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Production of doubled haploid plants from anther cultures of borage (*Borago officinalis* L.) by the application of chemical and physical stress

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Abstract

Anther culture can be used as a powerful tool to produce doubled haploid (DH) lines in medicinal plants, thus accelerating breeding programs. In the particular case of borage (*Borago officinalis* L.), a method to produce DH plants has not been yet published. In this work we evaluated the effect of different culture media and of different chemical (colchicine and n-butanol) and physical stresses (centrifugation and electroporation) on androgenesis induction and plant regeneration in borage anther cultures. We found that the highest response can be obtained with culture medium containing B_5 salts and NLN vitamins, the addition of 200 mg/l colchicine during 4 days, a pretreatment of anthers with 0.2% n-butanol for 5 hours, or the application to anthers of single physical stresses (either centrifugation at 300 g or a 100 v electrical shock, but not combined). This is the first report on the production of DH plants in borage. Together, the results presented hereby can be used as a basic framework for large-scale generation of DH plants in this important medicinal species**.**

Keywords: androgenesis, anther culture, callogenesis, centrifugation, colchicine, electroporation, microspore embryogenesis, n-butanol

Introduction

For thousands of years, medicinal plant species have been used to treat human diseases due to the presence of active compounds with beneficial pharmacological properties. The origin of the majority of these plants is wild populations, which have inherent variability in the levels of their active compounds. The lack of uniformity in the levels of such active compounds may result in conflicting clinical trial reports, which may likely decrease the optimal efficiency of the product. This imposes a limitation in the use of wild resources from medicinal plants, and points to genetic research and breeding of these plant species to overcome this bottleneck. Doubled haploid (DH) technology would be beneficial for breeding of medicinal plant species (Ferrie 2009) and for producing uniform lines useful as parents in F1 hybrid production, or as starting materials to develop new varieties with high levels of medicinal compounds (Ferrie 2007). Induction of androgenesis is the most efficient and therefore used way to produce DHs, since it allows for full homozygosity in just one step, and thus reduces considerably the time needed for production of pure lines compared with conventional breeding methods (Germanà 2011). In recent years, androgenesis-based methods such as anther and microspore culture, have been frequently applied to produce DH plants in medicinal plants such as *Carum carvi* (Ferrie et al. 2011); *Gentiana triflora* (Pathirana et al. 2011); *Ammi majus*, *Anethum graveolens*, *Angelica archangelica* and *Foeniculum vulgare* (Ferrie et al. 2011).

Borago officinalis, commonly known as borage, is an edible medicinal plant extended along the Mediterranean basin, Western Asia, and certain regions of North Africa, South American and Continental Europe. Traditionally, it is used in some regions of Spain, Italy, France and Germany as a valuable culinary resource. Most importantly, borage seed is one of the most common commercial sources of gamma-linolenic acid (GLA). GLA is an omega-6 fatty acid

very useful for clinical and pharmaceutical applications due to, but not only, its anti-inflammatory, tumoricidal and anti-angiogenic properties (Das 2006; Kapoor and Huang 2006). However, seed companies usually sell borage seed from wild varieties only, since no breeding programs have been carried out to develop varieties with uniformly high levels of GLA (Montaner et al. 2000). Conceivably, DH technology may help to produce such new, uniform borage varieties.

Induction of androgenesis consists in the reprogramming of microspores from their original gametophytic pathway towards sporophytic development, becoming haploid or DH microspore-derived embryos and eventually plants (Seguí-Simarro 2010). For haploid individuals, additional steps of chromosome doubling may be required to become DHs (Seguí-Simarro and Nuez 2008). One of the major factors influencing such a switch is the application of a stress treatment to entire floral buds, excised anthers or isolated microspores, either before or during *in vitro* culture (Germanà 2011). The selection of the most effective stress is an empirical parameter that must be previously adjusted in order to fully exploit the androgenic potential of a given species. High or low temperatures and sugar starvation are the stresses most commonly used to induce microspore embryogenesis (Shariatpanahi et al. 2006). Aside of them, other stress sources including chemical substances such as colchicine, n-butanol, amiprophosmethyl, orizalin, trifluralin, and cytochalasin (Castillo et al. 2009; Castillo et al. 2014), and also physical stresses such as centrifugation and electroporation (Grewal et al. 2009; Ochatt et al. 2009; Ochatt 2013), have been also used to induce embryogenesis in different plant species. Another influencing factor is the composition of the culture medium (Ferrie 2013). Culture media such as B_5 (Gamborg et al. 1968), MS (Murashige and Skoog 1962), N6 (Chu 1978), NN (Nitsch and Nitsch 1969), and NLN

(Lichter 1982) are some of the basal media most commonly used for anther or microspore culture in different plant species.

Despite their advantages, DH technology has not been fully implemented in borage yet. In a previous study, we demonstrated that production of haploids in borage is possible, since we showed for the first time the production of borage embryo-like structures (ELSs) from anther cultures (Chardoli Eshaghi et al. 2015). However, optimization of the procedure and regeneration of haploid and DH plants remained pending. In the present study, we evaluated the effect of five different culture medium compositions and various chemical (colchicine and n-butanol) and physical (centrifugation and electroporation) stress treatments in the optimization of the protocol of borage anther culture. As a result of this, we were able to regenerate entire haploid and DH plants.

