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Prospects of Isolated Microspore Culture for Haploid Production in *Anemone coronaria* L.

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ABSTRACT

The aim of this study was to establish a procedure to obtain haploid plants from microspore cultures of *Anemone coronaria* L., an important ornamental crop known worldwide due to its commercial value in the cut flower industry. Microspores were isolated from two genotypes of *A. coronaria*: 'Blue' (plants obtained through one cycle of selfing) and 'Lilac'. The effect of different treatments to interrupt the gametophytic development of microspores and promote sporophytic development was evaluated. High temperature, culture media composition and developmental stage of microspores at the moment of isolation were the assessed factors. Achieved microspore-derived embryo formation was 0.53% for 'Blue' and 0.06% for 'Lilac'. Different treatments were tested for microspore-derived embryo germination. Organic supplements had a positive effect on triggering germination, while growth regulators were needed to complete the development of the plantlets. Germination percentage was 2.13 and 2.41 for 'Blue' and 'Lilac', respectively. The ploidy analysis revealed the existence of haploid and doubled haploid plants of both genotypes. We identified 18 haploid plants and 9 doubled haploid plants of 'Blue', and 4 haploid plants and 3 doubled haploid plants of 'Lilac'.

Keywords: *Anemone coronaria* L., embryogenic response, gametophytic development, microspore-derived embryos, sporophytic development
Abbreviations: DH, doubled haploid; **MS**, Murashige & Skoog; **NLN**, Nitsche-Lichter-Nitsche; **S1**, progeny obtained from one selfing cycle

INTRODUCTION

The genus *Anemone* belongs to the Ranunculaceae family, whose origin is registered in temperate zones of Europe and the Mediterranean basin. Around 120 species are part of this genus, and many of them are cultivated for ornamental purposes. The most used *Anemone* species for cut flower production is *Anemone coronaria* (Ball 1998; Laura *et al*. 2006).

Anemone is an herbaceous perennial plant that propagates through seeds or corms. Cross fertilization is effected by insects although self-pollination is also possible between different flowers of the same plant (Ball 1998; Laura *et al*. 2006). Since existing cultivars are highly heterozygous, to have an efficient seed propagation system it is necessary to develop pure or homozygous lines.

Although *A. coronaria* is produced worldwide, the existing cultivars have only few flower colours, flowers are small and stems are short. The requirement for new and different genotypes can turn this species into an important target for breeding (Ball 1998). The production of F1 hybrids would be of great benefit for breeding purposes in *A. coronaria*.

The creation of pure or homozygous lines in cross pollinated crops takes from 6 to 8 selfing generations. Using haploid technology this period can be reduced to 1 or 2 years (Pauls *et al.* 2006). A very commonly used technique to produce haploids is androgenesis via anther or microspore culture.

Microspore culture is based on the ability of a single haploid cell, the microspore, to de-differentiate and regenerate into a whole plant after being exposed to stress, such as low or high temperature treatments, nutritional or chemical stress, etc. (Seguí-Simarro 2008). The advantage of microspore culture over anther culture is that in the latter there is a huge possibility that plantlets regenerate from the diploid tissues of the anther (i.e., anther filaments, pollen sac integuments) (Pacini 1994).

At the moment, there are only few studies reported of androgenesis on *Anemone* species. One of the first publications was made by Johansson and Ericsson (1977). They succeeded in inducing embryo formation from anther cultures of several *Anemone* species, even though they presented no evidence of the ploidy of the plants obtained. The most recent work on Anemone was published by Laura *et al*. (2006), who obtained haploid lines from anther cultures of *A. coronaria* and confirmed its androgenic origin through genotypic and karyotypic analysis. Even though a relatively high proportion of plants tested were mixoploids, they obtained haploid lines suitable for breeding purposes. There are no publications about microspore culture in *A. coronaria*.

Although *A. coronaria* is a species with a great value for cut flower production, there is still a need for an efficient system for haploid production. Therefore, the aim of this study was to produce haploid plants from microspore culture of *A. coronaria* for the further creation of doubled haploid lines that would be used for the production of F1 hybrids.

MATERIALS AND METHODS

Plant material

Flower buds of plants cultivated in a well-ventilated greenhouse at 20°C/10°C (day/night), 12-h light and 130 µmol/m^2/s during day time, were harvested for microspore isolation. No chemicals were applied to the plants since the day before microspores isolation.

