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Additional Information

1 **Assessment of different anther culture approaches to produce doubled haploids in cucumber**
2 **(*Cucumis sativus* L.)**
3
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14 AA performed all the experimental work and analyzed the results. AZ, MRA and JMSS designed
15 the experimental work and analyzed the results. JMSS wrote the manuscript.
16

Abstract

Cucumber is one of the most important vegetable crops worldwide, which makes it a good candidate to produce doubled haploid (DH) lines to accelerate plant breeding. Traditionally, these approaches involved induction of gynogenesis or parthenogenesis with irradiated pollen, which carries some disadvantages compared to androgenesis. Despite this, studies on anther/microspore cultures in cucumber are surprisingly scarce. Furthermore, most of them failed to unambiguously demonstrate the haploid origin of the individuals obtained. In this work we focused on anther cultures using two cucumber genotypes, different previously published protocols for anther culture, different *in vitro* culture variants to make it more efficient, and most importantly, a combination of flow cytometry and microsatellite molecular markers to evaluate the real androgenic potential and the impact of anther wall tissue proliferation. We developed a method to produce DH plants involving a bud pretreatment at 4ºC, a 35ºC treatment to anthers, culture with BAP and 2,4-D, and induction of callus morphogenesis by an additional 35ºC treatment and sequential culture first in liquid medium in darkness and second in solid medium with light. We also found that factors such as genotype, proliferation of anther wall tissues, orientation of anthers in the culture medium and growth regulator composition of the initial anther culture medium have a remarkable impact. Our rate of chromosome doubling (81%) was high enough to exclude additional chromosome doubling steps. Together, our results present androgenesis as an improvable but yet more convenient alternative to traditional gynogenesis and parthenogenesis-based approaches.

Keywords: Androgenesis, callogenesis, chromosome doubling, cucurbits, embryogenesis, organogenesis.

Introduction

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable crops worldwide, being used as food for at least 3000 years. According to the most recent data from the Food and Agriculture Organization of the United Nations (FAOSTAT 2018), cucumber ranks 6th in area harvested, 3rd in production, 2nd in yield among all the vegetables cultured in the world, and accounts for 7.5% of the total world vegetable production. Within cucurbit crops, cucumber is the most important. Indeed, cucumber accounts for 51.8% of the total area harvested and 75.2% of the total production of cucurbits. Conceivably, such a global importance explains why this crop has been subjected to genetic breeding by numerous private seed companies and public institutions in order to produce new, improved varieties and highly homogeneous and vigorous F1 hybrid seed. As in any other crop, hybrid production relies on the previous existence of pure, highly homozygous parent lines. In other crops, breeders are taking advantage of doubled haploid (DH) technology to produce pure lines in a faster and cheaper way, as compared with classic breeding approaches (Seguí-Simarro 2016 and references therein). However, DH technology has not been fully explored in cucumber yet.

In the last years, two reviews have summarized the advances in DH technology in cucumber, and in cucurbits in general (Gałązka and Niemirowicz-Szczytt 2013; Dong et al. 2016). These reviews reveal that compared to the efforts devoted to develop DH technologies in other species with similar or even lower agronomic interest, the number of publications addressing this issue in cucumber is surprisingly reduced. Basically, there are three different approaches to DH technology that have been applied to cucumber in the last decades: (1) *in vivo*-induction of parthenogenic embryos by pollination with irradiated pollen followed by *in vitro* embryo rescue, (2) *in vitro* induction of gynogenesis by culturing ovules or entire ovaries, and (3) *in vitro* induction of androgenesis by culturing entire anthers or isolated microspores (Gałązka and Niemirowicz-Szczytt 2013; Dong et al. 2016). Traditionally, the different attempts to develop useful DH technologies in cucurbits, including cucumber, have used approaches based on pollen irradiation or gynogenesis (Sauton and Dumas de Vaulx 1987; Ficcadenti et al. 1999; Lotfi et al. 2003; Claveria et al. 2005). These, indeed, are the most studied approaches, where most progress has been made. However, these approaches are technically challenging and have the additional limitation of the reduced number of egg cells present in a single female flower, as compared with the enormously higher number of microspores potentially inducible in the anthers of a single male flower. This limitation may explain why the efficiencies obtained in these cases are far from optimal. Despite this, very few studies reported

76 attempts to produce DH via androgenesis in cucumber (Ashok Kumar and Murthy 2004; Song et al. 2007; Hamidvand et al. 2013; Abdollahi et al. 2016; Xie et al. 2005; Zhan et al. 2009). It is easy to deduce that, considering the importance of cucumber, many researchers have tried it, but very few have succeeded. In other words, it seems that, in general, cucumber is extremely recalcitrant to DH induction via androgenesis, which prevents cucumber breeders from using a very powerful tool to speed up the release of new hybrid varieties in a cheaper and more sustainable manner. In addition, most of the few previous studies failed to unambiguously demonstrate the haploid nature of the plants obtained. In some cases, using the chromosome count technique, haploid cells were found in *in vitro*-regenerated plants, which led the authors to assume that these plants were DH. Since no microsatellite (SSR) molecular marker analysis was done in these studies, there are reasonable doubts in many of these cases about the true haploid origin of the plants obtained.

All this considered, we decided in this work to explore the possibilities of anther culture in two different cucumber backgrounds, a worldwide-known F1 hybrid and an Iranian landrace, in order to evaluate their current potential and most importantly, future directions to follow in order to optimize these techniques. For this, we first evaluated the efficiency of the previously published anther culture protocols in our genotypes, and then applied some variations to the protocols with the aim to make them more efficient. In addition, we evaluated the impact of anther walls in the production of somatic, non-DH, callus production by combining the use of the most powerful, unambiguous and resolutive techniques to assess the origin of anther-derived structures: flow cytometry and SSR molecular markers. This study is the first time that, to the best of our knowledge, these two techniques have been used to evaluate the occurrence of true haploids and DH plants in cucumber anther cultures. Together, our results demonstrate that anther culture is a convenient option to obtain DHs in cucumber, but there are specific limitations yet to overcome.

Materials and Methods

Plant materials

The cucumber (*Cucumis sativus* L.) Beta Alpha F1 hybrid and the Iranian landrace Esfahani were used in this study. Plants were grown in 30 cm pots under natural light and controlled temperature (20-25ºC) at the greenhouses of the COMAV Institute at Universitat Politècnica de València (Spain) during the months from October 2016 to July 2017.

Anther culture

110 As a general procedure for all anther culture experiments, male flower buds containing microspores
111 at the mid to late uninucleate (vacuolate) stages (10–15 mm in length) were collected, placed into
112 double layered, moistened cheesecloth, deposited in a glass plate and cold pretreated at 4°C for 2
113 days, unless otherwise stated in each particular experiment. Buds were surface sterilized with 70%
114 ethanol for 30 s and then with 4 g/l sodium hypochlorite for 5 min, followed by three rinses in
115 sterile water. Then, anthers were excised and plated in 100×15 mm petri dishes containing basal MS
116 salts and vitamins (Murashige and Skoog 1962), 3% sucrose and 0.8% agar, pH 5.8. Anthers were
117 exposed to 35ºC for one hour, cultured, and then subcultured every 21 days with freshly prepared
118 medium until they produced calli and/or embryos. Based on this general scheme, the following
119 variations were assessed:

120

121 *Experiment 1: Assessment of previously published protocols*

122 We evaluated the efficiency in our cucumber genotypes of 10 different specific protocols previously
123 used for anther culture in different cucurbit species. For each method and genotype, three replicate
124 dishes (10 anthers/replicate) were performed. The different combinations of time, temperature,
125 basal medium, supplements and growth regulators, as well as their respective references, are
126 detailed in Table 1.

127

128 *Experiment 2: Assessment of different growth regulators in the induction medium*

129 We tested in Esfahani and Beta Alpha the effect in callus induction of 25 different combinations of
130 BAP and 2,4-D concentrations, and of 10 different combinations of BAP and NAA concentrations,
131 as described in Table 2. All other medium parameters were kept fixed as follows: basal MS salts
132 and vitamins (Murashige and Skoog 1962), 3% sucrose and 0.8% agar, pH 5.8. Three replicates (20
133 anthers/replicate) were performed. All other experimental parameters were kept as described above.
134 After six weeks of culture, the number of induced calli was counted.

