together with the callus proliferation, there are shoot regeneration in the 46.6 and 57.1 % of calli cultured in PM5 and PM6 respectively, even some of these shoots are rooted during the incubation in this PM media. So, the use of the PM5 and PM6 in the anther culture supposes an important advantage in plantlet regeneration, shortening the time needed and the number of subcultures, reducing the possibilities of somaclonal variation (Bairu et al. 2011). The rooting of the unrooted shoots in the PMs media, the multiplication of the PRACs and the acclimatization of plantlets reach high success rates following the methods previously developed for our group (Carmona-Martin et al. 2014; Regalado et al. 2015a).

As mentioned in the introduction, one of the drawbacks of anther culture methods is the recovery of heterozygous males, which are not "super-males" (Falavigna et al. 1983; Riccardi et al. 2011). This makes very important the characterization of the plantlets regenerated from anther culture (PRACs), especially the determination of the callus origin from which the plantlets have been regenerated. The PRACs developed in this work have been characterized considering different aspects (ploidy, sex and EST-SSRs) to know if the males recovered were "super-males" (Table 6).

The microspores of the tetraploid genotype HT156 are diploid, so the PRACs originating from these microspores should be diploids. However, we only detected diploid plants ($2n = 2x = 20$) among the plantlets belonging to the PRAC1, while the other plantlets analyzed were tetraploid ($2n = 4x = 40$) or octoploid ($2n = 8x = 80$). Furthermore, different ploidy levels in the plantlets

regenerated from the same anther were detected. The regeneration of tetraploid and octoploid plantlets from diploid microspores, as well as the mix of ploidy levels, can be possible due to the high endoreduplication rate occurring in the callus culture in *A. officinalis* (Kunitake et al. 1998; Raimondi et al. 2001; Pontaroli and Camadro 2005; Regalado et al. 2015b), also described in the anther culture of *A. officinalis* (Feng and Wolyn 1993; Wolyn and Feng 1993; Shiga et al. 2009). Other important result obtained in this ploidy analysis is the increase of the spear diameter of the plantlets associated with ploidy level (Fig. 3). A bigger diameter of spears of polyploid plants respect to the original plants has been previously described in asparagus by different authors (Braak and Zeilinga 1957; Kunitake et al. 1998; Carmona-Martin et al. 2015; Regalado et al. 2015b).

The second question analyzed was the sex of the plantlets regenerated, using the sex-linked marker Asp1-T7 (Table 6). All plantlets regenerated from the same anther were catalogued with the same sex: 3 PRACs female and 11 PRACs males. There are no males among the plantlets of the PRACs characterized as females. This indicates that a single female microspore *(mm)* carried out the symmetric division originating the callus from which the plantlets of this PRAC were regenerated. The possibility that each of the eight plantlets analyzed comes from the symmetric division of eight independent female microspores is statistically insignificant.

Finally, the PRACs were characterized with four different EST-SSRs (AAT1, AG10, TC1 and TC5) selected by amplify at least three alleles in the tetraploid genotype HT156. Castro et al. (2013) indicated that EST-SSRs could amplify fragments until four different sizes in a tetraploid genotype. Each size of the fragments amplified by one EST-SSR can be treated as an allele and these alleles are inherited as Mendelian characters (Litt and Luty 1989). So the diploid microspores of the tetraploid HT156 may only receive during their formation through meiosis a maximum of two different alleles for each EST-SSR. The results of this characterization allowed us to determine the origin of the callus from which the different male PRACs were regenerated. The PRACs originated from somatic cells amplified the same fragments than HT156 in each EST-SSR while the PRACs originated from male microspore amplify a fragment of two sizes as maximum for each EST-SSR. Our results indicated that seven PRACs were regenerated from somatic cells (*Mmmm*) and four from male diploid microspores (*Mm*). As expected the female PRACs presented a maximum of two alleles in each EST-SSRs, since their origin is a female microspore. All plantlets of each PRAC with origin in male microspores amplify fragments of the same sizes, supporting the idea that the callus is originated from a single microspore. It is important to carry out this characterization with different EST-SSRs, since the plantlet regeneration from callus can induce somaclonal variation in some EST-SRRs (Regalado et al. 2015b) and could make confusing the origin of the PRAC. This is the case of PRAC8, TC5 suggested that the origin of PRAC8 is a microspore, but the others EST-SSRs indicated that the origin of PRAC8 is a somatic cell (Table 6; Fig. 4).

As far as we know, this work is the first in which the EST-SSRs have been used to determine the origin of the regenerated plantlets from anther culture in *A. officinalis*. Eimert et al. (2003) have used RAPDs with the same purpose. We opted by the use of EST-SSRs in this work because in our opinion it is difficult differentiate if the change in the pattern obtained by Eimert et al. (2003) is due to the homozygosis of the plantlets recovered from the anther culture or to the somaclonal variation induced during the plantlet regeneration from callus. This somaclonal variation has been detected with RAPDs (Raimondi et al. 2001), producing a changes similar to the obtained by Eimert et al. (2003). The EST-SSRs have been used to determine the origin of the regenerated plantlets from anther culture in different species such as rice (Lapitan et al. 2009), apple (Höfer et al. 2002; Vanwynsberghe et al. 2005; Okada et al. 2009), corn (Aulinger et al. 2003), soy (Rodrigues et al. 2004), potato (Veilleux et al. 1995; Chani et al. 2000), coconut (Perera et al. 2008), orange (Cao et al. 2011) or eggplant (Salas et al. 2011).

After the characterization of the PRACs we concluded that 50 % of the PRACs (7 of 14) are regenerated from microspores (four from male microspores and three from female microspores). These plants are not homozygous, since they come from a diploid microspore non homozygous. Nevertheless, the tetraploid and octoploid plants obtained are di-diploid and tetra-diploid respectively, having a maximum of two alleles for each character, instead of the four and eight that can be present in the natural tetraploid and octoploid genotypes. The plants obtained could be used as parental lines to produce more homogeneous varieties of the landrace "Morado de Huétor", due to with anthers culture the variability present in this line was reduced. To obtain DH belonging to "Morado de Huétor" it would be necessary to repeat this process from the di-diploid males obtained in this work. With respect to the utility as "super-males" of these di-diploid males (*MMmm*) and tetra-diploid males (*MMMMmmmm*), the first type of males would generate a ratio male:female of 5:1 (83.3 %) and the second type of male a ratio male:female of 69:1 (98.6 %). The tetra-diploid males (*MMMMmmmm*) can be considered as "super-males". So the objective of this work, the development of "super-males" of the tetraploid "Morado de Huétor" landrace, has been achieved.

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