Material and Methods

Plant material

Seeds of a local Iranian borage variety were provided from Pakan Bazr Company, (Isfahan, Iran) and used to obtain anther donor plants. Donor plants were grown in 25 cm pots in a greenhouse from March 2016 to September 2016 under natural light and temperatures ranging between 28–32ºC during the day and 18–24ºC at night. Plants were fertilized with nitrogen, phosphorus and potassium (15:12:24) every three weeks. Male flower buds were harvested from 50-60 day-old plants.

Anther culture

Flower buds were removed from donor plants, surface sterilized and cultured according to Chardoli Eshaghi et al. (2015). Briefly, 5–7 mm-long buds, containing mid-to-late microspores, were excised and surface sterilized in a laminar flow hood by immersion in 70% (v/v) ethanol for 30 s, followed by immersion in 2.5% (v/v) sodium hypochlorite solution for 10 min, and finally rinsed in sterile distilled water three times. After sterilization, petals were aseptically removed using small forceps and anthers were carefully excised and immediately placed in 100 × 15 mm culture dishes containing 20 ml of autoclaved induction medium. 20 anthers were plated per culture dish. Different induction media were used in the different experiments performed, as explained below. In all experiments, anthers were kept at 4^0C in darkness for 5 days, and then transferred to a growth chamber at 24^0C, 16/8 h photoperiod and light intensity of 40 µmol m^{-2} s^{-1}. During this period, anthers were checked on a daily basis. After four weeks, calli and ELSs were placed on regeneration medium containing half strength B_5-NLN, 0.1 mg/l 1-naphthaleneacetic acid (NAA), 1 mg/l benzyl aminopurine (BAP), 30 g/l sucrose and 8 g/l agar, and were kept in the growth chamber with the light and temperature conditions mentioned above.

Experiment I: Type of induction medium

Five different induction media, referred to as KA medium (Keller and Armstrong 1977), AT_3 medium (Touraev et al. 1996), B_5-NLN medium (Chardoli Eshaghi et al. 2015), EDM medium (Phillips and Collins 1979) and NN medium (Nitsch and Nitsch 1969), were tested for callus and ELS induction. All culture media were supplemented with 2 mg/l 2-dichlorophenoxyacetic acid (2,4-D), 1 mg/l BAP, 800 mg/l L-glutamine, 30 mg/l glutathione, 100 mg/l L-serine, 100 mg/l casein hydrolysate, 5 mg/l silver nitrate and 30 g/l maltose. All media were adjusted to pH 5.8 and solidified with 8 g/l agar.

Experiment II: Colchicine treatment

In order to elucidate the influence of colchicine on the androgenic responses of borage anthers, we added filter-sterilized colchicine to the induction medium at different concentrations, including 0 (control), 50, 100 and 200 mg/l. For this and the rest of experiments described below, the induction medium used was B_5-NLN (Chardoli Eshaghi et al. 2015) supplemented, adjusted and solidified as described in *Experiment I.* Anthers in culture dishes with each of the colchicine concentrations used were incubated for 2 and 4 days, and then transferred to fresh B_5-NLN medium without colchicine.

Experiment III: n-butanol pretreatments

To determine the optimal concentration of n-butanol to promote androgenic induction and plant regeneration, we soaked male flower buds at the right developmental stage in liquid B_5-NLN culture medium supplemented with 2 mg/l 2,4- D, 1 mg/l BAP, 90 mg/l maltose and different concentrations of n-butanol, including 0 (control), 0.1, 0.2, 0.3 and 0.4% (v/v). Buds were incubated at 4^0C for 5 h and then surface sterilized as described above. Next, anthers were excised from buds and placed on induction medium without n-butanol.

Experiment IV: Centrifugation and electroporation treatments

Centrifugation treatments were applied to borage anthers according to Grewal et al. (2009). Anthers containing microspores at the stage most suitable for induction were centrifuged at 0, 150, 300 and 600 g for 6 min in 1.5 ml microtubes containing 1 ml of RM-IK liquid culture medium prepared as described by Grewal et al. (2009). A VS-180CFI centrifuge (Vision Scientific Co., LTD) was used for anther centrifugation. Centrifuged anthers were then electroporated at various voltages (0, 100, 150 and 200 v) in 1.5 ml Eppendorf microtubes containing 800 µl of RM-IK liquid culture medium. Three successive exponential pulses of

each voltage were given by a hand-made analogue pulse generator with 2 mm electrodes, 25 lF capacitance and 25x resistance. Anthers were then dried on a sterile Whatman filter paper and transferred to induction medium. The rest of the culture proceeded as described above for the general anther culture procedure.

