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Induction of *In Vitro* Androgenesis in *Asparagus officinalis* L., *A. maritimus* (L.) Miller and in a Few Bred Diploid and Tetraploid Lines Obtained from Asparagus Interspecific Crosses

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ABSTRACT

Researches on androgenic plant production were carried out for two years in different male bred materials of asparagus. Four types of genetic materials were used as donors:

1) two all male hybrids of *A. officinalis* L., one diploid (2n) and one tetraploid (4n);

2) A tetraploid line called "OMAO" having genetic background {*A. officinalis* x [(*A. officinalis* x *A. maritimus*) x *A. acutifolius* L.]};

3) A tetraploid line called "96/1" originated from [*A. maritimus* x (*A. officinalis* x OMAO)]; 4) nine 2n lines obtained by crossing a few diploid androgenic lines derived from OMAO with different diploid androgenic clones of *A. officinalis*. The best anther culture response was observed in the line 96/1 which produced, in the year 2016, 194 androgenic embryoids per 100 cultured anthers; OMAO and the two all male hybrids of *A. officinalis* gave discrete results (from 6 to 44 %), whereas all other materials gave scarce (0.5-1.8 %) or no results. Three novelties were obtained from the overall study on the above vegetable material:

a) The possibility to produce several embryoids from microspore culture which were isolated from a single anther after removing at least one embryoid.

b) A discrete production (5.93 %) of androgenic calli giving plantlets from cultured anthers of *A. maritimus* (L.) Miller;

c) Most of calli made of vacuolated cells and issued from the internal part of the anthers, previously supposed to be of somatic origin, resulted androgenic.

Introduction

The genus Asparagus includes 240 species of herbaceous perennial and tender woody shrubs and vines, among which there is garden Asparagus (*Asparagus officinalis* L.) which is cultivated for its edible shoots. *A. officinalis* is one of the most economically important horticultural crops worldwide. In the genus, the different species form a polyploid series with 2n, 4n, and 6n somatic number of chromosomes (n=10). They exhibit hermaphrodite plants, with complete and perfect flowers, or dioecious, with male and female flowers on distinct individuals as in *A. officinalis* [diploid (2n), and tetraploid (4n)], *A. maritimus* (4n), *A. acutifolius* (4n), *A. prostratus* (4n), etc..Breeding activity carried out in allogamous plants always needs to recombine new desired traits and quickly fix them in homozygous condition for an immediate use in hybrid development. In *A. officinalis* this need is even greater since this species shows very reduced genetic variability Mercati, et al. [1]. Thus, for an innovative breeding plan, it is necessary to develop genetic

backgrounds containing a wider range of genetic variability from which it would be possible to draw and obtain new recombinants.

The development of new genetic backgrounds coming from the introgression of many genomes of different species, might be a basic target, not only for a new breeding activity but also for advanced molecular studies Falavigna, et al. [2,3]. The application of the anther culture technique, appears in this context an obligate way for the production of recombined homozygous lines since microspores are the first and direct result of genetic recombination and because *A. officinalis* is a dioecious species. In garden *Asparagus*, androgenesis was described for the first time in 1972 by Cateland, et al. [4-6]. Further results were reported by Doré, et al. [7-16]. Peng and Wolyn published interesting data on the obtainment of haploid plants from direct culture of isolated microspores. Later, Falavigna, et al. [2] tried to apply the in vitro anther culture technique to *A. maritimus* and to a few new materials coming from the interspecific 4n cross OMAO {*A. officinalis* 4n ♀ x [(*A. officinalis* 4n x *A. maritimus* 4n) ♀ x *A. acutifolius* L. 4n ♂] ♂}. The accomplishment of other crosses vs the 4n *A. officinalis* "Violetto d'Albenga" led to the production of highly heterozygous 4n genotypes. Their submission to anther culture produced new di-haploid (DI, 2n) recombinants, which were described by Falavigna, et al. [2]. Their androgenic response, however, resulted rather low, hence we planned a few experiments of androgenesis focused to:

1) Improve the yield of androgenic embryos obtainable from bred interspecific materials of Asparagus.

2) Induce the growth of many embryoids from microspores removed from single anthers that previously responded to androgenesis.