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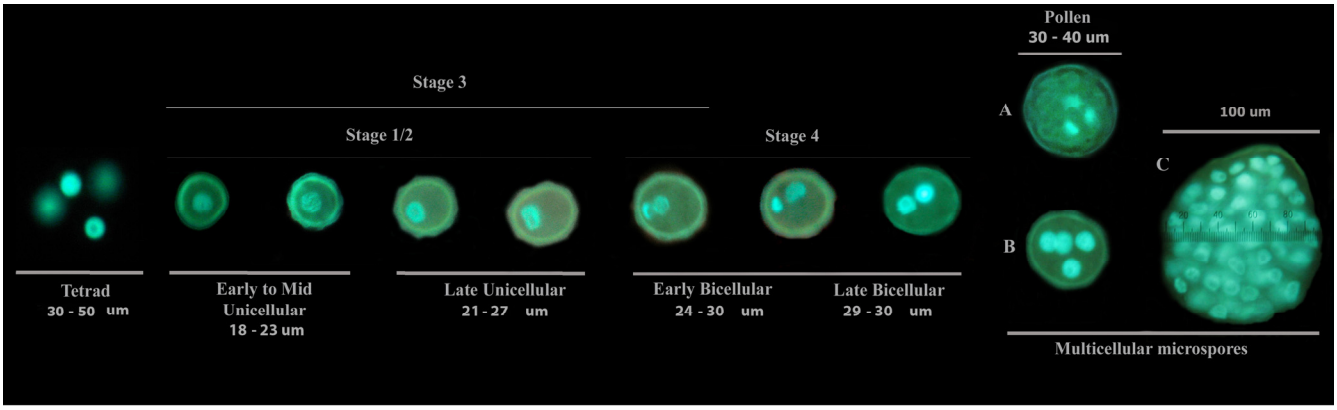

Fig. 1 Microspore development of *Anemone coronaria* L. from initial stage of monad liberation until pollen or microspore formation. (**A**) Pollen developed under 25°C, eight days after culture. (**B**) Microspore of eight days in culture; equal sized nuclei inside going through symmetrical divisions as a result of heat shock treatment. (**C**) Globular embryo 15 days after heat shock treatment.

Two heterozygous diploid genotypes of *A. coronaria* were tested: 'Blue' and 'Lilac'. Genotype 'Blue' originated from one cycle of self-pollination (microspores were isolated from S1 plants of this genotype).

Characterization of the microspore population

Flower buds from different levels of maturity were harvested to study anther morphology and its correlation with the developmental stage of microspore population. Size and color of the anthers, as well as flower stalk curvature and color of the center of the flower buds were observed.

The developmental stage of microspore populations from different types of flower buds and anthers were analyzed using DAPI (4',6-diamidino-2-phenylindole) staining. From these analysis we determined four developmental stages to be evaluated further: Stage 1: 80% early to mid unicellular and 20% late unicellular; Stage 2: 15% early to mid unicellular and 85% late unicellular; Stage 3: 15% early to mid-unicellular, 83% late unicellular and 2% early bicellular; Stage 4: most of the microspores were bicellular, only a few unicellular microspores (**Fig. 1**).

Isolation and microspore culture

Collected anthers were surface sterilized for 10 min in a solution of 2% NaOCl and rinsed in sterile distilled water for 1, 5 and 10 min. For microspore isolation, anthers were gently macerated in two sterile liquid media supplemented with sucrose 13% as washing solutions (pH adjusted to 5.8): EB4: NLN (Nitsche-Lichter-Nitsche, Lichter 1982) basal salts and vitamins, used for *Brassica napus* (Custers *et al.* 1994) and EB5: ¼MS (Murashige and Skoog 1962) basal salts and NLN vitamins, based on a medium used for *Tulipa gesneriana* (Van den Bulk *et al*. 1994). The resulting suspensions were aseptically filtered through a 50 µm nylon screen and centrifuged at 4°C for 4 min at 100 × *g*. The centrifugation procedure was repeated twice, re-suspending the microspores in fresh medium every time. Microspore density was adjusted to 4×10^4 microspores/ml and microspores were cultured in 6 cm diameter plastic Petri dishes containing the same culture media used for isolation (EB4 or EB5).

As heat shock treatments, stages 1, 2, 3 and 4 were incubated at different temperatures to induce changes in microspore developmental pathway. We evaluated 25, 32 and 34°C, for 1, 2 and 4 days. After heat shock treatments, microspore cultures were kept in darkness at 25°C until the embryos reached torpedo stage, approximately 1-2 mm long.