135

136 *Experiment 3: Assessment of different anther orientations*

137 We performed cultures where anthers were oriented in six different positions with respect to the
138 solid medium. According to the anther regions defined in Figure 3A, we oriented anthers facing the
139 solid medium by (1) their outer side (the side close to the petals), (2) their outer long arm, (3) their
140 inner side (the side opposite to the petals), (4) their inner short sac, (5) their proximal end (the end
141 close to the filament), and (6) their distal end (the end opposite to the filament). In all cases, 30
142 anthers (three replicates of 10 anthers/replicate) per genotype and anther orientation were cultured
143 on solid (0.8% agar) basal MS culture medium supplemented with 0.91 mg/l BAP and 0.25 mg/l

2,4-D and 3% sucrose, pH 5.8. A second culture batch of 10 anthers was performed in the same culture conditions, with anthers facing the solid medium by their long sac. After three days of culture, a scratch on the long sac wall was made with a scalpel.

Assessment of plant regeneration medium composition and culture conditions

For these experiments, we cultured a second batch of anthers, distributed in different dishes containing M18-M25 media. Three dishes for each of the eight media were used. As soon as 1-2 calli were produced in each medium, a fragment of each was excised and analyzed by flow cytometry. Those showing a diploid/DH DNA content were fragmented and subcultured to produce clonal callus populations to serve as experimental material. For plant regeneration from anther-derived calli, we tested different culture media including:

- Factorial combinations of NAA (0.05 and 0.1 mg/l) and BAP (2, 3 and 4 mg/l), and of liquid (without agar) and solid medium (adding 0.8 g/l agar). The rest of culture media conditions were fixed as follows: basal MS salts and vitamins, 3% sucrose and pH 5.8. For all combinations, calli were exposed to an additional heat shock at 35ºC during 1 h and kept at 25ºC for 20 days under a 12/12 light photoperiod. Three replicate dishes per combination were used. In each dish, eight different diploid/DH calli were cultured, coming from each of the eight different callus populations obtained from the eight different anther culture media (M18-M25). After 20 days of culture, all the calli of each dish were fragmented in two halves. Eight halves of a dish were transferred to freshly prepared liquid medium, and the other eight halves to freshly prepared solid medium (0.8 g/l agar), keeping unchanged the rest of culture conditions. These dishes were cultured for 20 additional days. This way, for each growth factor combination we produced four additional culture variables: (1) culture first in solid and then in solid media, (2) culture first in solid and then in liquid media, (3) culture first in liquid and then in liquid media, and (4) culture first in liquid and then in solid media.
- Factorial combinations of NAA (0.05 and 0.1 mg/l) and BAP (2, 3 and 4 mg/l). Three replicate dishes per combination were used. In each dish, we cultured five different diploid/DH calli coming from the clonal callus populations. Dishes were cultured for 20 days in liquid medium and darkness, and then transferred to solid (0.8 g/l agar) medium and cultured for 20 additional days under a 12/12 light photoperiod. All other experimental conditions were kept fixed as described above.
- Two different BAP concentrations (0.68 and 0.91 mg/l). For this experiment, we used diploid/DH calli coming from each of the eight different clonal callus populations obtained from M18-M25. Fifteen calli of each population were independently cultured during 2-3 weeks in media with the same BAP concentration originally used to produce them by anther culture. Then, the embryos produced were transferred to growth regulator-free MS basal medium.

178

179 *Design and statistical analysis*

180 For all anther culture experiments, we used three dishes (replicates) per experiment, unless
181 otherwise stated for a particular experiment. All the experiments were performed in parallel with
182 the Beta Alpha and Esfahani cultivars. For each experiment, the following parameters were
183 recorded: number of induced calli, percentage of anthers producing callus, percentage of
184 embryogenic (embryo-producing) calli, percentage of anthers producing embryogenic callus and
185 number of regenerated plants. Data were subjected to analysis of variance (ANOVA) and Duncan's
186 Multiple Range Test using the SAS software program.

187

188 *Flow cytometry*

189 We used flow cytometry to assess the ploidy level of donor plant leaves (as standards for 2C DNA
190 content), induced calli and regenerated plants as described in Corral-Martínez et al. (2011). Small
191 pieces of cultured calli and leaves were processed using the CyStain UV Precise P kit (Partec
192 GmbH, Münster, Germany). Briefly, samples were gently chopped in 400 µl of nuclear extraction
193 buffer (NEB) and incubated for 1 minute. Then, we added 1.6 ml of staining buffer and incubated
194 for 2 additional minutes. Extracted nuclei were filtered through 30 µm non-sterile Partec CellTricks
195 filters and immediately analyzed in a Partec CyFlow Ploidy Analizer.

196

197 *Microsatellite (SSR) analysis*

198 Total genomic DNA was isolated from fresh leaf material using the method described by Saghai-
199 Maroof et al. (1984). Quality and quantity of the isolated DNA was determined with a
200 biophotometer and with 1% (w/v) agarose gels, by comparing bands with known concentrations of
201 DNA. The SSR markers used in this study were first developed by Danin-Poleg et al. (2001) and
202 their primer sequences and annealing temperatures (Tm) are shown in Table 3. Amplification
203 reactions were carried out in an Eppendorf Mastercycler Gradient using a reaction mixture
204 containing 2 µl genomic DNA (400 ng/µl), 0.1 units of Taq DNA polymerase, 0,5 µl forward and
205 reverse primers (both at 100 pmol/µl), 1 µl dNTPs (2.5 mM), 0.7 µl $MgCl_2$ 50 mM, 1.8 µl PCR
206 buffer 10x, and ddH_2O to complete the final volume (20 µl). Amplification products were separated
207 by electrophoresis on 2.5% agarose gels. Amplified bands/alleles were scored as present (1) or
208 absent (0) for each genotype and SSR, and then compared with those of donor plants. Among the 15
209 primer combinations we used (Table 3), 12 were found monomorphic in donor plants and two
210 (CMAG59 and CMCTT144) were found polymorphic, and therefore useful to identify DHs among
211 the population of anther-derived plants. As expected, according to the original description of Danin-

Poleg et al. (2001), donor plants showed three amplification bands for CMAG59, whereas anther-derived plants presented one/two (for DHs) or the three original bands (for somatic regenerants). For CMCTT144, donor plants presented two bands and anther-derived plants presented just one (for DHs) or the two original bands (for somatic regenerants).

Results

Evaluation of previously published protocols for anther culture in cucurbits

Our first attempt to promote androgenesis in our cucumber cultivars was to apply some of the few protocols previously published for cucumber anther culture, as well as others published for other cucurbit species (Table 1). Three of the protocols developed for squash anther culture (Shalaby 2006; Metwally et al. 1998; Mohamed and Refaei 2004) produced identical results in both Beta Alpha and Esfahani: after two weeks of culture, the 30 cultured anthers swelled and turned yellowish white, but no calli were observed to emerge, becoming necrotic after 6 culture weeks. The protocols developed for squash (Kurtar et al. 2016) and for different cucurbit interspecific hybrids (Rakha et al. 2012) produced very few calli (1-3) from the 30 anthers cultured per genotype. However, the protocols previously tested in cucumber (Ashok Kumar and Murthy 2004; Song et al. 2007; Hamidvand et al. 2013; Abdollahi et al. 2016; Xie et al. 2005) produced calli in all 30 anthers (1 callus/anther), and for both genotypes. In most cases, the calli obtained by these protocols became yellowish after 4-6 weeks, then green, and eventually necrosed after 8 weeks of culture. None of them progressed in growth nor developed any differentiated structure (embryos, shoots or roots) on their surface after repeated subcultures. Only three calli among those produced by the Song et al. (2007) protocol developed shoot-like primordia after 6 weeks of culture, and could be eventually regenerated into five entire plants following the corresponding protocol. Flow cytometry analysis revealed that, as compared to diploid donor plants (2C DNA content, Figure 1A), 304 out of the 308 total calli produced from both genotypes (98.7%) were tetraploid (4C DNA content, Figure 1B), and the remaining 4 (1.3%) had a 2C DNA content compatible with a diploid or DH nature (Figure 1C). Consistent with this, four of the five plants regenerated from the Song et al. (2007) protocol were found to be tetraploid and one was diploid. SSR analysis with the CMAG59 and CMCTT144 markers (revealed as heterozygous for donor plants, as described in Materials and methods) evidenced identical band profiles for donor plants and all five regenerants, confirming their somatic nature, most likely coming from proliferation of anther wall tissues. In conclusion, the

245 ten protocols tested evidenced a relative ability to promote somatic proliferation from anther wall
246 tissues, but an inability to produce DH calli, embryos and plants in our genotypes.
247