3) Extend the application of possible improved protocols (obtained from first and second aim) to other species of the genus *Asparagus* including *A. officinalis*.

Materials and Methods

Anther Donors

Two all-male *A. officinalis* hybrids [one 2n (H884) and one 4n (AM914)], ten male heterozygous genotypes (eight 2n and two 4n) originating from different interspecific crosses, and one male plant of a wild *A. maritimus* (4n) were used as the anther donors. A list of these genetic materials, together with their ploidy level and genetic background, is reported in Table S1 of the "Online Resource".

Anther Culture, Plant Development and Plant Multiplication Media

The composition of the media used in this work is reported in Table 1. The medium "A2", previously described by Qiao, et al. [11] and routinely used from 1975 to 2015 to induce androgenesis in asparagus anthers (Dr. Agostino Falavigna, personal communication), was used as the control in all the experiments. The pH of all the media was adjusted to 5.8 with a 1 M aqueous solution of KOH. When 5g^{-1} of active charcoal were added, pH was later adjusted to 5.8 with a 1 M aqueous solution of HNO_3. Plant Agar (Duchefa Biochemie product no. P1001.1000) was added to all media at 6.0g l^{-1} after pH adjustment and before autoclaving at 118 °C for 20 min.

Table 1: Growth regulator and sugar composition of the media (all solid) used in the experiments of *in vitro* androgenesis.

Component	MEDIA				
	A2[a]	A3[b]	A4	N85[c]	N102[d]
2,4D (mg l^{-1})	0.5	–	0.5	–	–
6BA (mg l^{-1})	0.5	1	0.5	0.1	0.1
NAA (mg l^{-1})	0.1	2	0.1	0.2	0.2
Kinetin (mg l^{-1})	–	–	–	0.2	0.1
Ancymidol (mg l^{-1})	–	–	–	0.1	0.5
Glucose (g l^{-1})	20	–	–	–	–
Saccharose (g l^{-1})	20	60	35	30	60
Sorbitol (g l^{-1})	–	–	25	–	–

The basal formula used for all media was: MS Murashige, et al. [19] salts + vitamins [thiamine HCl + pyridoxine HCl + Ca pantothenate + nicotinic acid (each one at 1mg l^{-1}) + biotin (0.01 mg l^{-1})] + glycine 1mg l^{-1} + myo-inositol 100mg l^{-1}. When required, 5g l^{-1} of active charcoal were added to N102 medium before pH adjustment.

Abbreviations: 2,4D = 2,4-Dichlorophenoxyacetic acid; 6BA = 6-Benzylaminopurine; NAA = α-Naphthaleneacetic acid.

Note:

a. Growth regulator and sugar composition of the medium A2 was defined by Cateland [4], reported later also by Pelletier, et al. [5] and Doré [7].

b. According to Peng , et al. [15,16] formula.

c. Medium used to grow androgenic calli/embryoids into plants and, subsequently, multiply them Riccardi, et al. [17].

d. Five g l^{-1} of active charcoal were added to the medium N102 only in the last step of plant development (*in vitro* induction and formation of strong plant roots and stems, before transplanting to the soil – Riccardi, et al. [17].

Anther Culture Experiment Management

Flower buds were collected when microspores reached the early-middle uninucleate stage; their size and shape varied depending on their genetic background (being in general tetraploids lightly bigger than diploids). They were sterilized in a 0.6% sodium hypochlorite aqueous solution for 15-20 min, then rinsed three times in sterile water and placed on solid media inside sterile Petri dishes Ø 90 mm. All cultures were incubated under gro-lux light from fluorescent lamps (approximate photon flux density: 25µmol m^{-2} s^{-1}) for 16 h at 23/21°C (day/night). Two independent series of experiments were carried out. The *first series* was performed in 2016 by cultivating 15,322 anthers, removed from the flowers of twelve genotypes out of the thirteen listed in Table S1 (*A. maritimus* was excluded). It was addressed to explore preliminarily (without replications) possible large effects of four "*flower preculture → anther culture*" pathways (Table 2).

Table 2: "Flower preculture → anther culture" pathways applied to the first series of experiments in asparagus anther culture carried out in 2016 (related results are reported in Table 3).