Statistical analysis

For all the experiments presented in this study, a complete randomized design was implemented. Each experiment consisted of 4 replicates, and each replicate consisted of one culture dish containing 20 anthers. The percentage of anthers producing callus (C%), the mean number of ELSs per anther (ELS/A) and per callus (ELS/C) were calculated after 30 days of culture initiation. For each experiment, we also assessed the viability of induced calli (CV, measured as the number of days in which calli were still viable) and the percentage of plant regeneration per 100 anthers (PR%). Data were analyzed by analysis of variance (ANOVA) using SPSS v. 16.0. Non-normally distributed percentage data in residual plot analysis were square root transformed before ANOVA. Mean values were evaluated at $p \leq 0.05$ and compared using Duncan's multiple range test (DMRT).

Ploidy analysis

Ploidy analysis was conducted using two parallel methods: flow cytometry and measurement of stomatal guard cells. For flow cytometry, we used a Partec Ploidy Analyzer (Partec GmbH, Germany) as described by Chardoli Eshaghi et al. (2015). Leaf pieces (~1 cm^2) excised from anther-derived plantlets and control diploid borage plants were chopped with a razor blade and incubated at 8ºC with 2 ml of DAPI staining solution (Partec GmbH, Germany) in a plastic culture dish. The solution was filtered through a 50 µm nylon filter to remove large cellular debris and the filtrate was loaded into the Ploidy Analyzer. For measurement of

stomatal guard cells, the epidermal layer from the abaxial leaf surface of diploid donor and anther-derived plantlets was separated with a scalpel and tweezers under a binocular microscope. Aside of the diploid control plantlet, ten anther-derived plantlets were randomly chosen for analysis. From each of them, five stomatal guard cells were observed with a Nikon YS2-T light microscope at 100x magnification, and their width and length were measured.

Results

As a basis for the different experiments shown hereby, we used the standard medium previously set up by Chardoli Eshaghi et al. (2015). In these reference conditions, borage anthers swelled five days after plating on induction medium (Fig. 1A). After approximately two weeks of culture, anthers burst and calli popped out of them (Fig. 1B). Then, they continued growing to produce large, irregular callus masses (Fig. 1C). Two weeks after callus emergence, globular ELSs were observed to generate on the surface of necrosing calli (Fig. 1D). After two additional weeks (Figs. 1E-G), these ELSs became elongated and more differentiated embryos, where hypocotyl (Fig. 1E), cotyledon primordia (Fig. 1F) and radicles (Fig. 1G) could be identified. Transformation of ELSs into shoots (Figs. 1H) and plantlets (Fig. 1I) occurred upon transference to regeneration medium as described in Materials and Methods. Next we present the results obtained after modifying some of the factors involved in this morphogenic process.

Effect of culture medium composition

In the first experiment, we evaluated the androgenic response of borage anthers cultured using five different basal culture media (Table 1). The highest percentage of calli was obtained

when using AT3 medium. However, B_5-NLN medium produced the maximum number of ELSs (both in terms of ELS/A and ELS/C). This medium also provided the highest callus viability (up to 30 days) compared to other media. Therefore, we used B_5-NLN medium as the basis for all next experiments.

Effect of colchicine pretreatment

Colchicine at different concentrations and durations was applied to borage anthers (Table 2). Media containing 200 mg/l colchicine for 2 and 4 days and 100 mg/l colchicine for 4 days resulted in the highest percentages of callogenesis compared to control and other colchicine treatments. Calli produced with 200 mg/l colchicine during 4 days continued growing after 60 days from anther culture initiation. This medium significantly enhanced the frequency of ELS formation (0.22 ELS/A and 0.49 ELS/C) and plant regeneration, producing 10 regenerated, entire plantlets (0.17 PR%). In control and other colchicine treatments, calli died with very few or no embryo formation.

Effect of n-butanol pretreatment

Treatment of borage anthers with 0.2% and 0.3% n-butanol resulted in a significant increase in callus induction compared to the rest of assays (Table 3). The highest production of ELSs was achieved with 0.2% n-butanol, yielding 0.24 ELS/A, and 0.61 ELS/C. In addition, this concentration of n-butanol significantly enhanced the viability of induced calli (up to 50 days from culture initiation) compared to the rest of assays, and gave rise to two regenerated plants (0.03 PR%).

Effect of centrifugation and electroporation

In this experiment, a combination of two physical stresses, centrifugation and electroporation, were tested to evaluate their effects on androgenesis induction (Table 4). The highest percentages of callogenesis were obtained when anthers were centrifuged at 300 rpm for 10 min without electroporation (56.25%), or with an electrical current of 100 v and no centrifugation (47.50%). A 100 v discharge alone gave also rise to the highest values of ELS/A (0.80), ELS/C (1.81), callus viability (CV= 70) and plant regeneration, with 17 entire plants regenerated (PR%= 0.21). Centrifugation of anthers at 300 rpm without electric discharge also improved significantly ELS/A, ELS/C, CV and PR% rates, eventually yielding 14 regenerated plantlets, but they were below the results obtained with 100 v and centrifugation. Therefore, we could conclude that the most effective treatment is the application of a 100 v electric discharge.

