Eshaghi, ZC.; Abdollahi, MR.; Moosavi, SS.; Deljou, A.; Seguí-Simarro, JM. (2015). Induction of androgenesis and production of haploid embryos in anther cultures of borage (Borago officinalis L.). Plant Cell, Tissue and Organ Culture. 122:321-329. doi:10.1007/s11240-015-0768-5.

Induction of androgenesis and production of haploid embryos in anther cultures of borage (*Borago officinalis* L.)

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Abstract

Borage (*Borago officinalis* L.) is an important medicinal plant with different culinary, pharmaceutical and industrial properties. Unfortunately, there are no published reports on the establishment of protocols to produce DHs in this species up to now. In this work, we show for the first time the induction of borage microspores to become embryogenic calli, from which haploid embryos are produced. In addition, we evaluated the effect of using different flower bud sizes, carbon sources, concentrations of 2,4-D and BAP, cold (4ºC) pretreatments and heat shock treatments. Production of total calli, embryogenic calli and callus-derived embryos was differently affected by the different parameters studied. Our results showed that the use of 5-7 mm-long flower buds, a cold (4ºC) pretreatment during 4 days, a 32ºC heat shock for 3 days, and the addition of 3% maltose and 2 mgl^{-1} 2,4-D and 1 mgl^{-1} BAP to the culture medium, was beneficial for embryo production. Overall, this work demonstrates that DH technology is possible in borage, and opens the door for future improvements needed to finally obtain borage DH plants.

Keywords in vitro culture, microspore embryogenesis, plant growth regulators, temperature treatment

Introduction

Borage (*Borago officinalis* L., *Boraginaceae*) is an herbaceous annual species native to Europe, Asia Minor and North Africa. In the Mediterranean region, principally in France, Italy and Spain, borage leaves are used in salads and soups due to their delicate flavor. For centuries, it has been used as an herb of purported medicinal value (Simon et al. 1984). This species is one of the best known sources of gamma linolenic acid (GLA), an essential and unusual fatty acid (Guil-Guerrero et al. 2003). Indeed GLA, a prostaglandin precursor, has been reported to be beneficial in the suppression of inflammation and thrombosis, as well as in the treatment of a number of disorders including mild hypertension, elevated cholesterol levels, premenstrual syndrome, diabetic neuropathy, and certain cancers, such as malignant human brain glioma, among others (Horrobin, 1984; McDonald and Fitzpatrick 1998). Borage has a multifactorial self-incompatibility system. Such systems may have a high level of ineffective pollination (Leach et al. 1990), resulting in a limited production of pure (homozygous) lines by self-pollination. Doubled haploid (DH) technology is an economical and efficient alternative to avoid the limitations of self-incompatibility in these systems and in general, to speed up the process of producing homozygous lines for breeding programs (Forster et al. 2007). Indeed, androgenic DH lines produced either by anther culture or by isolated microspore culture have the potential to provide breeders with pure lines over the course of a few months, rather than the several (7-10) generations typically required using conventional selfing and selection strategies (Ferrie and Caswell 2011; Snape 1989). Despite that production of DH plants has been reported for many crop plants (reviewed in Maluszynski et al. 2003), there has been comparatively very little work conducted on the production of DHs in medicinal species (reviewed in Ferrie 2013). When available, this technology would be able to support medicinal plant breeding programs just as it

has been helping breeders for other crop species. Unfortunately, there are no published reports on the establishment of protocols to produce DHs in *Borago officinalis* L. This is the main goal of this study.

Aside of the genotype, there are three critical groups of factors that influence the deviation of a microspore towards embryogenesis. The first group includes the conditions of the microspores prior to isolation and culture. This group includes several factors related to the cultivation of the donor plants, but the most critical one is the developmental stage of the microspore at the moment of isolation. There is a wide consensus about the late (vacuolate) microspore and the young pollen grain as the sensitive stages for embryogenesis induction (Seguí-Simarro 2010; Maraschin et al. 2005). Thus, the proper identification of the flower buds containing these developmental stages is critical for the success of embryogenic induction. The second group of factors is the type of stress applied. It is commonly accepted that microspore embryogenesis is induced through the application of a stress treatment (Maraschin et al. 2005). Although the most used stress is thermal (heat or its absence, cold), many different stressors may be used, since different species are sensitive to different stresses (reviewed in Shariatpanahi et al. 2006). Thus, when trying to induce microspore embryogenesis in a new species for the first time, it is mandatory to find the most effective stress treatment. The third critical group of factors is culture conditions. As in other types of *in vitro* culture, medium composition including carbon and nitrogen sources, salts, vitamins and growth regulators, among others, are critical to promote growth, development and differentiation.

In this work, we aimed to determine a series of key parameters necessary to produce borage DHs through anther culture, included in the three groups above mentioned: the identification of the

most suitable developmental stage to excise and culture the microspore-carrying anthers, the carbon source, the type and concentration of plant growth regulators, and the use of low and high temperature treatments. Our results show that haploid embryos can be produced from borage anther cultures, and shed light on the different impact that the factors studied exert on the rate of embryo production.