Pathway Type	Pathway Description
Control	Flower preculture on A2 medium → anther removal and culture on fresh A2 within 8 hours
A	Flower preculture on "H_2O + Agar" for 2 days → anther removal and culture on A2
B	Flower preculture on A2 for 6 days → anther removal and culture on N85
C	Flower preculture on A2 for 2 days → anther removal and culture on A2 + Adenine SO_4 (60mg l^{-1})
D	Flower preculture on A2 for 1, 2, 3, 5, 7, 9 days → anther removal and culture on A2

Table 3: Results obtained in asparagus anther culture in preliminary tests performed in 2016 (first series of experiments). Total number of anthers cultured: 15,322. Total number of calli/embryoids obtained: 1,416. The pathway A, applied to the genotypes "96/1" and "116/1", yielded 12.44 and 0.00% of androgenic calli/embryoids, respectively; The pathway B yielded 0.00% of androgenic calli/embryoids in all the genotypes where it was applied (45/5, 116/1 and AM914). Preculture of flower buds on A2 → removal and culture of anthers on A2 within 8 hours was used as the control.

Genotype	Total no. of Anthers cultured	Total No. of Calli + Embryoids obtained	"Flower precult. → Anther cult." pathways applied (see M&m)	Callus / Embryoid Yield in the control (%)	Best Flower Preculture Treatment (callus/Embryoid yield %)	Calli / Embryoids Developing to plants (%)
37/1	340	4	Control	1.18	–	75.0
45/5	1435	1	Control, B, D	0.07	Control (0.07)	0.00
56/1	675	0	Control, D	–	–	–
96/1	1072	845	Control, A, D	77.00	D = 41 hours (194.02)	59.2
103/1	776	0	Control	–	–	–
116/1	1679	21	Control, A, B	1.25	Control (1.25)	66.7
130/2	630	0	Control	–	–	–
145/1	3575	43	Control, C, D	1.20	Control = C = D D = 5 days (1.20)	32.6
149/2	1440	3	Control, D	0.21	Control (0.21)	100.0
OMAO1/5	1235	83	Control, D	6.72	Control = D D = 2 days (6.72)	67.5
H884	1465	287	Control, D	6.66	D = 3 days (39.23)	85.7
AM914	1000	129	Control, B, D	4.20	D = 9 days (44.49)	75.2

Table 4: Results obtained in the second series of experiments carried out in 2017 with the genotypes 96/1 (Table 4A) and OMAO1-5 (Table 4B), and with a male plant of wild *Asparagus maritimus* Mill. (Table 4C). The total number of cultured anthers was 4,446. In each experiment, the type of medium used for flower preculture was used also later for another culture. "Percentage of responsive anthers" and "Number of embryoids per responsive anther" are the two components of "Androgenic embryoid yield" (see M&m).

Table 4A. 96/1

Factors Examined	Androgenic Embryoids (%) (C.V.: 9.37%)	P level
Best flower preculture time	69.5 (0 days) > 13.3 (2 days) and 12.9 (7 days)	0.0001
Best medium	46.1 (A3) > 17.7 (A2)	0.0068
Best combination	104.4 (0 days on A3)	
Factors Examined	**Responding Anthers (%) (C.V.: 5.26%)**	**P level**
Best flower preculture time	31.7 (0 days) > 4.7 (2 days) and 6.1 (7 days)	0.0000

Best medium	17.6 (A3) = 10.8 (A2)	ns
Factors Examined	**Andr. Embryoids / Respond. Anther (no.) (C.V.: 16.16%)**	**P level**
Best flower preculture time	2.08 (0 days) = 2.45 (2 days) = 2.10 (7 days)	ns
Best medium	2.91 (A3) > 1.50 (A2)	0.0390

C.V. = Coefficient of variation; ns = not significant.

Experiment design: (0; 2; 7 days) x (A2; A3) x 3 replications = 18 data cases x 60 anthers = 1,080 anthers.

Main results: androgenic calli/embryoids produced = 344; developing to plants 195 (56.7%).

Table 4B: OMAO1-5

Factors Examined	Androgenic Embryoids (%) (c.v.: 48.60%)	P level
Best flower preculture time	16.7 (6 days) = 10.5 (0 days)	Ns
Best medium	18.4 (A2) = 12.8 (A3) = 9.6 (A4)	Ns

Experiment design: (0; 6 days) x (A2; A3; A4) x 3 replications = 18 data cases x 97 anthers = 1,746 anthers;

Main results: androgenic calli or embryoids produced = 236; developing to plants 154 (65.3%).