248 **Assessment of different growth regulators in the induction medium**

249

250 We assayed the effect on callus production of different combinations of growth regulators in the
251 culture medium (Table 2). Media excluding the auxin (M2-M5) produced no callus, and anthers
252 necrosed after 4 weeks of culture. The same happened for combinations excluding the cytokinin
253 (M6-M9) and for those excluding both growth regulators (M1). Anthers cultured (Figure 2A) in MS
254 media with M10-M25 growth regulator combinations became swollen after three days and produced
255 a small, cream-colored callus that emerged from the anther, deforming it in some cases (Figure 2B).
256 One week later, the macroscopic callus was clearly visible (Figure 2C). After four weeks, all media
257 induced production of large callus masses (Figure 2D) in most of the cultured anthers (Table 4).
258 Among them, the best growth regulator combination in terms of percentage of callus production
259 was M22 (0.91 mg/l BAP + 0.25 mg/l 2,4-D), which induced callus response in 19.7 of the 20
260 (98.5%) anthers cultured in each replicate for Beta Alpha and 19.6 (97.8%) for Esfahani (Table 4).
261 However, no embryos were observed to arise neither directly from the anther locule nor indirectly
262 from the callus surface. Media with different combinations of BAP and NAA (M30-M39) showed
263 callus response too, but they were less abundant (0-16 callus/treatment), smaller than with BAP and
264 2,4-D combinations, and necrosed and died after six weeks of culture (Figure 2E). In total, 841 and
265 898 calli were produced for Beta Alpha and Esfahani, respectively. Among them, 27 and 34 calli,
266 respectively, corresponded to the experiments with BAP and NAA combinations. All of them were
267 found to have a 4C DNA content compatible with a tetraploid nature (Table 4). BAP and 2,4-D
268 combinations produced 814 and 864 calli for Beta Alpha and Esfahani, respectively. Their ploidy
269 levels were evaluated by flow cytometry (Table 4), revealing that most of them were tetraploid
270 (1642, 97.8%, Figure 1B), whereas one (0.06%) was triploid (data not shown), and 35 (2.1%) had a
271 putative haploid origin, being either haploid (Figure 1D) or diploid/DH (Figure 1C). In general, the
272 best results in terms of total and of diploid/DH callus induction were obtained with the media
273 containing 0.68 and 0.91 mg/l BAP, combined with different concentrations of 2,4-D (M18-M25 in
274 Tables 2 and 4).
275

276 **Anther culture at different anther orientations**

277

In view of the high rate of calli developed from anther somatic tissues with the different protocols tested, and the convoluted morphology of cucumber anthers, we considered the possibility that anther orientation in the culture medium might have an influence in the ratio of induction of somatic calli versus induction of microspore-derived calli. Since the region facing the culture medium is more exposed to nutrients and growth regulators, this might have an influence on the cell types that proliferate to give rise to calli. To test this possibility, we designed an experiment consisting in culturing 30 anthers in each of the six possible orientations. According to the anatomy shown in Figure 3A, we cultured anthers contacting the culture medium by the six different regions described in Materials and methods. The 30 anthers cultured produced one callus each, for a total of 30 calli per orientation. All of them were found tetraploid after flow cytometry, with the exception of one haploid callus from an anther cultured by their proximal end. In general, calli from anthers cultured by the distal end were smaller than the rest. It was also observed that, irrespective of the orientation, most calli were produced from the inner side, which is the side of filament insertion. These facts suggested that this anther region is especially prone to proliferate. On the opposite side (the outer region), anther walls were thinner and microspores were even visible through the walls (Figure 3B). Thus, we decided to make a scratch on this wall after three days of culture (Figure 3C), with the hope that this would help release the confined microspores and therefore facilitate the production of microspore-derived calli. Among the ten additional anthers with a scratch in the long sac wall, six produced one tetraploid callus on the inner side. However, four of them produced one haploid callus on the outer side and no callus on the inner side (Figure 3D). Thus, it appeared that a premature opening of the long sac wall by mechanical means facilitated the emergence and proliferation of haploid calli.

Plant regeneration from callus cultures

As explained in Materials and methods, we tried different combinations of growth regulators and liquid and solid media to promote callus growth and embryo/plant differentiation. For this, we produced a second batch of anthers cultured in M18-M25 media, as they produced the best overall results in our first batch. The calli produced were analyzed by flow cytometry and the diploid/DH calli were selected and cloned to produce a clonal diploid/DH callus population. Combinations involving exposure to light showed different embryogenic and/or organogenic responses, depending on the combinations of growth regulators and agar concentrations used. In general, calli grew and became yellow and friable (Figure 4A), with the exception of the combinations including the use of solid medium first, which at high BAP concentrations, produced green and hard, non-friable callus

masses (Figure 4B). No morphogenesis was observed on the surface of these calli. In contrast, the use of liquid medium first and then transference to solid medium produced no embryos when used with Beta Alpha-derived calli, but induced the development of embryo-like structures on the callus surface of Esfahani-derived calli, and for all growth regulator combinations. As seen in Figures 4C, D, these structures were morphologically similar to embryos, although they failed in all cases to develop a clearly identifiable shoot apex. Consistent with this, callus-derived embryos were transferred to basal medium with no growth regulators, but no plant regeneration was observed after 6 weeks.

In light of these experiments, we decided to repeat them with a new batch of callus from Beta Alpha and Esfahani anther cultures, but adding an initial stage of calli growing in darkness, and using only the first liquid and then solid media, since they were the only conditions where embryo production was observed. Results are summarized in Table 5. After heat shock, callus culture in liquid medium was performed in darkness for 20 days. Then, calli were transferred to solid medium and exposed to light. After one week, abundant green organogenic nodules were seen on the callus surface of Beta Alpha-derived calli (Figure 4E). Around 20 days later, some of them transformed into primordium-like structures (Figure 4F), then shoot-like structures (Figure 4G), and 2-3 weeks later, 47 entire plantlets regenerated from them. Plantlets were transferred to basal MS medium for completion of their *in vitro* stage (Figure 4H). The ploidy level of regenerated plantlets was analyzed by flow cytometry, and all 47 were found to have a 2C DNA content, identical to donor plants, and compatible with a diploid or DH nature. No other ploidies were found. To discriminate between diploids and DHs, SSR analysis with the CMAG59 and CMCTT144 primer pairs was performed (Figure 5). Nine plantlets (19%) presented the same band profile than donor plants, compatible with a diploid (therefore somatic) nature. The remaining 38 (81%) showed only one of the possible alleles present in donor plants, and were therefore considered as true DHs. On the other hand, Esfahani-derived calli showed no structures developed on their surface, neither embryogenic nor organogenic.

Induction of indirect embryogenesis by auxin removal

Since our best conditions for DH plant production involved plant regeneration through organogenesis but no embryogenesis, we designed alternative approaches to induce indirect embryogenesis from the diploid/DH clonal callus population coming from the second batch of anthers cultured in M18-M25 media. In this case, instead of using calli obtained from different

346 media for the same experiment, we transferred 15 subcloned calli obtained from the same medium
347 to each of the eight freshly prepared media with the same BAP concentration of their initial media,
348 but without 2,4-D. Three weeks after transference, the first embryo-like structures could be
349 observed (Figure 6A). Cotyledonary-like embryos were identified after seven culture weeks (Figure
350 6B). These embryos were transferred to basal MS medium (without growth regulators), germinating
351 and giving rise to entire plantlets (Figure 6C), which were successfully acclimated in pots with
352 substrate (Figure 6D). As seen in Table 6, all calli from M18-M25 media produced embryos,
353 although at a variable rate. This rate was not dependent on the BAP concentration used for
354 embryogenesis, but on the previous growth regulator ratios used to induce calli. Indeed, for the
355 same BAP concentration in callus culture media, the number of embryos induced was progressively
356 higher in calli previously induced with higher 2,4-D concentrations. This was observed in both Beta
357 Alpha and Esfahani cultivars.
358
359 Plant regeneration was also apparently affected by the initial conditions of callus induction in Beta
360 Alpha (Table 6). Thus, higher initial 2,4-D concentrations during the callus induction phase
361 produced not only more embryos from calli, but also more germinating embryos and therefore more
362 plants. In total, 14 plants were obtained. After flow cytometry and SSR analysis, ten plants (71.4%)
363 were found DH and four (28.6%) heterozygous, being two of them diploid and two tetraploid. From
364 Esfahani embryos, however, no plants were obtained in any of the conditions that promoted embryo
365 germination in Beta Alpha.
366
367 **Discussion**
368
369 **The genotype and conditions of donor plants are key factors for protocol reproducibility in**
370 **cucumber anther cultures**
371
372 We started this work trying to apply the protocols previously reported as most effective to promote
373 callus growth in different cucurbit species, including cucumber. All these works used chromosome
374 counts in few, randomly selected root tip cells to determine the ploidy level of regenerated plants,
375 and only one of them (Song et al. 2007) complemented this test with the use of molecular markers
376 (AFLPs in this case), which raises some concerns about the true gametophytic nature of the
377 individuals claimed as DHs. However, it is also true that all these previous studies documented the
378 occurrence of abnormal, but embryo-like structures growing on the callus surface. In our materials
379 and conditions, the protocols published for squash (Kurtar et al. 2016; Shalaby 2006; Metwally et