Material and Methods

Plant material

A local Iranian variety of borage (*Borago officinalis* L.) was used as the experimental donor material in the present study. Seeds were kindly provided by Pakan Bazr Company, Isfahan, Iran. Donor plants were grown in experimental plots using standard agronomic practices under a natural photoperiod of 28-32°C during the day and 18-24°C at night, from March 2014 *to* July 2014. 50-60 day-old plants were selected as donor material.

Determination of microspore developmental stages

To check the microspores contained in anthers at different developmental stages, anthers were excised from flower buds of different lengths (5, 7 and 9 mm), deposited in a glass slide and squashed with a coverslip under a drop of acetocarmine staining (1 g carmine in 100 ml glacial acetic acid 45%). The mounted slides were observed under a microscope to characterize the microspore stage.

Standard anther culture conditions

Flower buds (5–7 mm in length) containing anthers with microspores at the mid-to-late uninucleate stage were collected from the donor plants between 9:30 and 10:30 a.m., placed in water and brought into the laboratory. Buds were surface sterilized with 70% (v/v) ethanol for 30 s, followed by 2.5% (v/v) sodium hypochlorite for 10 min with gentle shaking, and rinsed three times with sterile distilled water. The explants were prepared by removing the petals by forceps. Excised anthers were cultured in 90-100 mm plastic dishes containing 20 ml of autoclaved culture medium. The standard culture medium (pH 5.8) consisted of B5 medium salts (Gamborg et al. 1968), NLN medium vitamins (Lichter 1982); 0.8% agar, 3% sucrose, 2 $mg.l^{-1}$ 2-dichlorophenoxyacetic acid (2,4-D) and 0.5 $mg.l^{-1}$ benzyl aminopurine (BAP). Dishes containing the cultured anthers were sealed with parafilm and kept at 4°C in darkness for 5 days, and then transferred to 25°C in a growth room under a 16/8 photoperiod with a light intensity of 40 $\mu mol.m^{-2}.s^{-1}$ for 2 weeks. These standard conditions were modified for the different experiments performed as described below. Four weeks after anther inoculation, induced calli and embryos were transferred to B5 germination medium containing 2.0 $mg.l^{-1}$ BA, 0.5 $mg.l^{-1}$ NAA, 3% sucrose and 0.8% agar (pH 5.7). Culture dishes were examined daily.

Evaluation of different culture parameters

Using the standard culture conditions described above as a control reference, we evaluated the effect of altering different culture parameters in the production of embryos and/or calli. For this, we designed four different experiments where, except for the altered parameter, the culture conditions were kept unchanged, as follows: In the first experiment, the effect of using different carbon sources, combined with the use of different flower bud sizes, was evaluated. Flower buds of different sizes (5, 7 and 9 mm) were collected, and the anthers were excised and cultured in standard culture medium modified to include either 3% maltose or 3% sucrose. For the second

experiment, the excised anthers or the donor flower buds (before excising their anthers) were subjected to a cold pretreatment. Buds were placed onto wet filter paper, and excised anthers were wrapped with aluminum foil. Both buds and anthers were kept at 4°C in darkness for 2, 4 or 7 days. For the third experiment, anthers inoculated in standard culture medium were subjected to different heat treatments: 30°C for 7 and 14 days, 32°C for 1, 2 and 3 days, or 35°C for 1 day. After the heat treatment, culture dishes were transferred to 25°C in a growth room, being kept in darkness at all time. For the fourth experiment, the standard culture medium was supplemented with different combined concentrations of 2,4-D (0, 1, 1.5 and 2 $mg.l^{-1}$) and BAP (0, 0.5 and 1 $mg.l^{-1}$).

Experimental design and statistical analyses

All the experiments were designed with a complete randomized design. For each experiment, a minimum of 3 replicates (4 for the first experiment) were prepared, each replicate consisting of one culture dish containing 20 anthers (15 for the fourth experiment). Each experiment was repeated three times. Callus and/or embryo production was measured after 12-14 days of culture. Results were expressed as the percentage of anthers producing calli (C%), the percentage of anthers producing embryogenic calli (EC%) and the mean number of embryos per anther (ME/A). Quantitative data in all experiments were analyzed by analysis of variance (ANOVA). Significant differences between means were assessed by Duncan's multiple range test (DMRT) at $p \leq 0.05$. SPSS 16 software was used to test the significant differences among levels of treatment but residual plot analysis showed that the percentage data were not normally distributed. These variables were therefore square root transformed before the analyses of variance.

157 *Ploidy analysis*

158 For flow cytometric analysis, anther-derived embryos, calli or leaf pieces (~1 cm^2) were chopped
159 with a razor blade into small pieces in 2 ml of cold (8°C) DAPI staining solution (5 $\mu g.ml^{-1}$,
160 Partec GmbH, Germany) and passed through a nylon filter (50 µm mesh size). The filtrate was
161 loaded in a Partec Ploidy Analyzer (Partec GmbH, Germany) and analyzed at a par gain FL1 of
162 400–415 (relative fluorescence). To calibrate the equipment, a tomato DNA standard was loaded
163 together with the diploid reference (borage seedlings) used in this study.