Table 4C: *A. maritimus* (L.) Mill.

Factors Giving Response	Androgenic Embryoids Obtained
Best response	1day x A3 = 16 embryoids (5.93 embryoids / 100 anthers = 5.93 %)
2nd combination	1day x A2 = 2 embryoids (0.74 %) → one of which regenerated plants
3rd combination	5 days x A3 = 1 embryoid (0.36 %)

Experiment design: (1; 5; 7 days) * x (A2; A3) x 3 replications = 18 data cases x 90 anthers = 1,620 anthers;

Main results: androgenic calli produced 19; calli regenerating plants 10 (52.6%).

* In all experiments it was impossible to apply the treatment "0 days" of flower preculture so we considered the "1 day" as the control.

The second series of experiments was carried out in 2017 by cultivating 4,446 anthers removed from the male genotypes 96/1, OMAO1-5 and from a male plant of wild *A. maritimus* Mill. It was addressed to evaluate the effect of three "preculture → culture media" pathways (on the media A2, A3 and A4 – Table 1) at different times of preculture (from 1 to 7 days depending on the genotype tested). In all the experiments of the second series the type of medium used for flower preculture was also used later for anther culture. The experiments were carried out by applying a "two factor randomized complete block design". Experiment details for each genotype are reported with results in Table 4. The control test used in the first series of experiments (Table 2) was also applied to the second one.

Culture of Isolated Microspores Extracted Per Single Anther

The whole lot of "micro-embryoids and induced microspores", still present in one anther after the removal of the first visible androgenic embryoid/s, was extracted by using a scalpel tip and immediately placed and kept on fresh solid N85 medium until embryoids were developed. This new test was done in 2016 in the genotypes 116/1 and OMAO1-5. As soon as embryoids were grown enough, they were singly subcultured on N85 fresh medium for plant development. In 2017 this technique was repeated and applied to all genotypes.

Anther Derived Calli/Embryoids

Any callus or embryoid clearly issuing from the inside part of the anther, was removed and placed on N85 fresh medium for plant regeneration/development. Plantlets were further multiplied once or twice in the same medium and finally transferred to N102 to form stronger roots and stems according to Riccardi, et al. [17]

Ploidy Analysis of the Regenerated Plants

Very small pieces of green tissues were collected from plantlets regenerated from anther derived calli or embryoids and submitted to ploidy analysis through the use of a flow cytometer (Sysmex CyFlow Space) with 532nm laser light source and a parameter of orange–red fluorescence emission (> 590nm). The ploidy determination was done by applying the chopping method treated with CyStain® PI absolute P-kit (Sysmex, art. No 05-5002).

Statistical Analysis of the Data

Statistical analysis was applied to the data obtained from the anther culture of the genotypes 96/1 and OMAO1-5 used in the 2nd series of experiments. The normality condition of the data distribution was checked by applying the skewness and kurtosis test (https://www.wessa.net/rwasp_skewness_kurtosis.wasp), for each one of the variables examined (Androgenic embryoid yield %, Responding anthers %, No. of Androgenic embryoids / No. of responding anthers). "Percentage of responsive anthers" and "Number of embryoids per responsive anther" are the two components of "Androgenic embryoid yield". Transformed data

and those already normally distributed were submitted to ANOVA analysis [Two factor complete randomized block design model (MSTATC software)], in order to evaluate, for each variable, the effect (P value) produced by three factors: replication (effect not shown in Tables), flower preculture time and culture medium. The data obtained from the anther culture of *A. maritimus* were not submitted to ANOVA analysis (see the Results chapter). "Duncan's multiple range test" was applied to rank means (only when they were more than two in a variable) and compare their difference values at a significance levels for $P \leq 0.05$.