380 al. 1998; Mohamed and Refaei 2004) and for different cucurbit interspecific hybrids (Rakha et al.
381 2012) produced very few or no calli at all. The only protocols that consistently promoted callus
382 growth in all anthers of both genotypes were those published for cucumber (Ashok Kumar and
383 Murthy 2004; Song et al. 2007; Hamidvand et al. 2013; Abdollahi et al. 2016; Xie et al. 2005).
384 However, in nearly all cases, calli arrested and died after an initial growing stage. Only in three out
385 to 30 calli produced with the Song et al. (2007) protocol, organogenesis could be induced, although
386 from somatic tissues, as revealed by SSR analysis. In summary, these protocols were able to induce
387 proliferation of anther wall tissues, but failed to promote any growth from microspores. This may
388 be expected for the protocols not developed for cucumber. However, this was also found for the
389 protocols developed for other cucumber cultivars, and even for the same cultivars, but whose donor
390 plants were kept under different growth conditions in different facilities and countries.
391
392 We showed that in order to promote differentiation in microspore-derived calli, one of the most
393 critical factors was the maintenance of callus under light or dark conditions, and the implementation
394 of an initial stage of callus culture in liquid medium. Indeed, the only media that promoted
395 differentiation were those combining an initial stage of callus growth in liquid medium for 20 days,
396 followed by transference to solid medium. Under continuous exposure to light, these media induced
397 embryogenesis in Esfahani calli, although at low rates and with no further embryo growth. Possibly,
398 a refined adjustment of growth regulators could have led to further growth and germination.
399 However, it is important to remark the need for light of Esfahani calli. On the other hand,
400 continuous light exposure was detrimental for Beta Alpha calli, which needed the initial culture in
401 liquid medium to proceed in darkness. However, these conditions promoted organogenesis, instead
402 of embryogenesis. In conclusion, these results evidence important differences between the needs of
403 these two cucumber genotypes for promotion of different morphogenic pathways. These needs are
404 not related to minute differences in growth regulator concentrations or balances, but to the
405 genotype-dependent beneficial or inhibitory effects of light or liquid/solid culture. Together, these
406 facts highlight the importance of adapting *in vitro* protocols to each particular genotype and donor
407 plant growing conditions, in order to obtain the best performance.
408

409 **Anther orientation influences haploid callus production**

410
411 We showed that in nearly all cases, only one callus was produced per anther. Interestingly, in four
412 out to ten anthers where a scratch was made on the long sac wall, the only callus produced emerged
413 through the outer (scratched) side and was haploid, whereas in anthers producing tetraploid

414 (somatic) calli, they were produced at the inner side. In light of this, it seems that, as proposed for
415 other species (Parra-Vega et al. 2013), there is a competence of the different anther tissues for
416 resources and space, and the first cell type to proliferate and produce callus seems to have
417 preferential access to them, preventing the growth of other tissues. Thus, strategies that promote
418 rapid induction of microspore-derived callus should be adopted. The inner side is the side of
419 filament insertion. It is known that the wound left after filament excision is especially prone to
420 proliferate, producing high rates of somatic calli in anther cultures of different species (Seguí-
421 Simarro and Nuez 2006; Corral-Martínez et al. 2011). Thus, it is quite possible that filament
422 remnants are also exerting a strong influence in this case. However, the particular anatomy of
423 cucumber anthers allows us to prevent this. We recommend to place anthers facing the solid
424 medium by their long sac, which is where most microspores are located. This might prevent
425 proliferation of somatic cells. In addition, we showed that an opening in the wall of this sac was
426 helpful to increase the production of microspore-derived calli from this sac, possibly by releasing
427 them from the space constriction imposed by the wall, and facilitating their rapid emergence. These
428 anthers did not show any callus on the inner side, suggesting that the emergence of a haploid callus
429 prevents the occurrence of other, somatic calli from the inner region. In conclusion, in order to
430 increase the ratio of microspore-derived vs somatic callus production, we propose to remove
431 filament tissues from the inner side as much as possible, and then culture the anther by their outer
432 side for three days, after which a scratch should be made in the long sac.
433
434 **The occurrence of somatic calli is a major limitation that requires proper study of each callus**
435
436 Our results showed that cucumber anther cultures are useful to regenerate haploid and DH plants.
437 However, the only way to regenerate them at present is through a previous step of callus induction,
438 from which organogenesis and/or embryogenesis can be induced, depending on the *in vitro* culture
439 conditions and the genotype of the donor plants. In this sense, our work is in line with most
440 previous studies (Ashok Kumar and Murthy 2004; Song et al. 2007; Hamidvand et al. 2013;
441 Abdollahi et al. 2016; Xie et al. 2005), where this was the only androgenic technique successfully
442 implemented, with the single exception of the Zhan et al. (2009) work on microspore culture. The
443 use of the way hereby explored implies the possibility of having callus originated from anther wall
444 tissues coexisting with microspore-derived calli. Up to now, the real impact of this possibility was
445 not estimated, since previous studies did not routinely use molecular markers to discriminate
446 between homozygous and heterozygous calli. In our study, we demonstrated that this is not just a
447 theoretical possibility, but a real limitation of this way, since somatic calli occur at a non-negligible

448 rate. Suprunova and Shmykova (2008) proposed that these calli might arise from proliferation of
449 endothecium anther wall cells. An alternative way to overcome this would be the use of isolated
450 microspore cultures, but this approach is still far from efficient in cucumber. As far as we know,
451 only Zhan et al. (2009) has shown evidences of the production of germinating embryos directly
452 derived from isolated and cultured microspores, although at a low rate and only from three out to
453 ten genotypes evaluated. Thus, as long as anther culture is the only way consistently proved to
454 obtain androgenic DHs in cucumber, the implementation of a methodology (flow cytometry + SSR
455 molecular markers) to discriminate true DHs from somatic diploids is strongly advised in order to
456 rely on the results obtained.
457

458 **The growth regulator composition of the initial medium for callus induction appears critical**
459 **for all the process**
460

461 Our results also showed that in order to obtain cucumber DHs through anther culture, the use of
462 growth regulators to promote initial cell divisions is mandatory, as was previously shown for other
463 cultivars (Ashok Kumar and Murthy 2004). As seen in Table 4, all the different growth regulator
464 combinations tested induced callus proliferation from anther tissues, although the most effective
465 were those combining BAP and 2,4-D. They were also the only media producing haploid and DH
466 calli. However, in our experimental conditions and design, none of them clearly showed to be more
467 efficient for induction of microspore-derived calli. It is reasonable to think that these growth
468 regulator combinations induce cell division and proliferation with different efficiencies, but
469 similarly in somatic and haploid cells, with no distinction. Our results also suggested that, although
470 some auxin had always to be included for callus proliferation, the effect of 2,4-D concentration or
471 of the general hormonal balance is less relevant than BAP concentration. Indeed, the highest
472 efficiencies of callus induction were obtained within the range of 0.68-0.91 mg/l BAP, irrespective
473 of the 2,4-D concentration and the balance between both growth regulators, for both genotypes and
474 for both somatic and microspore-derived calli. However, 2,4-D concentration appeared very
475 important for further induction of embryogenesis on the callus surface. Table 6 shows that,
476 independently for both BAP concentrations, the number of embryos produced in both genotypes
477 increased progressively with higher auxin concentrations. According to this, the most suitable
478 medium to induce the initial callus proliferation and further embryogenesis induction should include
479 a high 2,4-D concentration (at least the highest we tried, 1 mg/l), although it was not the
480 concentration that produced most calli. These results suggest that there seems to be a sort of "*auxin*