Microspore-derived embryo germination and plantlet development

From day 45 until day 60, torpedo embryos were collected, transferred to 9 cm diameter Petri dishes (max. 12 embryos per Petri dish) and incubated at 22°C under indirect light (±8.1 µmol/m^2/s) with a 16-h photoperiod. We evaluated five media to promote embryo germination and growth of plantlets: the two media used for microspore culture (EB4 and EB5), and three culture media commonly used for embryo germination in several species: EB1: MS basal salts, supplemented with MS vitamins, 3% sucrose, 0.02 mg/l BA (6-benzyladenine), 0.04 mg/l NAA (α-naphthalene acetic acid), solidified with Plant Agar (Duchefa cod. 1001, The Netherlands) 0.7% and pH adjusted to 5.8 (Esmeralda Breeding BV, unpublished data). EB2: B5 basal salts, supplemented with Kao vitamins, sucrose 3%, coconut milk (purchased as bottled coconut water in the supermarket) 20 ml/l and hydrolyzed casein 2500 µl/l, solidified with 0.7% Plant Agar and pH adjusted to 5.8 (Kao and Michayluk 1975). EB3: ½MS basal salts, supplemented with MS vitamins, 2% sucrose, 1% activated charcoal, solidified with 0.8% Plant Agar and pH adjusted to 5.8 (Johansson 1983).

The ploidy of all the plants obtained was determined by flow cytometry. Three samples per plant were analyzed. Analyses were performed by Plant Cytometry Services (PCS), The Netherlands.

Experimental design and statistical analyses

Average number of embryos per treatment were analyzed to determine the success of the heat shock treatments. Embryo germination data were analyzed with one factor ANOVA using SPSS 16.0 General Linear Model for Windows. Differences among treatments were studied with Tukey significance test at $P = 0.05$.

RESULTS

Identification of suitable flower and anther stage for microspore culture

Unicellular microspores (Stages 1 and 2, **Fig. 1**) of 'Blue' and 'Lilac' genotypes gave the best embryogenic response. Microspores at this stage of development were found in 1.5-2.0 mm white-cream anthers in flower buds with a pale yellow center. Dark pigmentation both in the center of the flower bud or in the anthers was an indicator of maturity. We found no relation between the curvature of the flower stalk and the stage of development of microspores.

There was no embryogenic response of bicellular microspores (Stages 3 and 4) of both cultivars; not even the microspores that received the heat shock at the highest temperature responded. Instead, these microspores completed their gametophytic development and formed pollen grains.

Effect of culture media on the embryogenic response of microspores

Microspores developed into multicellular structures and converted into torpedo embryos in EB5 medium. In EB4 medium (NLN salts with Sucrose 13%) there was an initial embryogenic response of *A. coronaria* microspores, however, multicellular microspores stopped their development after the second week in culture.