Ploidy analysis

From the different experiments described above we obtained a total of 43 regenerated borage plantlets. Unfortunately, all of them died after transplanting, once in the greenhouse, due to an uncontrolled pest. Nevertheless, we were able to study with flow cytometry and light microscopy to analyze their ploidy before losing them. For flow cytometry analysis, we took a young plantlet as a diploid reference, showing G1 and G2 peaks at gains of ~121 and ~242, respectively (Fig. 2A). The analysis of the 43 regenerated plants (Table 5) showed that 28 of them (65.12%) were haploid, with G1 and G2 peaks at ~61 and ~122 gains, respectively (Fig. 2B), and the remaining 15 plants (34.88%) were diploid, with histograms equivalent to that shown in Fig. 2A for the diploid reference. To test whether microscopic analysis of leaf

stomata could also serve as an estimator of ploidy, we measured the length and width of leaf stomata from haploid and diploid plants, finding that they were significantly different in size. Stomatal guard cells of diploid regenerant plantlets (Fig. 2C) had an average length and width of 26.24 μm and 17.46 μm, respectively, whereas those of haploid leaves (Fig. 2D) had smaller stomata, averaging 19.82 μm length and 16.21 μm width.

Discussion

In this work, we evaluated the effect of different chemical and physical factors potentially affecting the efficiency of DH production in borage anther cultures. Chemical factors include culture medium composition, colchicine and n-butanol, while physical factors include centrifugation and electroporation. Among the five culture media tested in this work, the most effective to promote callus induction and ELS formation in borage anther culture was B_5-NLN, containing B_5 salts and NLN vitamins (Chardoli Eshaghi et al. 2015). Although there were other medium compositions (AT3) that resulted in higher callus percentages, B_5-NLN was, by far, the medium that produced calli with highest survival times (about 30 days), which most likely accounted for the highest rate of ELSs obtained with this medium. Aside of other subtle differences, the principal differences between B_5-NLN and the other media used relate to an increased KNO_3 concentration, and reduced NH_4NO_3 and $CaCl_2.2H_2O$ concentrations. Although in general, medium composition uses not to be the most determinant factor, the levels of these three salts may be better adjusted to the particular needs of borage microspores, thereby accounting for the improvement observed in terms of callus viability and ELS formation.

Colchicine, a microtubule-depolymerizing drug, is typically used after induction to block cytokinesis, allowing for nuclear coalescence and eventual fusion, and therefore promoting chromosome doubling in haploid embryos (reviewed in Seguí-Simarro and Nuez 2008). Obviously, this later application has no effect on the initial induction rate. However, application of colchicine to anther cultures during the induction phase is known to enhance the rate of androgenesis induction in a wide range of species including rapeseed (Zhou et al. 2002), tomato (Seguí-Simarro and Nuez 2007), wheat (Szakács and Barnabás 1995; Soriano et al. 2007), rice (Alemanno and Guiderdoni 1994) and maize (Obert and Barnabás 2004). In borage, colchicine application during four days seemed to have a better effect than only two days. Our results showed that the most positive effect on callus induction, ELS formation, callus viability and plant regeneration rate was provided by 200 mg/l for 4 days. Different hypothesis have been proposed to explain such a beneficial effect. Some of them point to an increase in the frequency of symmetric cell divisions instead of the typical, asymmetric pattern of the first pollen mitosis (Szakács and Barnabás 1995). Other hypothesis relate to a more general cytoskeletal restructuration produced by colchicine-mediated microtubule depolymerization (Shariatpanahi et al. 2006). However, an impact of this early application on chromosome doubling has not yet been demonstrated. Indeed, our results showed that among the chemical stresses tested, only colchicine was able to produce DHs, but the rate, revolving around 35-40%, was comparable to that produced by the use of physical stresses as centrifugation or electroporation.

Similar to colchicine, n-butanol was proposed to induce androgenesis due to its ability to disrupt cytoskeletal microtubules (Soriano et al. 2008; Fábián et al. 2015). These authors also proposed that n-butanol would be related to an increased rate of autophagy which would also contribute to the switch from gametophytic to sporophytic development. Indeed, n-butanol

309 was previously used to increase the androgenic response in cereals such as wheat (Soriano et
310 al. 2008), maize (Fábián et al. 2015) and barley microspores (Castillo et al. 2014). In this
311 work, we demonstrated that a pretreatment of borage anthers with 0.2% n-butanol
312 significantly increased all the parameters measured, being the only concentration that gave
313 rise to regenerated plants. Indeed, this is the first report describing positive effects of n-
314 butanol in a dicot plant species in terms of androgenesis induction, but also in terms of callus
315 viability and plant regeneration.
316
317 Perhaps, the most remarkable results of this study pertain to the use of physical stresses to
318 induce androgenesis. The potential of electroporation in plant tissue culture seems largely
319 underexploited (Ochatt 2013). Indeed, in the context of androgenesis induction it is still a
320 neglected source of stress, having been successfully used only in chickpea anther cultures
321 (Grewal et al. 2009), asparagus isolated microspore cultures (Delaitre et al. 2001), and field
322 pea, grass pea and *Medicago truncatula* (Ochatt et al. 2009). Grewal et al. (2009) proposed
323 that the positive effects of electroporation on androgenesis induction would be due to the
324 transient formation of pores in the plasma membrane, through which the entry to the anther
325 locule of culture medium components, including hormones, would be facilitated. This implies
326 that this might potentially be useful for all the species. However, a particular adjustment to
327 each species is mandatory in order to achieve beneficial results. Application of an electrical
328 shock needs to be long enough to let cells form pores, but short enough to allow cells reseal
329 pores without cell disruption. In addition, it is possible that anther wall cells, more sensitive to
330 electrical current than exine-covered microspores, may die and release harmful or inhibitory
331 substances to the medium (Lulsdorf et al. 2011), thereby reducing the androgenic response.
332 Therefore, the optimum range and duration must be determined for each plant species. In our
333 work, we showed that electroporation with 100 v had a markedly beneficial effect for ELS