481 *memory effect"* whereby calli induced with higher concentrations of exogenous auxin will be more
482 prone to produce embryos when auxin is removed.
483
484 It has long been known that removal of exogenous auxin from the culture medium promotes
485 induction of *in vitro* embryogenesis. It was first shown in carrot (Steward et al. 1958), and more
486 recently in arabidopsis (Su et al. 2009; Bai et al. 2013). These studies demonstrated that the
487 exogenous addition of auxin increases ethylene levels, which in turn interferes with local expression
488 of *YUCCA* genes, which are directly responsible of inducing somatic embryogenesis by regulating
489 local auxin biosynthesis and subsequent distribution. This relationship between ethylene and auxins
490 may also be used to speculate that, perhaps, the "auxin memory" is related to the fact that high
491 auxin levels during callus proliferation keep cells in a proliferative, totipotent status that prevents
492 early differentiation while the callus is still growing, thereby increasing the odds of producing
493 embryos as soon as exogenous auxin is removed and endogenous levels of ethylene decrease.
494
495 We also observed that, while embryogenesis induction was similar for Beta Alpha and Esfahani, no
496 plants at all were obtained from Esfahani embryos using the conditions that promoted embryo
497 germination in Beta Alpha. As mentioned above, a similar scenario was observed in Esfahani
498 embryos obtained from calli grown in first liquid and then solid media exposed to light. In other
499 words, Esfahani seemed extremely recalcitrant in terms of conversion of embryos into plants. In our
500 opinion, we think that this difference between genotypes most likely relies on the fact that Beta
501 Alpha is a F1 hybrid whereas Esfahani is a local landrace. In other words, Esfahani has not been
502 subjected to such exhaustive breeding and, in addition to being less homogeneous in terms of
503 population, the passage of calli and/or embryos through a haploid stage may allow for the
504 expression of lethal or deleterious genes that may compromise further growth, and that were
505 eliminated from the pure lines used to produce Beta Alpha hybrids.
506
507 **Cucumber *in vitro* cultured cells undergo *spontaneous* chromosome doubling at high rates**
508
509 In light of the ploidy levels observed in the calli and plants analyzed, it is also important to remark
510 that most of the somatic calli produced were tetraploid at the moment of flow cytometry analysis,
511 and most of the microspore-derived calli were DH, while just very few haploid calli were identified.
512 For example, among the 47 diploid plants obtained by organogenesis in Beta Alpha calli, 19% were
513 somatic diploids whereas 81% were DHs, which implies the rapid and frequent occurrence in the
514 initially haploid cells of a chromosome doubling process, likely nuclear fusion since it is by far the

515 most widely reported (Seguí-Simarro and Nuez 2008). In addition, no mixoploid (chimeric) calli
516 were found in any case. Together, these data suggest that soon after induction of callus
517 proliferation, our *in vitro* culture conditions promoted *spontaneous* (in the sense that no additional
518 treatments are needed) chromosome doubling in all dividing cells, both somatic and haploid. This
519 may have positive implications in the implementation of an efficient DH technology in these
520 genotypes, since the high rate of DHs vs haploids observed may alleviate the need for a specific
521 step of chromosome doubling.
522
523 **Concluding remarks**
524
525 In light of our results and previous reports, it seems evident that cucumber is a strongly recalcitrant
526 species where conventional protocols involving mild stresses and few or no growth regulators in the
527 culture medium are unlikely to work. As also shown for other heavily recalcitrant species such as
528 tomato (Seguí-Simarro and Nuez 2007; Corral-Martínez et al. 2011), a combination of different
529 stresses together with growth regulators appear necessary to promote the initial microspore cell
530 proliferation. Thus, we propose that at least for the particular cases of Esfahani and Beta Alpha, the
531 most efficient protocol to produce DHs at present involves a first step of callus induction through
532 bud pretreatment at 4ºC and the application of a 35ºC heat shock to anthers, once inoculated in
533 medium with 0.68-0.91 mg/l BAP and 1 mg/l 2,4-D. With this, a maximum yield of 6.67% (6.67
534 diploid/DH calli/100 anthers) was achieved. The calli produced must be transferred to a different
535 medium to induce morphogenesis upon application of an additional short 35ºC heat shock. For Beta
536 Alpha, which is the only genotype where we obtained fully regenerated DH plants, organogenesis
537 should be induced by a combination of culture in liquid medium and darkness, and then solid
538 medium exposed to light. Organogenesis was found strongly dependent on the cytokinin (BAP)
539 concentration, which showed best performance at 3 mg/l, and to a lower extent, 4 mg/l. In these
540 conditions, a maximum yield of 80% (80 DHs/100 diploid/DH calli) was obtained. Combining both
541 yields (calli obtained per 100 anthers and DHs obtained from calli), a maximum theoretical yield of
542 5.34% (5.34 DHs/100 anthers would be possible with our protocol. We also showed that indirect
543 embryogenesis was possible, but we found it strongly dependent on the auxin concentration
544 previously used for callus induction and proliferation, although auxin must be removed for
545 embryogenesis induction. The maximum yield of indirect embryogenesis was 33% (33 DHs/100
546 diploid/DH calli), which is considerably lower than that of the organogenic pathway. Thus, at
547 present induction of organogenesis from microspore-derived calli would be the most efficient
548 approach to doubled haploidy at least in our cucumber backgrounds. Irrespective of the pathway

used to obtain DHs, the percentage of DHs obtained was high enough to discard the routine use of additional steps for chromosome doubling.

This is the first report, to the best of our knowledge, that combines the use of flow cytometry and SSR molecular marker analysis to assess the real occurrence of true DHs in cucumber. Thanks to this, we evaluated the real androgenic potential of a cucumber F1 hybrid and a local landrace, demonstrating that true DH production is possible at reasonable amounts. But most importantly, we also highlighted the importance of some factors, sometimes dismissed and not always related to the *in vitro* culture conditions, and demonstrated the occurrence of some phenomena that limit the efficiency of this technique. Among them, the most important one is the massive occurrence of proliferation from anther wall tissues, which makes mandatory a proper analysis of each callus obtained. Further improvement of the technique should focus on the limitation of this problem, perhaps by assessing a microspore culture approach.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Abdollahi MR, Najafi S, Sarikhani H, Moosavi SS (2016) Induction and development of anther-derived gametic embryos in cucumber (*Cucumis sativus* L.) by optimizing the macronutrient and agar concentrations in culture medium. Turk J Biol 40 (3):571-579

Ashok Kumar HG, Murthy HN (2004) Effect of sugars and amino acids on androgenesis of *Cucumis sativus*. Plant Cell Tissue Organ Cult 78 (3):201-208. doi:10.1023/b:ticu.0000025637.56693.68

Bai B, Su YH, Yuan J, Zhang XS (2013) Induction of Somatic Embryos in Arabidopsis Requires Local YUCCA Expression Mediated by the Down-Regulation of Ethylene Biosynthesis. Mol Plant 6 (4):1247-1260. doi:10.1093/mp/sss154

Claveria E, Garcia-Mas J, Dolcet-Sanjuan R (2005) Optimization of cucumber doubled haploid line production using in vitro rescue of in vivo induced parthenogenic embryos. J Am Soc Hortic Sci 130 (4):555-560

Corral-Martínez P, Nuez F, Seguí-Simarro JM (2011) Genetic, quantitative and microscopic evidence for fusion of haploid nuclei and growth of somatic calli in cultured *ms10*^{35} tomato anthers. Euphytica 178 (2):215-228. doi:10.1007/s10681-010-0303-z