164 Additionally, chromosome preparations were carried out by staining the plant materials with
165 alpha-bromonaphthalene acid for 3 h, followed by fixation in acetic acid: absolute alcohol (1:3
166 v/v) for 24 h. After fixation, the plant materials were washed in distilled water, and hydrolyzed
167 in 1 M HCl for 6 min at 60°C. The treated samples were squashed in 1% acetocarmine and 45%
168 acetic acid. Then, the prepared samples were observed and imaged under a light microscope to
169 count their chromosomes and to determine their ploidy level.

170

171 **Results**

172

173 **Bud, anther and microspore characterization**

174 We checked the developmental stage of the microspores contained in 5, 7 and 9 mm-long buds. 5
175 mm-long buds (Fig. 1A) presented green ~2.5 mm-long anthers (Fig. 1B). Anthers at this stage
176 contained mid-unicellular microspores (Fig. 1C), together with late-unicellular microspores. 7
177 mm-long buds (Fig. 1D) presented green ~3.5 mm-long anthers (Fig. 1E). Anthers at this stage
178 also contained principally mid-unicellular microspores and late-unicellular (vacuolate)
179 microspores (Fig. 1F). 9 mm-long buds mostly presented anthers with pollen grains (data not
180 shown), beyond the time window where embryogenesis can be induced. Thus, we concluded that

5-7 mm-long flower buds, containing 2.5-3.5 mm immature anthers with mid-to-late microspores, were the most suitable for borage anther culture.

Culture establishment

25-3.5 mm-long anthers were inoculated into dishes with standard culture medium (Fig. 2A), becoming pale after some days in culture. Then, callus-producing anthers began to swell and approximately 12-14 days after anther inoculation, callus-like structures emerged out of the anther (Figs. 2B-C). Some of these structures were pale green, round and compact (Fig. 2B), resembling the type of calli described as non-embryogenic in other species (Seguí-Simarro and Nuez, 2006). In parallel, intense green and irregular calli also emerged from these anthers (Fig. 2C). Over the surface of some of these calli, we observed the growth of small, rounded structures resembling globular embryos (arrowheads in Fig. 2D). After two more weeks, these globular structures became clearly identifiable torpedo embryos (Fig. 2E), defined by the presence of an elongated hypocotyl, two cotyledon primordia (arrowheads in Fig. 2E), and an epidermal layer with a embryo-typical texture. This pattern, however, was not found in all embryos, since many others presented abnormal shoot apices, defined by the total absence of any identifiable structure (arrowhead in Fig. 2F). All the embryos produced were transferred to germination medium, where they turned brown and eventually died.

Ploidy level of anther derived calli and embryos

In order to unambiguously verify the haploid origin of the embryogenic calli and the embryos produced by borage anthers, we checked the ploidy of 7 randomly chosen anther-derived calli and 3 callus-derived embryos by flow cytometry. As a reference, we used diploid borage seedlings (Fig. 3A) which produced G1 and G2 peaks at gains of ~120 and ~240, respectively.

To confirm this, we performed chromosome counts in root tip cells of borage seedlings (2n=2x=16), observing cells with 16 chromosomes (Fig. 3B). The three callus-derived embryos analyzed by flow cytometry and five out of seven anther-derived calli (71.43%), showed G1 and G2 peaks at gains of ~60 and ~120, respectively (Fig. 3C). In other words, they were haploid. The chromosome counts in cells of the root apex of these embryos confirmed the presence of a chromosome set half of the diploid seedlings (Fig. 3D). The flow cytometric analysis of the remaining 3 anther-derived calli (28.58%) showed peaks at gains similar to the diploid reference (data not shown), being considered as diploid too.

Effect of flower bud size and type of carbon source

Our next step was to attempt the improvement of the efficiency of embryo production by changing several culture parameters in order to know whether they have an effect on embryo yield. First, we evaluated the combined effect of the carbon source and flower bud size used. As seen in Table 1, calli were produce in all bud lengths and with both sucrose and maltose. However, the use of sucrose was clearly detrimental in terms of embryogenic callus and embryo production. On the contrary, maltose-containing dishes produced embryogenic calli and embryos in 5 and 7 mm-long anthers, at a similar rate.

Effect of different cold pretreatments

The next step was to determine whether the application of a cold (4ºC) pretreatment to the flower buds or to the excised anthers could be beneficial for embryo yield. As seen in Table 2, the application of a cold pretreatment to flower buds before anther excision did not improve the results obtained in control conditions neither in terms of calli (total or embryogenic) nor embryos. Moreover, longer exposures to 4ºC were clearly detrimental, indicating that bud

pretreatment at 4ºC was not useful. However, the exposure of the excised anthers to 4ºC for a limited time (four days) clearly improved significantly all the parameters measured, including the most important one, the embryo yield, which increased five fold.

Effect of different heat treatments

The last experiment involved the assay of different heat shock conditions, as shown in Table 3. Prolonged exposure (14 days) to mild heat (30ºC) produced the highest rate of calli (~71%). However, the most effective combination to increase the embryo yield was 32ºC during three days, which dramatically increased both the percentage of anthers with embryo-producing calli to ~13 and the mean number of embryos per anther to 0.13.