Results

Calli/Embryoids Obtained by Anther Cultures and Capacity To Regenerate/Develop Plantlets

Between 30 to 100 days after culture, responding anthers revealed their androgenic products issuing from a split generated through the anther wall. Already before or, sometimes only after removal of these structures, it was possible to see clearly the anther split from which they grew out (Figure 1a). This feature was always used as a key proof to define if a callus/embryoid produced from the anther was of androgenic origin or not. Other calli or structures of doubt origin were not considered androgenic (Figure 1b). The visual aspect of the androgenic products (Figure 1c) ranged from callus-like (made of vacuolated cells) to embryo-like structures (embryoids) depending in part on the donor genotype but above all on the year in which flowers were collected. The anthers of 96/1 and OMAO1-5 put in culture in 2016 produced mostly embryoids and very few calli. Those collected in 2017 from 96/1 (the same plant) produced much less of not well formed embryoids and mostly calli having highly vacuolated cells. The response of OMAO1-5 in 2017 was even worse producing almost all calli and very few embryoids (data not shown). The androgenic products of *A. maritimus* anthers, cultivated in 2017, were only callus-like structures made by vacuolated cells.

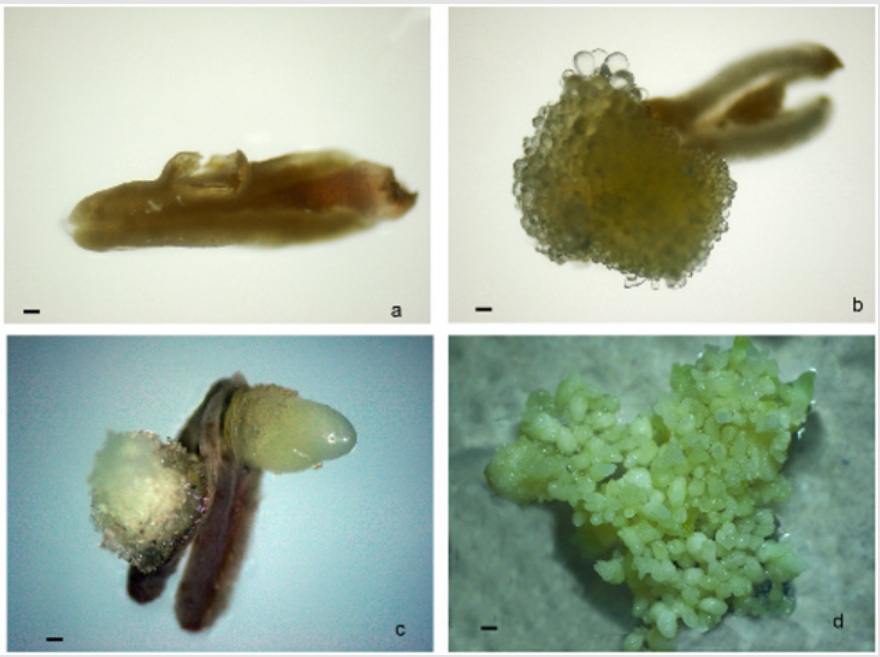

Figure 1: Asparagus anthers on culture medium and calli /embryoids. Scale bar 100 µm

a. Asparagus anther on culture medium after removal of an androgenic embryoid.

b. Anther callus of doubt origin.

c. Two different androgenic structures produced on the same anther. The left callus-like is made of vacuolated cells, the right embryo-like structure is an embryoid.

d. Micro-embryoids developed from isolated microspores extracted from a single anther after the removal of an androgenic embryoid.

First Series of Experiments

The results obtained in 2016 are summarized in (Table 3). Only three genotypes (96/1, H884 and AM914) out of eight submitted to the D pathway, produced visible superior results compared to the control. The C pathway, tested only in the genotype 145/1, gave comparable results to the control, whereas the pathways A (applied to the genotypes 96/1 and 116/1) and B (applied to the genotypes 45/5, 116/1 and AM914), produced lower or no responses.

Second Series of Experiments

The results obtained in 2017 from the second series of experiments are summarized in Table 4. In the genotype 96/1, highly significant differences in embryoid yield values were observed between the treatments of both preculture times and media. This result allowed to identify the optimal combination of treatments (preculture 0 days x medium A3) giving the highest yield of androgenic embryoids (104.4 %). A similar trend was

also lightly sketched in the genotype OMAO1-5 even if differences between means resulted not significant. In *A. maritimus*, only three combinations of treatments out of the eighteen tested gave a response. Considering the strong superiority of the combination "1-day x A3" in comparison to the others, we avoided to perform any statistical analysis for this genotype.