587 Danin-Poleg Y, Reis N, Tzuri G, Katzir N (2001) Development and characterization of
588 microsatellite markers in *Cucumis*. Theor Appl Genet 102 (1):61-72.
589 doi:10.1007/s001220051618
590 Dong Y-Q, Zhao W-X, Li X-H, Liu X-C, Gao N-N, Huang J-H, Wang W-Y, Xu X-L, Tang Z-H
591 (2016) Androgenesis, gynogenesis, and parthenogenesis haploids in cucurbit species. Plant
592 Cell Rep:1-29. doi:10.1007/s00299-016-2018-7
593 FAOSTAT (2018) http://faostat.fao.org [last checked July 2018].
594 Ficcadenti N, Sestili S, Annibali S, Di Marco M, Schiavi M (1999) *In vitro* gynogenesis to induce
595 haploid plants in melon *Cucumis melo* L. Genet Breed 53:255-257
596 Gałązka J, Niemirowicz-Szczytt K (2013) Review of research on haploid production in cucumber
597 and other cucurbits. Folia Horticulturae, vol 25. doi:10.2478/fhort-2013-0008
598 Hamidvand Y, Abdollahi MR, Chaichi M, Moosavi SS (2013) The effect of plant growth regulators
599 on callogenesis and gametic embryogenesis from anther culture of cucumber (*Cucumis*
600 *sativus* L.). International Journal of Agriculture and Crop Sciences 5 (10):1089
601 Kurtar ES, Balkaya A, Kandemir D (2016) Evaluation of haploidization efficiency in winter squash
602 (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) through anther
603 culture. Plant Cell Tissue Organ Cult 127 (2):497-511. doi:10.1007/s11240-016-1074-6
604 Lotfi M, Alan AR, Henning MJ, Jahn MM, Earle ED (2003) Production of haploid and doubled
605 haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance.
606 Plant Cell Rep 21 (11):1121-1128
607 Metwally EI, Moustafa SA, El-Sawy BI, Shalaby TA (1998) Haploid plantlets derived by anther
608 culture of *Cucurbita pepo*. Plant Cell Tissue Organ Cult 52 (3):171-176.
609 doi:10.1023/a:1005908326663
610 Mohamed M, Refaei E (2004) Enhanced haploids regeneration in anther culture of summer squash
611 (*Curcurbita pepo* L.). Cucurbit Genetics Cooperative Report 27:57-60
612 Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue
613 cultures. Physiol Plant 15:473-479
614 Parra-Vega V, Renau-Morata B, Sifres A, Seguí-Simarro JM (2013) Stress treatments and in vitro
615 culture conditions influence microspore embryogenesis and growth of callus from anther
616 walls of sweet pepper (*Capsicum annuum* L.). Plant Cell Tissue Organ Cult 112 (3):353-
617 360. doi:10.1007/s11240-012-0242-6
618 Rakha M, Metwally E, Moustafa S, Etman A, Dewir Y (2012) Evaluation of regenerated strains
619 from six *Cucurbita* interspecific hybrids obtained through anther and ovule *in vitro* cultures.
620 Aust J Crop Sci 6 (1):23-30
621 Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-
622 length polymorphisms in barley: mendelian inheritance, chromosomal location, and
623 population dynamics. Proc Natl Acad Sci USA 81 (24):8014-8018.
624 doi:10.1073/pnas.81.24.8014
625 Sauton A, Dumas de Vaulx R (1987) Obtention de plantes haploides chez melon (*Cucumis melo* L.)
626 par gynogenese indute par du pollen irraidié. Agronomie 7:141-148
627 Seguí-Simarro JM (2016) Androgenesis in solanaceae. In: Germanà MA, Lambardi M (eds) In vitro
628 embryogenesis, vol 1359. Methods in Molecular Biology. Springer Science + Business
629 Media, New York, pp 209-244. doi:10.1007/978-1-4939-3061-6_9
630 Seguí-Simarro JM, Nuez F (2006) Androgenesis induction from tomato anther cultures: callus
631 characterization. Acta Hort 725:855-861
632 Seguí-Simarro JM, Nuez F (2007) Embryogenesis induction, callogenesis, and plant regeneration
633 by *in vitro* culture of tomato isolated microspores and whole anthers. J Exp Bot 58 (5):1119-
634 1132
635 Seguí-Simarro JM, Nuez F (2008) Pathways to doubled haploidy: chromosome doubling during
636 androgenesis. Cytogenet Genome Res 120 (3-4):358-369. doi:10.1159/000121085

637 Shalaby TA (2006) Embryogenesis and plantlets regeneration from anther culture of squash plants
638 (*Cucurbita pepo* L.) as affected by different genotypes. J Agric Res Tanta Univ 32 (1):173-
639 183
640 Song H, Lou QF, Luo XD, Wolukau JN, Diao WP, Qian CT, Chen JF (2007) Regeneration of
641 doubled haploid plants by androgenesis of cucumber (*Cucumis sativus* L.). Plant Cell Tissue
642 Organ Cult 90 (3):245-254. doi:10.1007/s11240-007-9263-y
643 Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured cells. II.
644 Organization in cultures grown from freely suspended cells. Am J Bot 45 (10):705-708
645 Su YH, Zhao XY, Liu YB, Zhang CL, O'Neill SD, Zhang XS (2009) Auxin-induced WUS
646 expression is essential for embryonic stem cell renewal during somatic embryogenesis in
647 Arabidopsis. Plant J 59 (3):448-460. doi:10.1111/j.1365-313X.2009.03880.x
648 Suprunova T, Shmykova N (2008) *In vitro* induction of haploid plants in unpollinated ovules,
649 anther and microspore culture of *Cucumis sativus*. Cucurbitaceae 2008: Proceedings of the
650 IXth Eucarpia Meeting on Genetics and Breeding of Cucurbitaceae:371-374
651 Xie M, Qin L-y, Pan J-s, He H-l, Wu A-z, Cai R (2005) Flower morphogenesis and microspore
652 development versus anther culture of cucumber. Acta Bot Boreal-Occid Sin 25 (6):1096
653 Zhan Y, Chen J-f, Malik AA (2009) Embryoid induction and plant regeneration of cucumber
654 (*Cucumis sativus* L.) through microspore culture. Acta Hort Sin 36 (2):221-226

655

656

657

658 **Tables**

659

660 **Table 1.** Previously published protocols assessed in Experiment 1. For each medium, growth
661 regulator concentration is expressed in mg/l, whereas sucrose concentration is expressed in % (w/v).

662

Species	Pretreatment	Heat shock	Anther culture medium	Callus/embryo culture medium	Reference
Cucumber	4°C, 2 days	32°C, 1 day	B5+ 0.44 2,4-D + 0.23 BAP + 8.5% sucrose	B5 + 0.06 Kin + 0.055 NAA + 3.1% sucrose	Ashok Kumar and Murthy 2004
			B5+ 0.442 2,4-D + 0.225 BAP + 5% sucrose		Xie et al. 2005
		33°C, 1 h	MS+ 1 BAP + 1.1 KT + 0.5 2,4-D + 3% sucrose	MS + 0.5 BAP + 6% sucrose	Song et al. 2007
			MS + 0.44 2,4-D + 0.23 BAP + 0.37 KT + 2.5% sucrose	MS + 0.1 NAA + 3 BAP + 3% sucrose	Hamidvand et al. 2013
			MS + 0.5 2,4-D + 1 BAP + 1.15 Kt + 3% sucrose	MS + 0.1 NAA + 3 BAP + 3% sucrose	Abdollahi et al. 2016
Squash	4°C, 4 days	35°C, 7 days	MS + 5 2,4-D + 10% sucrose	MS + 0.05 Kin + 0.05 NAA + 3% sucrose	Shalaby 2006
			MS + 5 2,4-D + 15% sucrose	MS + 0.057 Kin + 0.05 NAA + 15% sucrose	Metwally et al. 1998
			MS + 6 2,4-D + 10% sucrose	MS + 0.05 Kin + 0.05 NAA, no sucrose reported	Mohamed and Refaei 2004
	4°C, 2 days	35°C, 2 days	MS + 3 BAP + 0.1 NAA + 12% sucrose	MS + 0.5 BAP + 12% sucrose	Kurtar et al. 2016
Different *Cucurbita* hybrids	4°C, 2 days	35°C, 7 days	MS + 1 2,4-D + 9% sucrose	MS + 0.05 Kin + 0.05 NAA + 9% sucrose	Rakha et al. 2012

663

664 **Table 2.** Plant growth regulator combinations tested for anther culture in Experiment 2. All
665 concentrations are expressed in mg/l.