Effect of different 2,4-D and BAP combinations

We next tried to improve the efficiency by modifying the amounts of 2,4-D and BAP added to cultures. We tried all possible combinations of 2,4-D used at 0, 1.0, 1.5 and 2.0 $mg.l^{-1}$, and BAP used at 0, 0.5 and 1.0 $mg.l^{-1}$. As seen in Table 4, all hormone combinations (including the addition of no hormones) gave rise to anther-derived calli. However, the percentage of embryogenic calli and the number of embryos produced per anther increased as higher concentrations of 2,4-D and BAP were used. The highest number of embryos (0.17 per anther) was obtained with the use of 2 mgl^{-1} 2-4-D and 1 mgl^{-1} BAP. This result was significantly different from the rest according to the ANOVA test.

Discussion

252 We have shown in this work that it is possible to produce haploid borage embryos. For this, we
253 have evaluated the role of different parameters known to influence the androgenic response. The
254 first parameter was to determine the right developmental stage of the microspore to be induced.
255 It is widely accepted that the embryogenesis can be induced around the first pollen mitosis, i.e. in
256 the vacuolate microspore and young bicellular pollen stages (Seguí-Simarro 2010). Before or
257 after these stages, it is remarkably difficult or impossible at all to achieve embryogenesis. We
258 showed that in borage, the inducible stage is also the vacuolate microspore, since the only
259 anthers that responded to embryogenesis induction were those containing this microspore stage.
260 In addition, we characterized the length of buds containing microspores at this stage. It is known
261 for many species that there is a significant relationship between floral bud length and the
262 different developmental stages of the microspores contained in the anthers (Lauxen et al. 2003;
263 Parra-Vega et al. 2013a; Salas et al. 2012; Skrzypek et al. 2008). Thus, the characterization of
264 the optimal bud length as 5-7 mm defines a reliable morphological marker to easily identify buds
265 and anthers containing vacuolate microspores.
266
267 Aside of the genotype and the microspore stage, the third main parameter that has a critical
268 influence on the success of embryogenesis induction is the *in vitro* culture conditions. Actually,
269 this is a large set of conditions that include pretreatments previous to culture, inductive (stress)
270 treatments, and the *in vitro* culture conditions *per se*. We altered several parameters related with
271 these three groups. As to the pretreatments, we evaluated the effect of exposing the anthers or the
272 entire flower buds to 4ºC during variable times. A cold pretreatment (0-10ºC, 0.5-7 days) has
273 been shown to be beneficial for many medicinal plant species (reviewed in Ferrie 2013).
274 Typically, cold pretreatments applied to the microspores are thought to induce cytoskeletal and
275 nuclear rearrangements, to increase intracellular ABA levels, to slow down degradation

processes in the anther tissues, and to assure survival of a greater proportion of microspores (reviewed in Maraschin et al. 2005; Shariatpanahi et al. 2006). It was found in triticale that a cold pretreatment could be useful to induce embryogenesis with efficiency lower than with heat shock, but without compromising cell viability, which eventually prevailed in the final embryo yield (Zur et al. 2009). In borage, we found that the application of a cold pretreatment at 4°C for 4 days to the excised anthers significantly increased the frequency of embryogenic calli and embryos produced. However, it is important to note that this pretreatment must be applied to anthers and not to flower buds, since our results in this respect were not significant. This adds to the number of reports showing that cold pretreatments applied to the flower bud are ineffective (Vagera and Havranek 1985; Tipirdamaz and Ellialtioğlu 1998; Ozkum and Tipirdamaz 2002, 2011; Irikova et al. 2011).

The application of a mild heat shock to cultured anthers is one of the most used stresses to induce microspore embryogenesis (Shariatpanahi et al. 2006). Indeed, a heat shock treatment alone is sufficient to deviate the microspore towards embryogenesis in a number of species such as rapeseed and pepper (Custers et al., 1994; Abdollahi et al. 2004; Parra-Vega et al. 2013b), among others. Our results showed that for borage microspores, heat shock is also effective to induce embryogenesis. In particular, the best combination to produce embryogenic calli and embryos was 32ºC during 3 days. However, it must be noted that other heat regimes, such as 30ºC during 14 days, dramatically increased the production of total calli (both embryogenic and non embryogenic) up to 71%, while the production of embryogenic calli and embryos was not affected likewise. It cam be deduced from this that most of the calli produced at 30ºC for 14 days were not derived from microspores, but most likely from anther wall tissues. In other words, a

299 prolonged exposure to heat may have a stimulating effect on anther wall tissues that may start
300 proliferation, as described in other species (Parra-Vega et al. 2013b). Obviously, this will be
301 detrimental for the purpose of producing microspore-derived embryos, and should be avoided.
302
303 The third large group of factors affecting embryogenesis induction is the *in vitro* culture
304 conditions. Among them, we evaluated the effect of replacing sucrose by equimolar
305 concentrations of maltose, and found that it was remarkably positive. This is not surprising, since
306 although sucrose is the most commonly used carbohydrate in plant tissue culture, several studies
307 have shown that the use of maltose improves the efficiency of embryo induction in different
308 species (Bohanec et al. 1993; Raquin 1983; Ferrie et al. 2011). It is believed that in addition to its
309 value as carbon source, maltose may have different osmotic properties (Calleberg and Johansson
310 1996), which might contribute to stress the microspores and therefore, to increase the induction
311 rate. Perhaps, the most important of the *in vitro* culture conditions is the type of growth
312 regulators used, together with their concentration and their relative proportions. Since
313 microspores of model species do not need hormones added to the medium to become embryos,
314 hormones are not considered essential for the androgenic switch (Seguí-Simarro 2010).
315 However, they are essential to sustain growth and to promote differentiation in those species
316 where culture conditions are not well optimized, as it is the case for borage, with no previous
317 studies available. Among the different combinations we tested, the use of 2 mgl^{-1} 2-4-D and 1
318 mgl^{-1} BAP was clearly shown the best to promote the growth of microspore-derived calli and to
319 sustain the growth of embryos from these calli.
320
321 In summary, we propose that in order to produce microspore-derived embryos in borage, the
322 standard protocol described in Materials and Methods should be used including the following