334 formation (0.8 ELS/A) and plant regeneration (21.25%). This treatment enhanced the
335 frequency of ELS induction up to four fold compared to the best results of the previous study
336 on borage (Chardoli Eshaghi et al. 2015). Considering its effects over all the parameters
337 studied, this is the most beneficial factor revealed by this study. However, it must be noted
338 that electroporation devices may not be accessible to all laboratories working on androgenesis
339 induction in recalcitrant species. Considering this, the use of 300 g centrifugation might be an
340 interesting alternative, since centrifuges are by far more available, and the results in terms of
341 regenerated plants (0.21% for electroporation vs 0.18% for centrifugation) were not
342 significantly different.
343
344 Centrifugation is one of the most widely used stresses, since its use is mandatory in isolated
345 microspore cultures. However, it is not clear to what extent it influences the induction rate. In
346 anther cultures, centrifugation is not mandatory. This is why it has been possible to test its
347 effects when added to the induction protocol. This way, positive effects have been reported in
348 anther cultures of tobacco (Tanaka 1973), lupin (Bayliss et al. 2004) and chickpea (Grewal et
349 al. 2009). As in the case of electroporation, it is important to adjust very well the parameters
350 involved, since very disparate g values have been shown to be effective in different species
351 and even different genotypes, ranging from 10,000-11,000 g (30 min) in tobacco (Tanaka
352 1973) to 671 g (3 min) and 168 g (6 min) for two different chickpea cultivars (Grewal et al.
353 2009). Our study showed that in the case of borage, the best conditions imply the use of 300 g
354 during 6 min. Above or below 300 g, no plants were regenerated by centrifugation alone. It is
355 also interesting to note that the best results with physical stresses were obtained when only
356 one stress was applied at a time. No centrifugation + electroporation combinations
357 overperformed the individual use of these stresses. In other recalcitrant species such as *Pisum*
358 *sativum*, it was shown that the combination of different abiotic stresses work additively to

359 elicit or enhance androgenesis induction (Ribalta et al. 2012). This might also be true for
360 borage. However, it seems that the combined use affects excessively to cell viability, as
361 revealed by the low values of the parameters studied in nearly all combinations. Thus,
362 although both stresses might possibly have additive effects for androgenesis induction, these
363 effects would be counteracted by the viability loss.
364
365 In a previous study about induction of borage androgenesis (Chardoli Eshaghi et al. 2015), all
366 the ELS and most of the calli analyzed were haploid. In this study, we showed that ~65% of
367 regenerated plantlets were haploid, and ~35% were diploid. Diploids may come either from
368 genome duplication of haploid ELSs, which would make them DHs, or from proliferation of
369 somatic anther tissues, which would make them useless for our purposes. Ideally, the best way
370 to find out the origin of regenerants is to use microsatellite (SSR) molecular markers.
371 Unfortunately, to the best of our knowledge there are no SSR available for borage. The only
372 available molecular markers are AFLPs (De Lisi et al. 2014) and RAPDs (Sales et al. 2008).
373 However, AFLPs and RAPDs are not codominant, so they are not useful to differentiate
374 between DHs and somatic diploids. Anyway, we can rely on the haploid origin of the diploid
375 regenerants and assume they are DH, because both haploid and diploid embryos are
376 remarkably similar in morphology, indicating that they seem to have a same origin. The fact
377 that haploids account for nearly 66% of the total make reasonable that some of them have
378 duplicated their genome spontaneously. Besides, in our experience with different recalcitrant
379 species, we never observed the occurrence of embryos on the surface of diploid, anther wall-
380 derived callus. Typically, diploid (somatic) plants regenerate through organogenesis, and the
381 only embryogenic process we have observed are either direct embryogenesis from
382 microspores or indirect embryogenesis from microspore-derived callus. This is why we think
383 our diploid plantlets are actually DH. This study also showed that, irrespectively of the stress

used, the haploid/DH ratio is approximately 2:1. This may be enough to produce DHs at a low scale for research purposes. However, it may be insufficient to implement this protocol at a large scale for industrial borage DH production. In this case, the addition of a chromosome doubling step to the protocol should be considered.