666

Medium	BAP	2,4-D	Medium	BAP	2,4-D	Medium	BAP	NAA
M1	0	0	M14	0.45	0.25	M30	1	0.05
M2	0.22	0	M15	0.45	0.5	M31	1	0.1
M3	0.45	0	M16	0.45	0.75	M32	1	0.2
M4	0.68	0	M17	0.45	1	M33	1	0.5
M5	0.9	0	M18	0.68	0.25	M34	2	0.1
M6	0	0.25	M19	0.68	0.5	M35	2	0.5
M7	0	0.5	M20	0.68	0.75	M36	3	0.1
M8	0	0.75	M21	0.68	1	M37	3	0.5
M9	0	1	M22	0.91	0.25	M38	4	0.1
M10	0.22	0.25	M23	0.91	0.5	M39	4	0.5
M11	0.22	0.5	M24	0.91	0.75			
M12	0.22	0.75	M25	0.91	1			
M13	0.22	1						

667

Table 3. SSR markers used in this study

Name	Tm (ºC)	Forward primer (5´-3´)	Tm (ºC)	Reverse primer (5’-3´)
CMAG59	65.3	TTGGGTGGCAATGAGGAA	54.1	ATATGATCTTCCATTTCCA
CMGA104	63.1	TTACTGGGTTTTGCCGATTT	57.1	AATTCCGTATTCAACTCTCC
CMCTT144	67.2	CAAAAGGTTTCGATTGGTGGG	65.5	AAATGGTGGGGGTTGAATAGG
CMTC47	56.5	GCATAAAAGAATTTGCAGAC	49	AGAATTGAGAAGAGATAGAG
CMAT141	67.1	AAGCACACCACCACCCGTAA	55.9	GTGAATGGTATGTTATCCTTG
CMCCA145	65.9	GAGGGAAGGCAGAAACCAAAG	60.6	GCTACTTTTGTGGTGGTGG
CMTC123	60.9	CGGATTGTACTTATTGCCAAG	63.7	CATGTGCATGTGTGCATGTAC
CMTA170a	65.6	TTAAATCCCAAAGACATGGCG	62.9	AGACGAAGGACGGTTAGCTTT
CMTA170b	58.6	ATTGCCCAACTAAACTAAACC	57.9	CACAACACAATATCATCCTTG
CMGA165	57.3	CTTGTTTCGAGACTATGGTG	62	TTCAACTACAGCAAGGTCAGC
CMTC160a+b	58.9	GTCTCTCTCCCTTATCTTCCA	65.5	GATGGTGCCTTAGTTGTTCCG
CMCT505	56	GACAGTAATCACCTCATCAAC	55.8	GGGAATGTAAATTGGATATG
CSCTTT15a	61.2	GTTTGATAATGGCGGATTGT	57.5	GTAGAAATGAAGGTATGGTGG
CSAT214	53.5	TTGAGTACCATTGTCATAGAT	49.9	TTAGTTTAATTTCATCTCTGT
CSAT425	55.9	TAGGGCAGGTATTATTTCAG	56.5	ACGGACTGATTTAGTATAGGC

Table 4. Callus induction response and DNA content of all calli obtained in MS-based media with different growth regulator combinations for Esfahani and Beta Alpha. Means and percentages (%) of three replicate dishes (20 anthers/replicate) are shown. Total calli obtained refer to all the calli obtained from the three replicates of each experiment, separated by ploidies.

	Beta Alpha								Esfahani						
				Total calli obtained								Total calli obtained			
Medium	Anthers /dish	Mean/ dish	%	1C	2C	3C	4C	Medium	Anthers /dish	Mean/ dish	%	1C	2C	3C	4C
M10	20	11.9	59.7	0	0	0	36	M10	20	15.5	77.5	0	1	0	46
M11	20	14.7	73.5	0	0	0	44	M11	20	17.5	87.5	0	0	0	53
M12	20	15.6	78.2	0	0	0	47	M12	20	17.8	88.8	0	0	0	53
M13	20	14.5	72.5	0	0	0	44	M13	20	16.3	81.3	0	0	0	49
M14	20	16.2	80.9	0	4	1	44	M14	20	16.9	84.4	1	2	0	48
M15	20	17.8	89.1	0	1	0	52	M15	20	19.4	96.8	0	0	0	58
M16	20	16.0	80.0	0	2	0	46	M16	20	18.0	90.0	0	0	0	54
M17	20	13.9	69.7	0	0	0	42	M17	20	16.3	81.3	0	0	0	49
M18	20	18.6	93.2	1	3	0	52	M18	20	17.5	87.5	0	4	0	49
M19	20	19.1	95.3	0	1	0	56	M19	20	18.5	92.5	0	2	0	54
M20	20	19.1	95.3	0	0	0	57	M20	20	19.0	95	0	0	0	57
M21	20	19.3	96.3	0	0	0	58	M21	20	19.0	95.1	0	0	0	57
M22	20	19.7	98.5	2	4	0	53	M22	20	19.6	97.8	0	1	0	58
M23	20	17.2	85.9	1	2	0	49	M23	20	19.1	95.3	0	2	0	55
M24	20	19.1	95.3	0	0	0	57	M24	20	19.6	98	0	1	0	58
M25	20	18.8	93.8	0	0	0	56	M25	20	18.3	91.3	0	0	0	55
M30	20	0.7	3.3	0	0	0	2	M30	20	0.3	1.7	0	0	0	1
M31	20	1.3	6.7	0	0	0	4	M31	20	1.0	5.0	0	0	0	3
M32	20	1.7	8.3	0	0	0	5	M32	20	1.7	8.3	0	0	0	5
M33	20	3.3	16.7	0	0	0	10	M33	20	2.7	13.3	0	0	0	8
M34	20	2.0	10.0	0	0	0	6	M34	20	1.3	6.7	0	0	0	4
M35	20	2.0	10.0	0	0	0	6	M35	20	1.7	8.3	0	0	0	5

M36	20	0.0	0.0	0	0	0	0	M36	20	0.0	0.0	0	0	0	0
M37	20	0.3	1.7	0	0	0	1	M37	20	0.3	1.7	0	0	0	1
M38	20	0.0	0.0	0	0	0	0	M38	20	0.0	0.0	0	0	0	0
M39	20	0.0	0.0	0	0	0	0	M39	20	0.0	0.0	0	0	0	0

675

676 **Table 5.** Characterization of plantlets regenerated through organogenesis from the second batch of
677 diploid/DH Beta Alpha calli, cultured with different NAA and BAP combinations in the 'first liquid
678 (+ darkness) and then solid (+ light)' media conditions. Growth regulator concentrations are
679 expressed in mg/l. Ploidy is expressed as multiples of haploid (1C) DNA content. SSR analysis
680 shows the number of homozygous (homo) and heterozygous (hetero) plants obtained from each
681 combination of growth regulators. See text for further details.

682

Growth regulators				**Ploidy**				**SSR analysis of 2C plants**	
NAA	**BAP**	**Calli**	**Plantlets**	**1C**	**2C**	**3C**	**4C**	**Homo (DHs)**	**Hetero**
0.05	2	15	1	0	1	0	0	0	1
0.05	3	15	15	0	15	0	0	12	3
0.05	4	15	8	0	8	0	0	7	1
0.1	2	15	0	0	0	0	0	0	0
0.1	3	15	13	0	13	0	0	11	2
0.1	4	15	11	0	11	0	0	8	2

683

684

685 **Table 6.** Induction of indirect embryogenesis from the second batch of diploid/DH calli. For each
686 callus induction medium, the corresponding combination of BAP and 2.4-D is shown in brackets. In
687 cultured calli, the BAP concentration used is expressed in mg/l. Percentages (%) refer to plants
688 produced from induced embryos.

689

Callus induction medium	**Cultured calli**		**Beta Alpha**			**Esfahani**		
	Number	**BAP**	**Embryos**	**Plants**	**%**	**Embryos**	**Plants**	**%**
M18 (0.68 – 0.25)	15	0.68	4	0	0	3	0	0
M19 (0.68 – 0.50)	15	0.68	6	0	0	4	0	0
M20 (0.68 – 0.75)	15	0.68	7	2 (DH, 2n)	28.6	4	0	0
M21 (0.68 – 1.00)	15	0.68	13	7 (5xDH, 2n, 4n)	53.8	10	0	0
M22 (0.91 – 0.25)	15	0.91	2	0	0	1	0	0
M23 (0.91 – 0.50)	15	0.91	5	1 (DH)	20	6	0	0
M24 (0.91 – 0.75)	15	0.91	7	1 (4n)	14.3	8	0	0
M25 (0.91 – 1.00)	15	0.91	9	3 (3xDH)	33.3	10	0	0

690

691

692 **Figure legends**

693

694 **Figure 1.** Histograms representative of the flow cytometry analysis of donor plants and calli
695 obtained from the different anther cultures performed in this work. A: Diploid donor plant. B:
696 Tetraploid callus. C: Diploid or DH callus. D: Haploid callus. Note the high level of background in
697 callus histograms as compared to donor plant, due to DNA degradation as a consequence of
698 exposure to *in vitro* culture conditions.

699

700 **Figure 2.** Cucumber anther cultures. A: Just plated anthers. B: Anther with an emerging callus.
701 three days after inoculation. C: Callus mass after one week of culture. D: Callus masses after four
702 weeks of culture. E: Necrosing callus masses after six weeks of culture. Bars: A: 1 cm; B-E: 5 mm.