Culture of Isolated Microspores Extracted Per Single Anther

The whole lot of micro-embryoids and induced microspores of both 116/1 and OMAO1-5 (tested in 2016) produced embryoids within 120 days (Figure 1d). Around 90 embryoids could be isolated from 116/1 and 310 from OMAO1-5. Among all the genotypes analyzed in 2017 only the content of one anther of the genotype 96/1 was able to grow into embryoids and plants whereas all others did not develop into anything within 120 days.

Ploidy Level of The Regenerated Plants

The results of ploidy level obtained from flow cytometer analyses are summarized in Table 5. Plant samples were distinguished based on related anther donor and on the type of medium used for anther culture (A2 or A3).

Table 5: Ploidy level surveyed on a small green piece of tissue taken from regenerated plants (each plant from one androgenic callus/embryoid) derived from asparagus anther culture carried out in 2016 and 2017. The androgenic plantlets obtained from the genotypes 45/5, 145/1, H884 and AM914 have not been analyzed yet. The genotypes 56/1, 103/1 and 130/2 did not produce any androgenic calli/embryoid. The plant issued from one androgenic callus of *A. maritimus* induced on A2 medium has been lost.

Anther donor (related ploidy level)	Anther Culture Medium Used	No. of Regenerated Plants Analyzed	Regenerated Plants (%)			
			n	2n	4n	> 4n, others
OMAO1/5 (4n)	A2	67		49.1	47.4	3.5
OMAO1/5 (4n)	A3	17		76.5	23.5	
37/1 (2n)	A2	2		100.0		
96/1 (4n)	A2	190		30.0	60.0	10.0
96/1 (4n)	A3	12		50.0	50.0	
116/1 (2n)	A2	32	3.1	81.3	15.6	
149/2 (2n)	A2	3		100.0		
A. maritimus (4n)	A3	8		25.0	62.5	12.5

Additional Observations

By a general view given to the anther cultures during incubation, it was possible to observe that the anthers of the low responding genotypes underwent to an apparent very early degeneration in comparison to those of high responding genotypes. Just after 30 days of culture, the anther wall of the recalcitrant genotypes became brown and the inside microspores had loosed their vitality.

Discussion

By applying heat treatment (32 °C) to the anthers and cold treatment (4 °C) to flower buds, Feng, et al. [12,13] Peng, et al. [15,16] increased remarkably the yield of androgenic embryos. Moreover, the addition of ancymidol to the liquid media, used to develop androgenic embryos to plants Feng, et al. [12] gave another important improvement to the plant regeneration, the last step of the technique. Unfortunately, those procedures have been studied and set up each one in only very few genotypes of *A. officinalis* whereas, for breeding purposes, their effectiveness should be exploited in a very wide range of different genotypes. Despite our efforts were done on a rather large number of genotypes repeated for two years, the number of materials that are unable to respond, still remains high. A first positive finding from the 2016 experiments was the very high androgenic response of the 96/1 anthers (194% from the combination "41 hours of flower preculture on A2 followed by anther culture on A2"). Another interesting finding, obtained from the second series of experiments (carried out in 2017), was the general higher androgenic yield produced on the medium A3 (Table 4) in comparison to that obtained on A2 (in two out of the three tetraploid genotypes tested 96/1, OMAO1-5, *A. maritimus*).

Now it should be interesting to verify if A3 medium will be more effective also in the *A. officinalis* genetic background deriving materials. All the anther-derived calli having highly vacuolated cells, obtained in our lab before 2015, were discarded because we thought they could be of somatic origin. Since 2016 we examined better their origin: at the time of removal from the anther we checked for the presence of a split visible on the anther wall and, later, we examined the ploidy level of related regenerated plantlets through the flow cytometer analysis. We could realize that more than 50% of these calli (coming from the inside part of the anther by splitting the anther wall) had a halved number of chromosomes (2n) in comparison to their anther donors (4n). This result showed us that also these types of calli (beyond the embryoids) were of androgenic origin. Finally, the very strong year effect on the androgenic response of the genotype 96/1 to different flower preculture applications (Table 3) compared to those of (Table 4) for this genotype, was an unexpected finding. A similar effect (again depending on the different year of anther culture and again unexpected) was also observed on the much higher percentage

of calli than embryoids, obtained in both A2 and A3 in 2017 in comparison to those of 2016. Both these results confirm that the environment where plant donors are grown is determinant for androgenesis response.