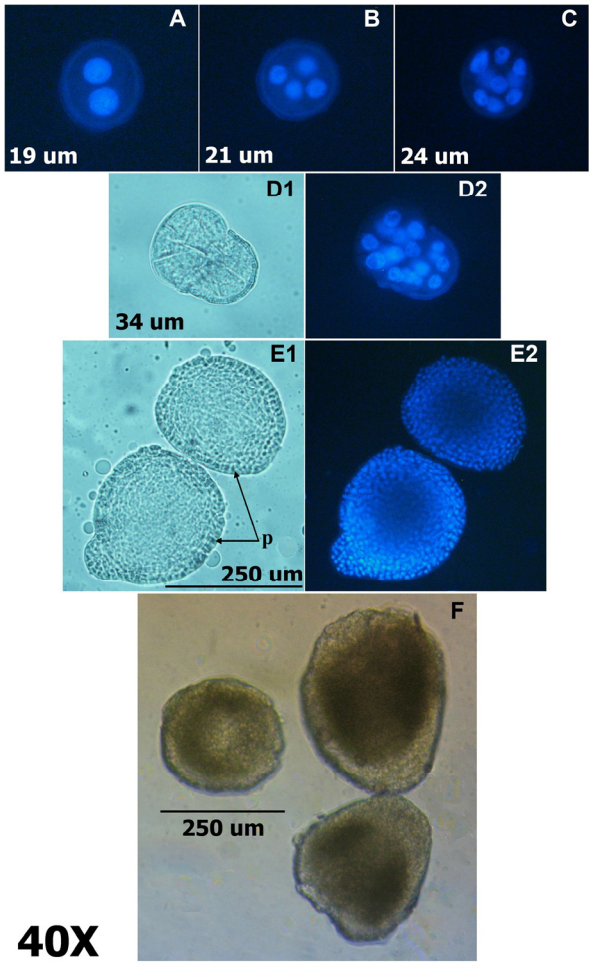

Fig. 2 Sporophytic development of *A. coronaria* microspores. (**A-C**) Microspores after two, four and eight days (respectively) after the inductive stress treatment (notice that the nuclei inside the microspore are the same size). (**D**) Multicellular microspore with visible protoderm (p). (**E**, **F**) Globular embryos after 30-45 days in culture at 25°C. (**A-E**) Structures seen through a fluorescence microscope; (**F**) Embryos seen through an inverted microscope.

Effect of heat shock treatment on the embryogenic response of microspores

The most effective heat shock treatment for both genotypes was the exposure of the microspore suspension cultures to 32°C for two days. Under these conditions we obtained 1266 torpedo embryos of genotype 'Blue' (0.53%) and 290 torpedo embryos (0.06%) of genotype 'Lilac' (**Table 1**).

Higher temperature (34°C) or longer exposure than two days gave negative results. In these treatments microspores stopped their development or continued to develop into pollen. As expected, at the control treatment at 25°C microspores completed its development into pollen grains.

General embryogenic response during microspore culture

Cellular growth, due to symmetric divisions of the nuclei inside the microspore, was visible from the fourth day after the application of the heat shock treatment. By day 15, globular embryos were observed (90-110 µm diameter). In these embryos, protoderm was visible in the perimeter of the microspore (**Fig. 2**). In a few days, only the microspore-derived embryos that developed a normal protoderm reached heart-shaped stage (approx. 200 µm diameter). From day 45 to day 60 in culture, bipolar embryos reached torpedo stage (**Fig. 3**).

Growth and development of microspores in culture was non-synchronous, even though similar anthers were used for microspore isolation. As a result, different cellular types were obtained at the same time: a) small size cells with thick exine that stopped their development, b) medium size cells with starch granules in the cytoplasm, c) big size cells with thin exine and clear cytoplasm; the latter microspores switched their gametophytic development to sporophytic development (**Fig. 4**).

Better response of microspore embryogenesis (**Table 1**) was observed with self-pollinated (S1) 'Blue' genotype compared to 'Lilac' genotype (1266 vs. 290 torpedo embryos obtained).

Microspore-derived embryo germination and plantlet development

From the five media evaluated, only EB1 and EB2 promoted embryo germination. For 'Blue' genotype, 8.46% and 32.46% of the embryos cultured in EB1 and EB2 germinated respectively. For 'Lilac' genotype embryo germination occurred only in EB2 medium (2.41%). However, not all the germinated embryos turned into normal plants, a high percentage showed further abnormal growth, became necrotic and died. Signs of hyperhydricity were also noticeable. Plantlets obtained were transferred to EB1 medium to complete its development.

Activated charcoal (EB3) did not promote microspore-derived embryo germination.

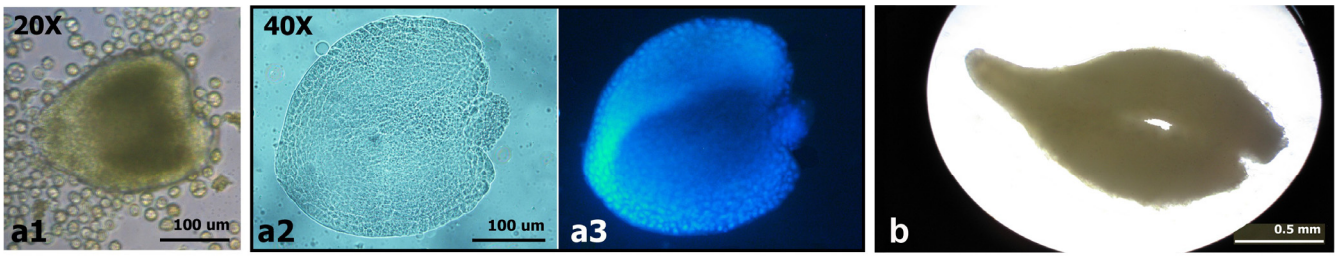

Fig. 3 Heart-shaped and torpedo stage embryos of *A. coronaria* 'Blue'. (**A**) Heart-shaped embryos after 30 days in culture (**A1**). Embryo seen through inverted microscope; **A2, A3.** Embryo seen through a fluorescence microscope). (**B**) Torpedo stage embryo after 45 days in culture, seen through an inverted microscope.