In parallel to flow cytometry, we checked the possibility of using stomatal size as a parameter to distinguish between haploids and diploids. Although this parameter is not universally applicable, it has been proven useful in some species (Uno et al. 2016). We confirmed that it can also be applied to borage, since all the stomata measured in haploid borage leaves were smaller than those of diploid or DH leaves. Thus, although flow cytometry is always faster and more reliable and accurate, the measurement of stomatal sizes could be a simpler, cheaper and more accessible approach to estimate ploidy in those labs where access to a flow cytometer is difficult or impossible.

Concluding remarks

Androgenesis induction in borage was first demonstrated in 2015 by Chardoli Eshaghi et al. (2015). Hereby we demonstrated for the first time the successful conversion of androgenic embryos into DH plantlets. In addition, we showed that efficiency can be improved by the use of different chemical and physical stresses, as follows: a 5-hour pretreatment of flower buds with 0.2% n-butanol, the addition of 200 mg/l colchicine for 4 days during the induction stage, a 6-min centrifugation of anthers at 300 g, or the application of a 100 v electrical shock to anthers. They open the door for a larger scale production of borage DH lines to be used in breeding programs aimed to improve, for example, GLA biosynthesis.

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Tables

Table 1. Effect of different anther culture media on the efficiency of androgenesis induction, expressed as percentage of anthers producing callus (C%), mean number of ELSs per anther (ELS/A) and per callus (ELS/C), and callus viability (CV). Different letters for each column denote significant differences (Duncan's multiple range test, $p \leq 0.05$).

Culture medium	C%	ELS/A	ELS/C	CV
KA	10.00 c	0.00 b	0.00 b	5.00 c
AT3	33.12 a	0.01 ab	0.05 b	12.00 b
B_5-NLN	23.75 b	0.04 a	0.16 a	30.00 a
EDM	2.50 d	0.00 b	0.00 b	1.50 d
NN	13.12 c	0.00 b	0.00 b	6.00 c

Table 2. Effects of different colchicine treatments on the efficiency of androgenesis induction and plant regeneration, expressed as percentage of anthers producing callus (C%), mean number of ELSs per anther (ELS/A) and per callus (ELS/C), callus viability (CV) and the percentage of plant regeneration (PR%). Different letters for each column denote significant differences (Duncan's multiple range test, $p \leq 0.05$).

Colchicine treatment		C%	ELS/A	ELS/C	CV	PR%
Control		23.33 c	0.02 b	0.08 b	12 cd	0.00 b
50 mgl^{-1}	(2 days)	28.33 c	0.00 b	0.00 b	7 d	0.00 b
100 mgl^{-1}	(2 days)	25.00 c	0.00 b	0.00 b	8 cd	0.00 b
200 mgl^{-1}	(2 days)	48.33 a	0.00 b	0.00 b	14 c	0.00 b
50 mgl^{-1}	4 days)	33.33 bc	0.00 b	0.00 b	8 cd	0.00 b
100 mgl^{-1}	(4 days)	45.00 ab	0.02 b	0.03 b	30 b	0.00 b
200 mgl^{-1}	(4 days)	45.00 ab	0.22 a	0.49 a	60 a	0.17 a

Table 3. Effect of different n-butanol pretreatments on the efficiency of androgenesis induction and plant regeneration, expressed as percentage of anthers producing callus (C%), mean number of ELSs per anther (ELS/A) and per callus (ELS/C), callus viability (CV) and the percentage of plant regeneration (PR%). Different letters for each column denote significant differences (Duncan's multiple range test, $p \leq 0.05$).

n-butanol treatment	C%	ELS/A	ELS/C	CV	PR%
Control	21.25 b	0.01 b	0.06 b	14 c	0.00 b
0.1%	26.25 b	0.01 b	0.05 b	17 c	0.00 b
0.2%	41.25 a	0.24 a	0.61 a	50 a	0.03 a
0.3%	41.25 a	0.05 b	0.11 b	30 b	0.00 b
0.4%	26.25 b	0.00 b	0.00 b	6 d	0.00 b

Table 4. Effect of combined centrifugation and electroporation on the efficiency of androgenesis induction and plant regeneration, expressed as percentage of anthers producing callus (C%), mean number of ELSs per anther (ELS/A) and per callus (ELS/C), callus viability (CV) and the percentage of plant regeneration (PR%). Centrifugation forces are expressed in g units, and electrical current in volts (v), Different letters for each column denote significant differences (Duncan's multiple range test, $p \leq 0.05$).