323 modifications: the use of 5–7 mm flower buds as donors of microspores, a pretreatment of the
324 excised anthers at 4°C for 4 days prior to the heat shock, which should consist of the application
325 of 32ºC during 3 days, and the use of 3% maltose, 2 mgl^{-1} 2,4-D and 1 mgl^{-1} BAP in the culture
326 medium.
327
328 **Concluding remarks and future perspectives**
329 In this work, we demonstrated for the first time the occurrence of haploid embryogenic calli from
330 which haploid embryos are formed. In addition, we improved the protocol in order to increase
331 the embryo yield. These results are especially relevant for this species, since to the best of our
332 knowledge, no previous reports have been published on the induction of any androgenic pathway
333 in *B. officinalis*. Given the important role of this species as a source of gamma linolenic acid and
334 in general, as a plant of medicinal and culinary interest, our study opens the way to the
335 production of DH plants to speed up breeding programs in borage. We demonstrated that
336 microspores can be reprogrammed to become haploid embryos, which is the most important step
337 when trying to develop a protocol to produce DHs in a new species.
338
339 However, this is just the beginning, and further work will be needed to finally obtain borage
340 DHs. This work should be mainly focused on three aspects: the induction of direct
341 embryogenesis, the improvement of shoot apical meristem quality and chromosome doubling.
342 Although the rate of secondary embryogenesis production may be enough for certain purposes, it
343 is clear that the production of embryos directly derived from microspores will surely increase the
344 efficiency of the process. As shown in Fig. 1F, many of the embryos produced were abnormal,
345 showing a malformed of absent shoot apex. This is a common feature of anther and microspore
346 culture in many species (reviewed in Seguí-Simarro et al. 2011) which has a direct and strong

impact in further embryo germination. Such embryo defects may explain the null rate of embryo germination we obtained and conversely, improvement of embryo quality will surely allow to obtain microspore-derived borage plants. Finally, our results indicate that the addition of a chromosome doubling step might be necessary in order to obtain true DH plants. It is expected that in the short-mid term, these studies are concluded and the possibility of producing borage DHs will come true.

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Tables

Table 1: Effect of bud size and carbon source in the percentages of anthers producing calli (C%), anthers producing embryogenic calli (EC%), and the mean number of embryos obtained per anther (ME/A). For each column, different letters denote significant differences (Duncan's multiple range test, $p \leq 0.05$).

Carbon source	Bud size	Anthers cultured	C%	EC%	ME/A±SE
3% sucrose	5 mm	80	27.5 b	0.0 b	0.0 b
	7 mm	80	36.25 a	0.0 b	0.0 b
	9 mm	80	16.25 b	0.0 b	0.0 b
3% maltose	5 mm	80	47.5 a	6.67 a	0.07±0.044 a
	7 mm	80	37.5 a	10.0 a	0.07±0.016 a
	9 mm	80	22.5 b	0.0 b	0.0 b

Table 2: Effect of different cold pretreatments in the percentages of anthers producing calli (C%), anthers producing embryogenic calli (EC%), and the mean number of embryos obtained per anther (ME/A). For each column, different letters denote significant differences (Duncan's multiple range test, $p \leq 0.05$).

Cold pretreatment	Anthers cultured	C%	EC%	ME/A±SE
Control	60	26.67 c	3.33 bc	0.03±0.033 bc
Buds, 4ºC - 2 days	60	33.33 bc	3.33 bc	0.03±0.033 bc
Buds, 4ºC - 4 days	60	10.00 d	0.0 c	0.0 c
Buds, 4ºC - 7 days	60	3.33 d	0.0 c	0.0 c
Anthers, 4ºC - 2 days	60	36.67 bc	0.0 c	0.0 c
Anthers, 4ºC - 4 days	60	50.00 a	15.00 a	0.15±0.050 a
Anthers, 4ºC - 7 days	60	45.00 ab	10.00 ab	0.1±0.0 ab

Table 3: Effect of different heat treatments in the percentages of anthers producing calli (C%), anthers producing embryogenic calli (EC%), and the mean number of embryos obtained per anther (ME/A). For each column, different letters denote significant differences (Duncan's multiple range test, $p \leq 0.05$).