Table 1 Embryogenic response of microspores of genotypes 'Blue' and 'Lilac' (day 60 of culture).

Cultivar	Microspore stage [a]	Culture media	Temperature (°C)	Time of incubation (days)	# Torpedo embryos	Total embryos	Percentage of embryogenic microspores
'Blue'	1	¼MS-13	32	2	1251	1266	0.53%
	2	¼MS-13	32	2	15		
'Lilac'	1	¼MS-13	32	2	117	290	0.06%
	2	¼MS-13	32	2	173		

[a] Stage 1: most of the microspores are at early to mid unicellular stage. Stage 2: most of the microspores are at late unicellular stage

Table 2 Germination of torpedo embryos of genotypes 'Blue' and 'Lilac' and percentage of haploid and double haploid plants obtained.

Cultivar	# Torpedo embryos	# Plantlets	% Germination	# Haploids	% Haploids	# DH	% DH
'Blue'	1266	27	2.13%	18	66.67%	9	33.33%
'Lilac'	290	7	2.41%	4	57.14%	3	42.86%

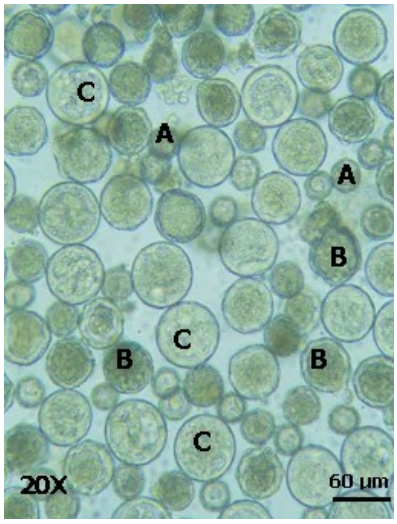

Fig. 4 Microspores of *A. coronaria* after 15 days in culture medium at 25°C. (**A**) Microspores that stopped their development, (**B**) Pollen grains, (**C**) Microspores that switched their developmental pathway and will become embryos.

Secondary embryo formation on the cotyledons of abnormal embryos was observed in both EB1 and EB2 medium. They developed into normal plantlets.

The overall success of embryo conversion into normal plantlets of *A. coronaria* was 2.13% for 'Blue' and 2.41% for 'Lilac' (**Table 2**).

Ploidy analysis

Ploidy analysis revealed the existence of 18 haploid plants and 9 doubled haploid plants of genotype 'Blue', and 4 haploid plants and 3 doubled haploid plants of genotype 'Lilac' (**Table 2**).

DISCUSSION

The results of this research reveal that it is possible to obtain haploid and doubled haploid plants from microspore cultures of *A. coronaria*.

As well as found in earlier studies on androgenesis of the genus *Anemone* (Johansson *et al*. 1982; Johansson 1983; Laura *et al.* 2006), in the present study the best response was observed in unicellular microspores. The stage of development of microspores is considered a main factor that influences the ability of microspores to switch their developmental fate in response to external stimulus (Van den Bulk *et al.* 1994; Custers *et al.* 1996; Bonet *et al.* 2000; Smýkal 2000; Indrianto *et al.* 2001; Touraev *et al.* 2001; Datta 2005; Ahmadi *et al.* 2011). Binarova *et al.* (1997) proposed that when bicellular microspores of *Brassica napus* are exposed to 34°C sporophytic development can be induced. This was not the case for *A. coronaria* since bicellular microspores at that temperature did not show a positive response.

Normal microspore-derived embryos of *A. coronaria* were formed in medium EB5. This result was also reported by Van den Bulk *et al.* (1994), who obtained embryos in EB5 medium for tulip, also a bulbous species.

As for *A. coronaria*, an essential factor for embryogenic induction on microspore cultures is stress caused by high temperature (Guo and Pulli 2000; Tashpulatov *et al.* 2002; Nomizu *et al.* 2004). When high temperature was applied to unicellular microspores of *A. coronaria*, it was possible to switch the gametophytic development into sporophytic development. High temperature has been reported to have an effect on the expression of proteins involved in embryogenic processes, *i.e.*, heat shock proteins (HSP) described by Cordewener *et al.* (1995). High temperatures are also involved in cytoskeletal alterations that prevent the movement of the nucleus towards the pole of the cell previous to the first mitotic division (Hause *et al*. 1993; Binarova *et al*. 1997), the activation of genes involved in the suppression of pollen development and the acquirement of embryogenic characteristics (Custers *et al.* 1999; Boutilier *et al.* 2002). Besides, high temperature may be related to the inhibition of apoptotic events that could be responsible for the death of microspores (Zorinniants *et al*. 2005).