Centrif.	Current	C%	ELS/A	ELS/C	CV	PR%
0	0	32.50 b	0.03 c	0.08 c	12 de	0.00 b
	100	47.50 a	0.80 a	1.83 a	70 a	0.21 a
	150	36.25 b	0.00 c	0.00 c	18 c	0.00 b
	200	28.75 bc	0.00 c	0.00 c	15 cde	0.00 b
150	0	33.75 b	0.00 c	0.00 c	17 cd	0.00 b
	100	28.75 bc	0.00 c	0.00 c	14 cde	0.00 b
	150	20.00 cd	0.01 c	0.08 c	14 cde	0.00 b
	200	17.50 de	0.01 c	0.08 c	10 ef	0.00 b
300	0	56.25 a	0.44 b	0.74 b	50 b	0.18 a
	100	36.25 b	0.23 bc	0.60 bc	11 ef	0.00 b
	150	31.25 b	0.03 c	0.06 c	10 ef	0.00 b
	200	21.25 cd	0.00 c	0.00 c	10 ef	0.00 b
600	0	37.50 b	0.04 c	0.08 c	18 c	0.00 b
	100	15.00 de	0.00 c	0.00 c	7 fg	0.00 b
	150	8.75 ef	0.00 c	0.00 c	5 g	0.00 b
	200	3.75 f	0.00 c	0.00 c	5 g	0.00 b

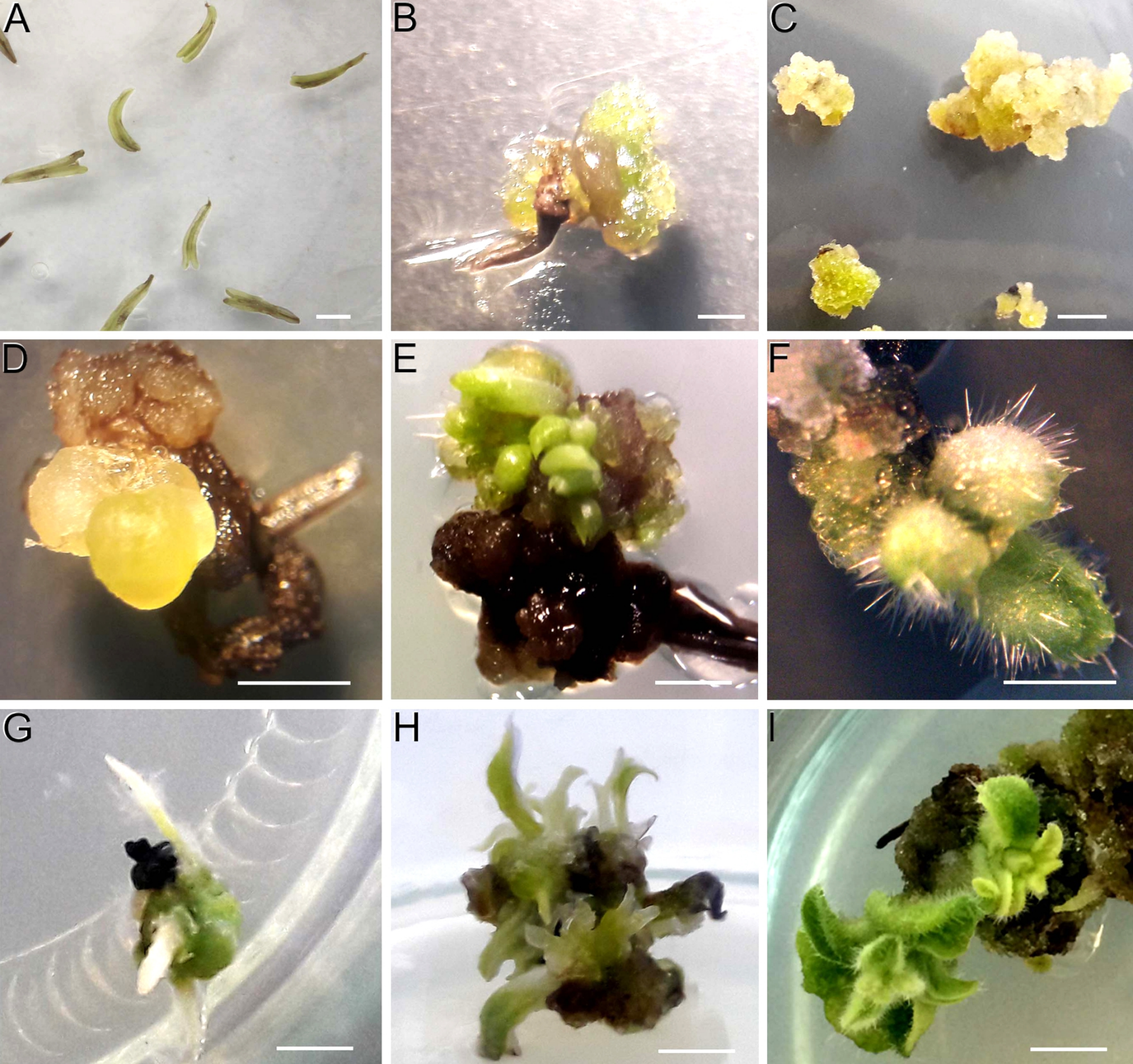
Table 5. Ploidy levels of anther culture-derived plantlets.