703

704 **Figure 3.** Cucumber anther regions. A: Cucumber anther image illustrating its different regions as
705 used for the experiment of different anther orientations. B: Detail of the long outer sac where
706 microspores (m) are visible through the anther wall. C. D: Anther with a scratch in the wall of its
707 long outer sac, revealing the microspores of the locule (C) and the production of a callus on its outer
708 side (D). The red box in D illustrates the area enlarged in C. Bars: A, D: 1 mm; B, C: 100 μm.

709

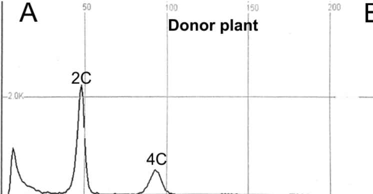
710 **Figure 4.** Induction of embryogenesis and organogenesis from microspore-derived calli. A: Yellow.
711 friable callus. B: Green, non-friable callus. C: Globular-like embryo emerging from the callus
712 surface of a Esfahani-derived callus. D: Earth-shaped embryo. E: Organogenic nodule on the callus
713 surface of a Beta Alpha-derived callus. F: Leaf primordium-like structures. G: Leaf-like
714 regenerating structure. H: Entire. *in vitro* regenerated plant. Bars: A, B, G: 5 mm; C-F: 1 mm.

715

716 **Figure 5.** Microsatellite (SSR) marker agarose electrophoresis. Lane 1 corresponds to the DNA
717 ladder. Lanes 2 and 3 correspond to Beta Alpha and Esfahani heterozygous donor plants,
718 respectively. Lanes 4-13 are examples of different regenerated plants. A: CMCTT144 primer pairs.
719 Note that heterozygous plants (somatic regenerants) present two amplification bands and
720 homozygous (microspore-derived DH) plants present just one. B: CMAG59 primer pairs. Note that
721 heterozygous plants (somatic regenerants) present three amplification bands and homozygous
722 (microspore-derived DH) plants present one/two bands.

723

724 **Figure 6.** Induction of embryogenesis from Beta-Alpha microspore-derived calli. A: Earth-shaped
725 embryo generated on a microspore-derived callus. Inset shows a detail of the embryo. B: Growing,
726 cotyledonary-like embryo. C: *In vitro* plantlet. D: Acclimated plant. Bars: 2 mm.
727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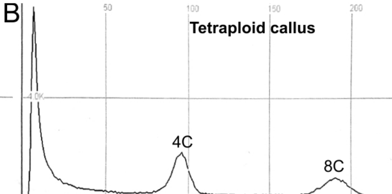
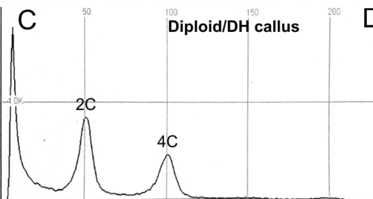
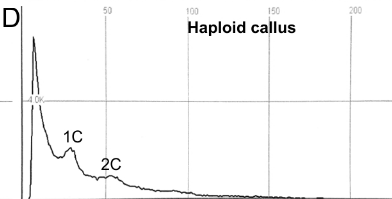
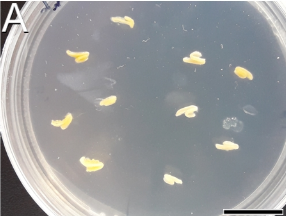
Donor plant
2C
4C
50
100
150
200
2.0K

B
Tetraploid callus
4C
8C
50
100
150
200
4.0K

C
Diploid/DH callus
2C
4C
50
100
150
200
4.0K

D
Haploid callus
1C
2C
50
100
150
200
4.0K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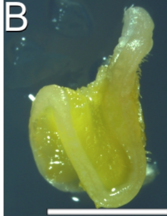
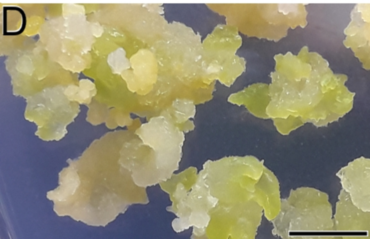
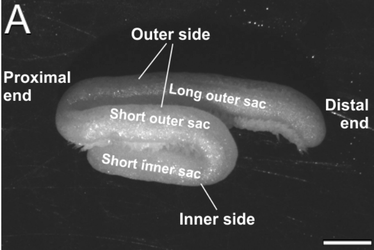
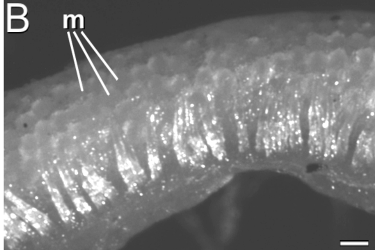
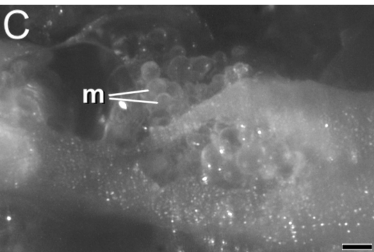

B

C

D

E


A
Outer side
Proximal end
Long outer sac
Short outer sac
Distal end
Short inner sac
Inner side


B
m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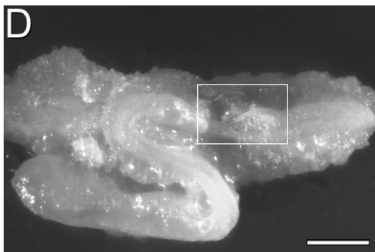
m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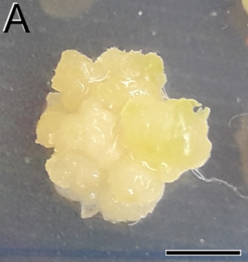
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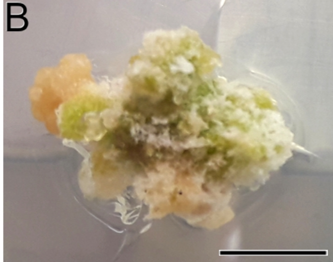
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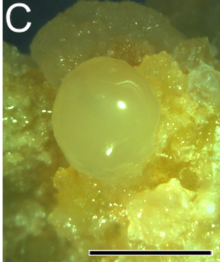
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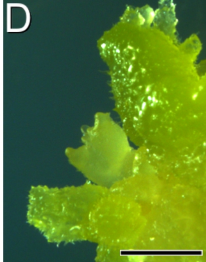
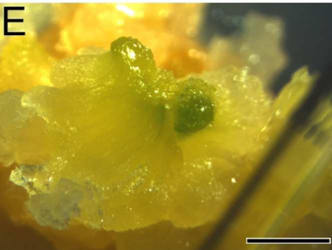

D

H

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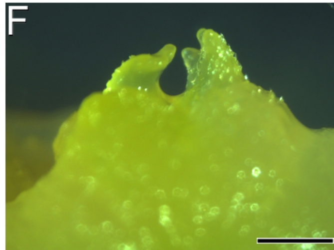
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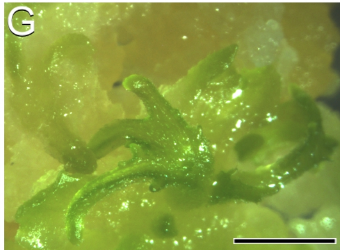
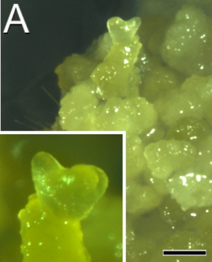
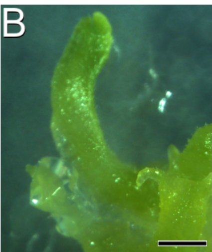
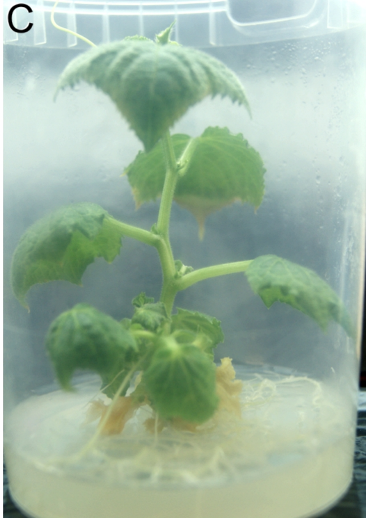

G

A 1 2 3 4 5 6 7 8 9 10 11 12 13

B 1 2 3 4 5 6 7 8 9 10 11 12 13







D