The non-synchronous development of microspores observed during the initial phases of culture has been commented also by other authors. According to Pauls *et al.* (2006), the non-synchronous development occurs because microspores are in different developmental stages inside similar morphological anthers, therefore there are different responses after the application of the inductive treatment. The non-synchronous event on microspore cultures is also related to the medium composition, specifically to the carbon source (Van den Bulk *et al.* 1994; Rao and Suprasanna 1996; Illic-Grubor *et al.* 1998).

During culture we observed that many microspores did not respond to the heat shock treatment and did not switch its developmental pathway. According to Van den Bulk *et al.* (1994), the rate of conversion in the developmental pattern is generally low and occurs because not all the microspores keep their viability after isolation. Also, it has to be considered that the presence of advanced developmental stage microspores (i.e. bicellular) can inhibit the development of microspores that are at the right stage at the moment of isolation (Binarova *et al.* 1997).

Organic supplements were needed to promote germination of *A. coronaria* microspore-derived embryos. These supplements were also used by Morgan *et al.* (1998) for embryo germination of interspecific hybrids of *Limonium*.

Laura *et al.* (2006) reported that activated charcoal stimulated embryogenesis in *A. coronaria* and even favoured androgenic plant multiplication. The same effect was reported by Johansson (1983) in his research about anther culture of *Anemone* species. However, our results differ from these authors because in our case activated charcoal did not promote embryo development.

Although we obtained a high number of torpedo embryos, 1266 of 'Blue' genotype and 290 of 'Lilac' genotype, only 27 and 7 respectively converted into normal plants. Choung and Beversdorf (1985) and Supena (2004), pointed out that germination rate is highly dependant on embryo quality, and only well-developed bipolar embryos germinate successfully. For this reason, it is essential to assure proper embryo formation from the initial phases of development. Scott *et al.* (1995) suggested that, as a consequence of reducing sucrose concentration or replacing it with maltose, it is possible to decrease ethanol accumulation and enhance microspore embryogenesis. Also, it can be considered that using high osmolarity of metabolizable carbohydrates before applying stress treatment may help microspores to divide and tolerate stress conditions (Shariatpanahi *et al.* 2006). These statements agree with Jan Custers (pers. comm.), who supports the fact that replacing sucrose for another less metabolizable carbon source can improve embryo quality and avoid hyperhydricity. The latter phenomenon was one of the main causes of mortality of *A. coronaria* microspore-derived embryos.

Secondary embryogenesis observed during this research has been also reported for *A. coronaria* by (Laura *et al.*

2006), for *Brassica napus* (Sangwan and Sangwan-Norreel 1990; Ahmadi *et al.* 2011) and *Tulipa gesneriana* (Van den Bulk *et al.* 1994).

The influence of the genotype in the success of microspore cultures has been reported by many authors (Seguí-Simarro *et al.* 2007; Abraha *et al*. 2008; Ćalić-Dragosavac *et al*. 2010; Kahrizi *et al*. 2011; Wen *et al*. 2012). The different responses observed in our study between both genotypes of *A. coronaria* support this fact. We found a better embryogenic response in 'Blue' genotype that went through one selfing cycle (S1), what may be an indicator that a certain level of homozygosis could be beneficial for the production of haploid embryos. This hypothesis is supported by Khush and Virmani (1996), who declare that in species that can be selfed, lines with some level of homozygosis give better results for haploid production because they have less deleterious recessive genes (or do not have it at all).

Ploidy analysis of the androgenic progeny revealed that not only haploid plants were obtained but also doubled haploids. These results agree with several authors that have seen that the inductive treatment itself causes spontaneous chromosome doubling, either through nuclear fusion or endomitosis (Custers *et al.* 1994; Gu *et al.* 2003; Testillano *et al.* 2004; Shim *et al.* 2006; Kahrizi *et al.* 2009; Zhang *et al.* 2012).Six or more years are necessary to obtain homozygous lines of *A. coronaria* by selfing. The availability of this protocol shortens that period remarkably and represents a great benefit for the breeding of this species and the creation of homozygous lines for the production of improved F1 hybrids.

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