Experiment (treatment)	Number of regenerated plants		Haploid/Diploid %
	Haploid	Diploid	
Exp1 (Culture medium)	0	0	0
Exp 2 (Colchicine)	6	4	60/40
Exp 3 (n-butanol)	2	0	100/0
Exp 4 (Centrifugation and electroporation)	20	11	64.52/35.48
Total	28	15	65.12/34.88

Figure legends

Fig. 1. Callus production, ELS development and plantlet regeneration from borage anther cultures. A: 5-day old anther culture. B: Callus emerged from anther after14 days of culture. C: Individualized calli 28 days after anther inoculation. D: Globular ELS formed on the surface of a necrosing callus, 2 weeks after callus induction. E-G: ELSs arisen from calli 4 weeks after callus induction. Note the presence of differentiated hypocotyls (E), cotyledons (F) and radicles (G). H: Shoots emerging from a necrotic callus. I: Individualized plantlet growing *in vitro*. Bars: A-G: 1 mm; H, I: 1 cm.

Fig. 2. Ploidy levels in leaves of control diploid (A, C) and haploid (B, D) borage plantlets, analyzed by flow cytometry (A, B) and measurement of the size of stomatal guard cells (C, D). Bars: 20 µm.


A
B
C
D
E
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G
H
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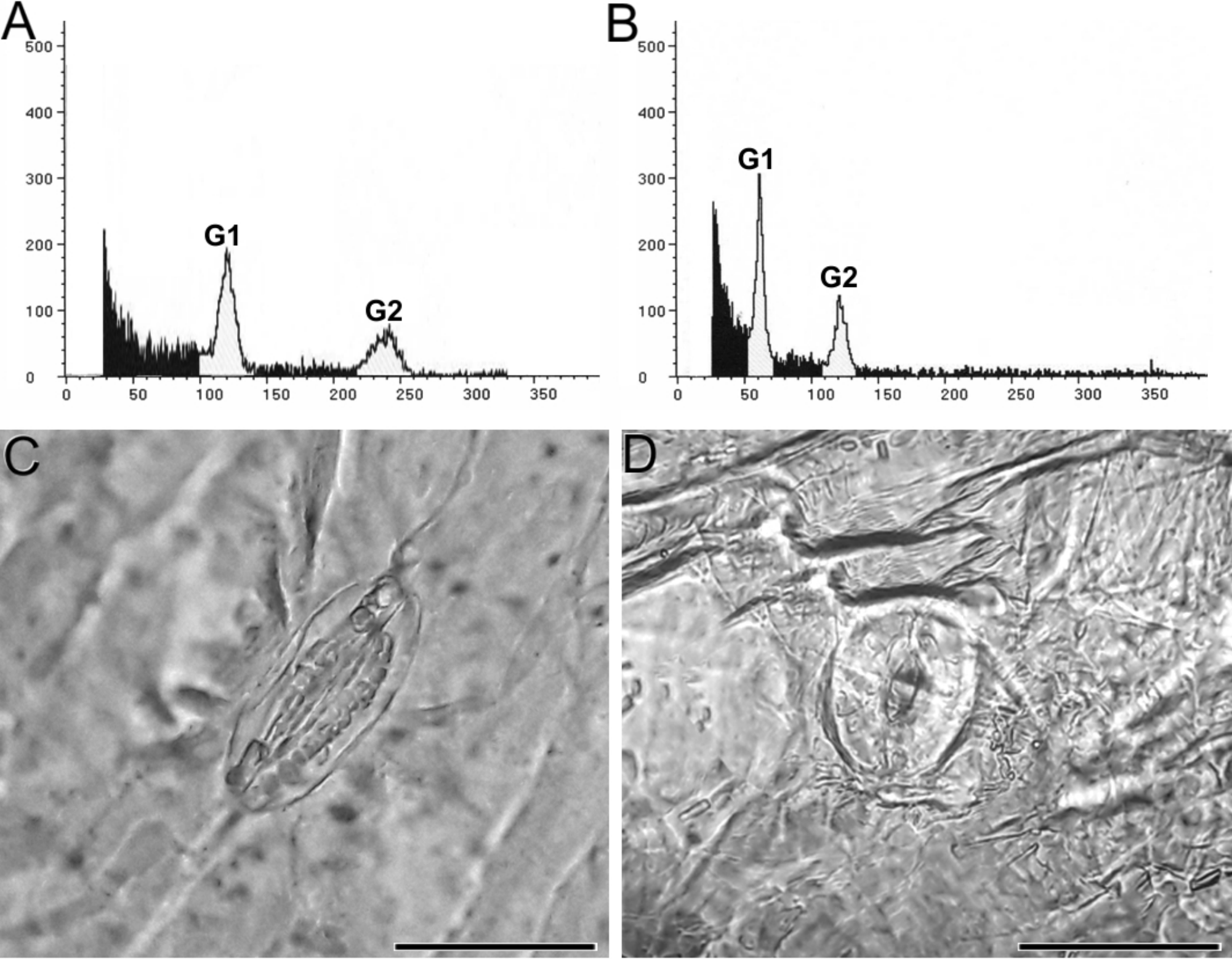
A
500
400
300
200
100
0
G1
G2
0
50
100
150
200
250
300
350
B
500
400
300
200
100
0
G1
G2
0
50
100
150
200
250
300
350
C
D