Heat pretreatment	Anthers cultured	C%	EC%	ME/A±SE
Control	45	24.44 d	0.0 b	0.0 b
30°C - 7 days	45	35.55 cd	2.22 b	0.02 ± 0.023 b
30°C - 14 days	45	71.11 a	2.22 b	0.02 ± 0.023 b
32°C - 1 day	45	53.33 b	2.22 b	0.02 ± 0.023 b
32°C - 2 days	45	37.77 cd	2.22 b	0.02 ± 0.023 b
32°C - 3 days	45	44.44 bc	13.33 a	0.13 ± 0.037 a
35°C - 1 day	45	28.88 d	0.0 b	0.0 b

Table 4: Effect of different 2,4-D and BAP concentrations in the percentages of anthers producing calli (C%), anthers producing embryogenic calli (EC%), and the mean number of embryos obtained per anther (ME/A). For each column, different letters denote significant differences (Duncan's multiple range test, $p \leq 0.05$).

Medium	2,4-D	BAP	Anthers cultured	C%	EC%	ME/A±SE
M1	-	-	60	25.00 ab	0.0 b	0.0 b
M2	-	0.5	60	38.33 ab	3.33 b	0.03±0.033 b
M3	-	1	60	23.33 b	0.0 b	0.0 b
M4	1	-	60	28.33 ab	0.0 b	0.0 b
M5	1	0.5	60	30.00 ab	3.33 b	0.03±0.033 b
M6	1	1	60	33.33 ab	3.33 b	0.03±0.033 b
M7	1.5	-	60	33.33 ab	0.0 b	0.0 b
M8	1.5	0.5	60	36.66 ab	0.0 b	0.0 b
M9	1.5	1	60	40.00 a	3.33 b	0.07 ± 0.066 b
M10	2	-	60	25.00 ab	0.0 b	0.0 b
M11	2	0.5	60	25.00 ab	3.33 b	0.03 ± 0.033 b
M12	2	1	60	40.00 a	16.66 a	0.17 ± 0.033 a