Culture of Isolated Microspores Extracted Per Single Anther

Foisset, et al. [18,19] demonstrated that when the number of androgenic events is low (this occurs normally in androgenesis), we obtain an androgenic plant population whose distribution of genetic variability is distorted in comparison to that potentially generated by the anther donor genotype when submitted to several selfings. The influence exerted by the in vitro conditions on the cultured anthers interacts also with the singular different genetic backgrounds of microspores, generating many different and substantial responses. This problem could be theoretically overcome if we are able to obtain androgenic plants from all the microspores present inside an anther. As a result of numerous observations made on our anther cultures of asparagus, we realized that below the main visible callus/embryoid splitting the anther wall, often there were many micro-embryos and hundreds microspores that, most probably, were induced to develop new individuals. This gave us the idea to remove and cultivate them on solid medium N85, as we routinely did for normal androgenic embryoids.

Our results showed that such a technique worked in a chance way (deeper investigations are needed), however, in our positive response, the number of embryoids obtained had been really very high (310 in OMAO1-5, (Figure 1d). The range of genetic variability present in the population of the plantlets obtained from these embryoids has not yet been examined but, it is likely to think that a possible distortion of variability segregation, in this case, would be much less high than that we could obtain from the use of a conventional anther culture technique. Anyhow, considering the great potentiality of this technique, we think that it might be extremely important for the future of androgenesis, even more than that of isolated microspore culture extracted from many anthers where the percentage of individuals obtained is generally still too low.

Androgenesis in *Asparagus maritimus* Miller

The obtainment of a few androgenic embryoids able to develop to plant was described for the first time in this tetraploid species by Falavigna, et al.[2] Unfortunately, the information reported in this issue are too limited and, after this, no further works have been published. In our experiments we could obtain nineteen calli (no embryoids), ten of which were able to regenerate plantlets. This number is not so high, but we should consider that these calli were mostly obtained from only one combination of treatments (preculture 0 days x medium A3) which yielded 5.93% (Table 4). Moreover, two of them regenerated di-haploid (2n) plants (Table 5), confirming their androgenic origin. As in *A. officinalis*, also androgenic callus of *A. maritimus* grows until splitting the anther wall, becoming visible and removable to be subcultured on N85 medium for plant regeneration and multiplication.

Ploidy Level of Androgenic Plants

Results of ploidy analysis are in line with those of previous Authors. As previously reported by Peng, et al. [15,16] also in our work the absence of 2,4D in the medium (A3 medium) seems to lower in general the tetraploid/diploid plant ratio obtained by anther culture (Table 5). This could be a very positive aspect because the obtainment of diploid androgenic plants is preferred than tetraploids when we apply anther culture on 4n materials.

Conclusion

The best anther culture response was observed in the line 96/1 which produced, in the year 2016, 194 androgenic embryoids per 100 cultured anthers. Discrete results (from 6 to 44 %) were also obtained in the 4n genotype OMAO1-5 (derived from interspecific crosses) and in two *all-male* hybrids of *A. officinalis* (H884 2n and AM914 4n). Unfortunately, the advances obtained with the new technique, did not allow all other materials to give a better response than the scarce one (0.5-1.8 %, or no results) obtained before this study. Anyhow, three novelties were obtained from the overall work:

a) The possibility to potentially produce several embryoids from microspore culture which were isolated from a single anther after removing at least one embryoid

b) A discrete production (5.93 %) of androgenic calli giving plantlets from cultured anthers of *A. maritimus* (L.) Miller

c) Most of the calli made of vacuolated cells and issued from the internal part of the anthers, previously supposed to be of somatic origin, resulted androgenic.

Key Message

In garden asparagus, the application of the anther culture technique to produce double haploids (DH) is the main way to obtain pure lines for hybrid development. In this manuscript we present the results of a study focused to set up an androgenesis protocol to be applied to interspecific and wild asparagus genotypes.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author Contribution

All the Authors have contributed equally to this work.

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