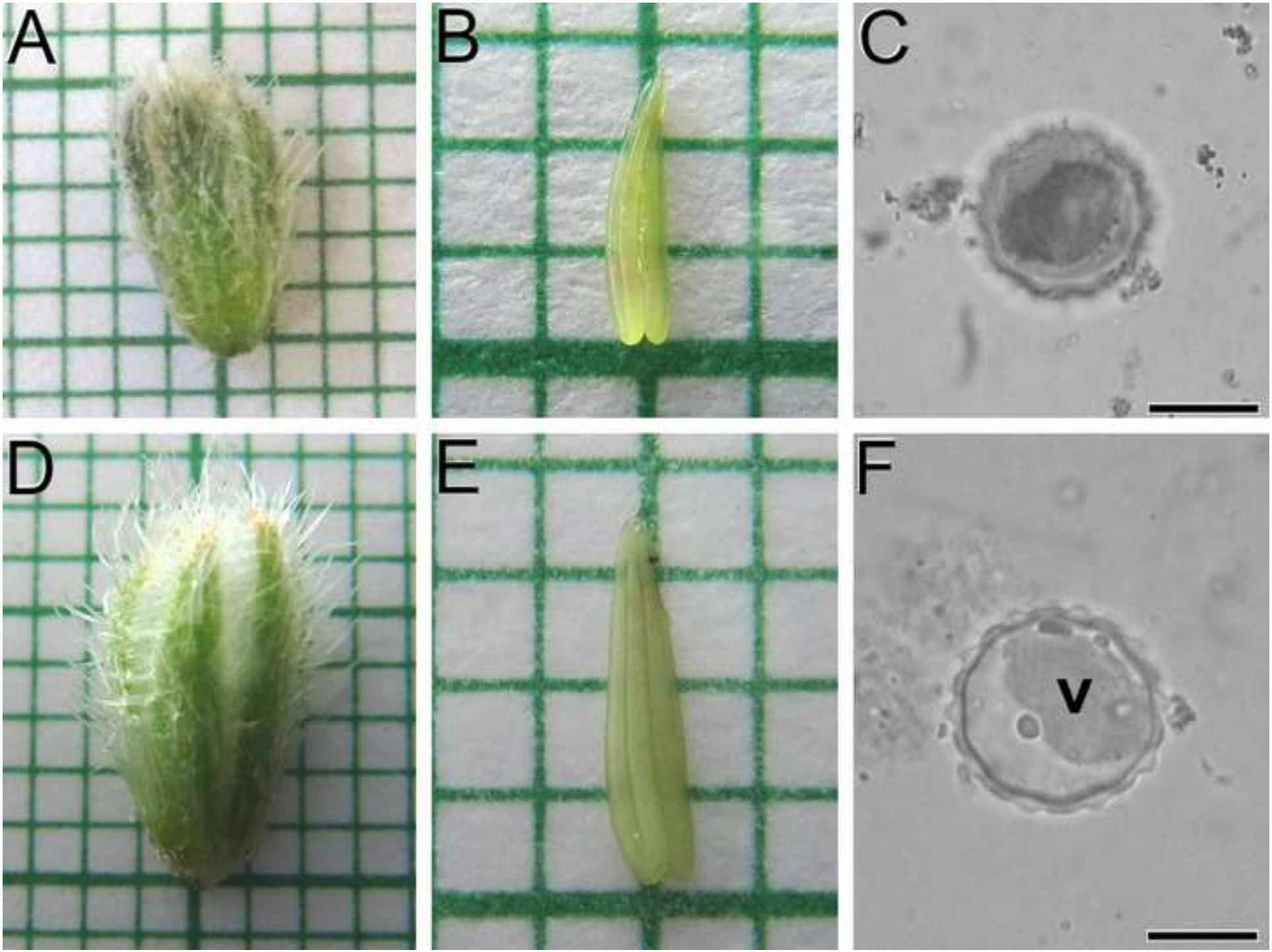
Figure legends

Fig. 1: Bud, anther and microspore characterization. A: 5 mm-long bud. B: 2.5 mm-long anther dissected from the bud shown in A. C: mid-uninucleate microspore. D: **7** mm-long bud. E: 3.5 mm-long anther dissected from the bud shown in D. F: late-uninucleate microspore. Note the large vacuole (v) typical from this stage. Squares in A, B, D and E background are 1 mm-wide. Bars in C, F: 10 µm.

Fig. 2: Culture establishment and progression. A: 2.5 mm-long borage cultured anther. B: Pale green callus, presumably non-embryogenic, emerging from a cultured anther. C: Intense green callus, presumably embryogenic, emerging from a cultured anther. D: Globular embryo-like structures (arrowheads) arising from the surface of an embryogenic callus. E: Elongated torpedo embryo arisen from an embryogenic callus. Arrowheads point to the two cotyledonar primordia. F: Elongated embryo arisen from an embryogenic callus. The arrowhead points to the putative shoot apical end, where no identifiable organs can be observed. Bars: 1 mm.

Fig. 3: Determination of the ploidy level in borage seedlings (A, B) and anther-derived embryos (C, D) by flow cytometry (A, C) and chromosome preparations (B, D). A: Flow cytometry histogram of a diploid borage seedling. To calibrate the system, DNA of a diploid tomato leaf was used as standard. B: Chromosome preparation of a root tip cell of a borage seedling (2n=2x=16), where 16 chromosomes can be distinguished. C: Flow cytometry histogram of a haploid embryo. D: Chromosome preparation of a haploid embryo cell, where 8 chromosomes can be distinguished. Bars: 10 µm.

Key Message: We report here, for the first time, the production of haploid embryos via anther culture in borage, an important medicinal plant


A
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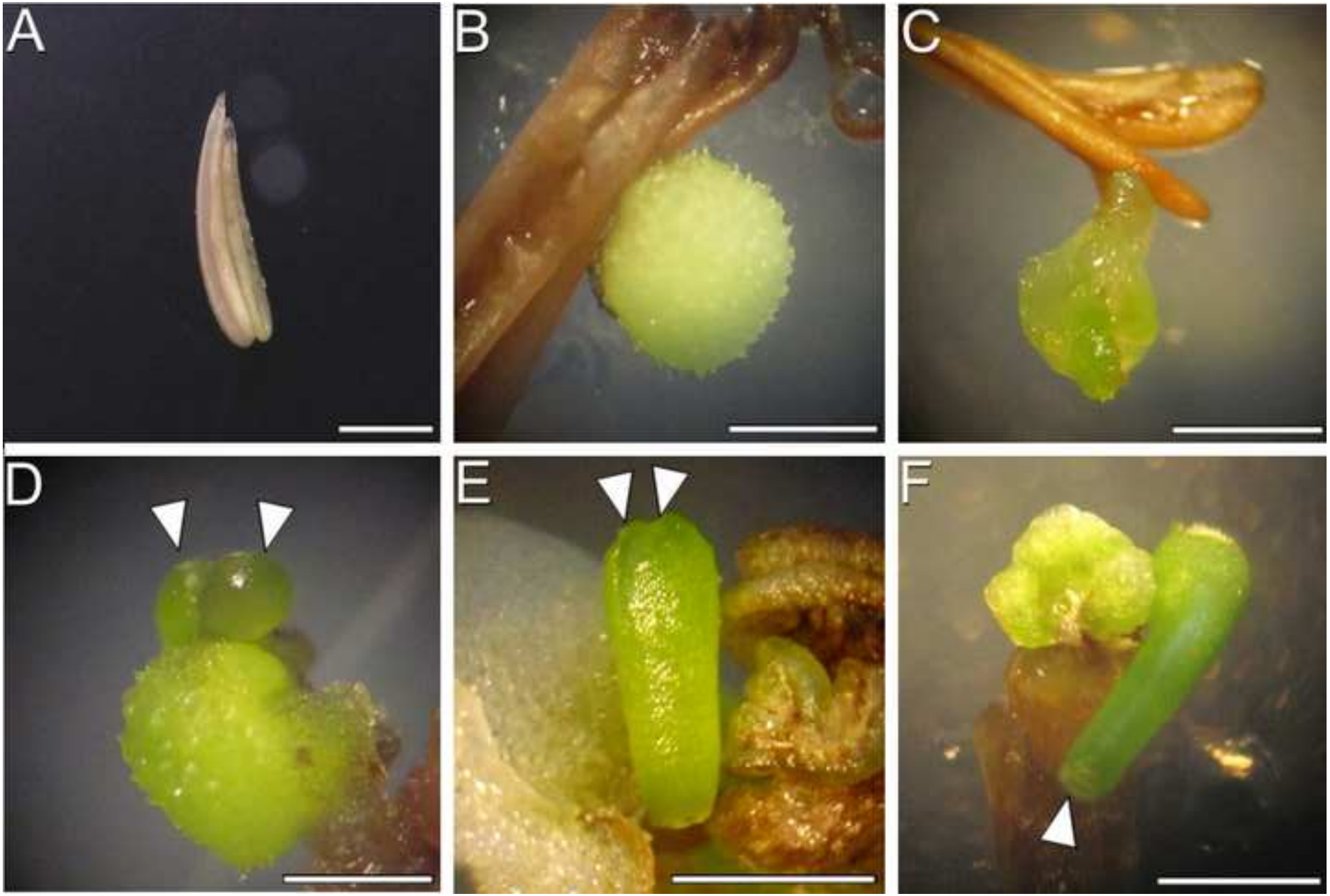
A
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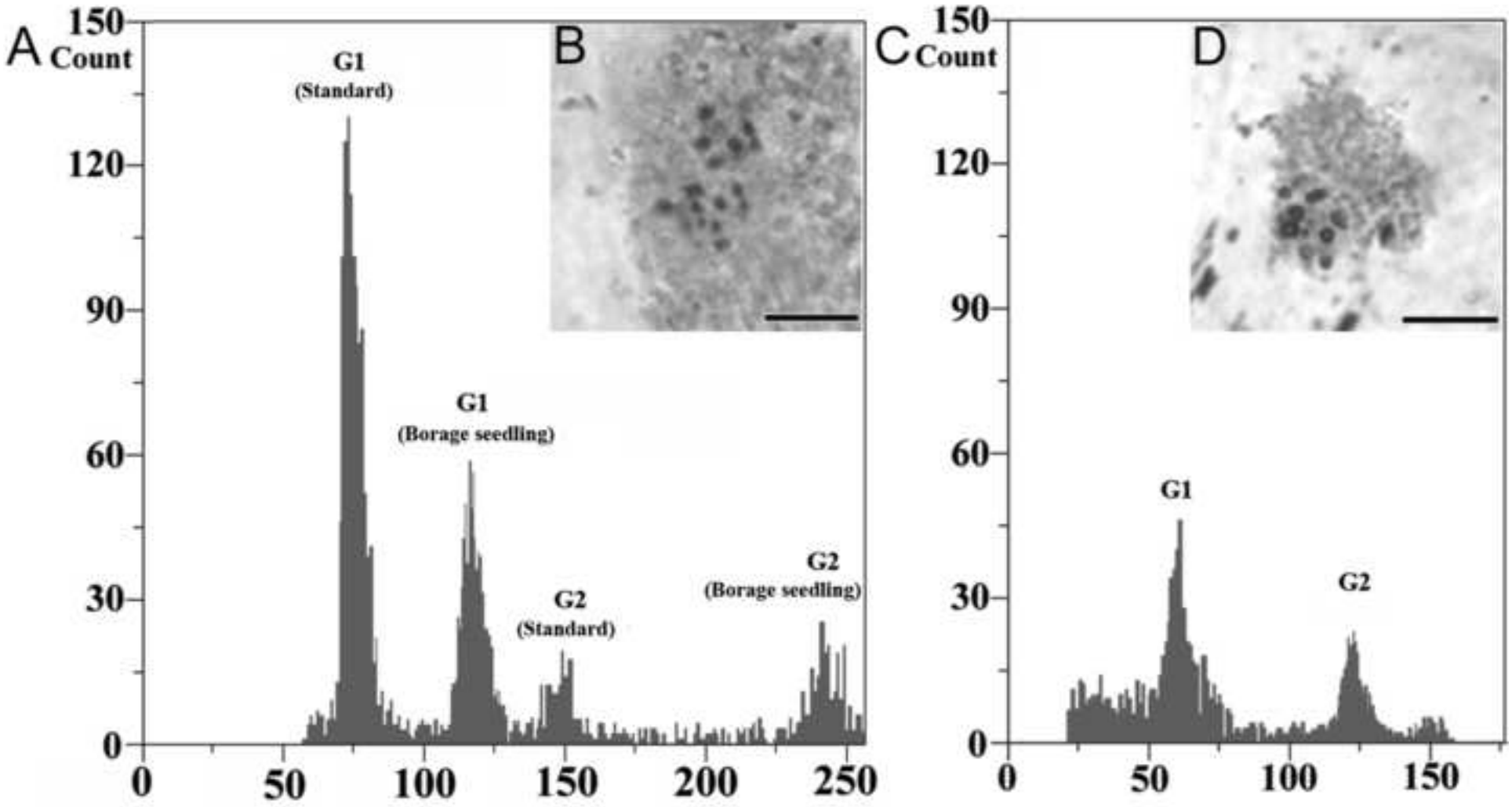
A
Count
150
120
90
60
30
0
G1
(Standard)
G1
(Borage seedling)
G2
(Standard)
G2
(Borage seedling)
0
50
100
150
200
250
B
C
Count
150
120
90
60
30
0
G1
G2
0
50
100
150
D