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Additional Information

1 **Development and characterization of an eggplant (*Solanum melongena*) doubled**
2 **haploid population and a doubled haploid line with high androgenic response**
3

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10

11 **Abstract**
12 We developed an eggplant doubled haploid (DH) population from a commercial hybrid
13 through androgenesis in microspore culture. Morphological variation, reproductive
14 ability and androgenic responsiveness were evaluated. The DH population showed
15 segregation in vegetative traits related to leaf, flower and fruit, and in reproductive traits
16 such as fruit and seed setting or germination rate. The DH population and subsequent
17 generations also presented variation in the androgenic response, with null, low and high
18 response lines. From this population, we were able to identify the first eggplant highly
19 androgenic DH line (DH36), remarkably similar to the donor hybrid in terms of
20 morphology and reproductive ability, but stably producing four times more calli than
21 the hybrid. The segregating DH population is potentially useful for genetic studies and
22 mapping of several traits, whereas the highly androgenic line DH36 may be used as a
23 model line to facilitate the study of eggplant androgenesis and embryogenesis for both
24 basic and applied research.

25 **Keywords: androgenesis,** anther culture, DH36, microspore culture, microspore
26 embryogenesis.

27 **Introduction**

28

29 DH technology is of core importance in breeding programs, since it allows for the
30 production of true breeding (pure) lines, homozygous for all traits, in a way faster and
31 cheaper than classical breeding procedures. This reduces considerably the number of
32 generations needed to produce a pure line, thus decreasing the costs of breeding
33 programs (Germanà 2011b). In addition, the fully homozygous condition of DHs makes
34 them a useful tool to facilitate the establishment of chromosome maps, mapping of
35 genetic markers, marker-trait association and identification of recessive mutations
36 (Forster et al. 2007).

37

38 One of the androgenic alternatives to obtain DH individuals is microspore
39 embryogenesis, an experimental pathway by which microspores are redirected from
40 their original gametophytic fate towards a new embryogenic program, giving rise to
41 haploid individuals that are converted to DHs either spontaneously or with genome
42 duplication techniques (Seguí-Simarro and Nuez 2008). Microspore embryogenesis is
43 induced *in vitro* by applying specific stresses to microspores either still enclosed inside
44 the anther (anther culture), or isolated and inoculated in liquid culture (isolated
45 microspore culture). Anther culture is the most widely applied method for DH
46 production due to its simplicity (Germanà 2011a). However, isolated microspore
47 culture, although more technically demanding, presents additional advantages including
48 a higher efficiency and the possibility to avoid the occurrence of regenerated somatic
49 plants from anther tissue. Therefore, it constitutes a better way to investigate the
50 biological processes involved in microspore embryogenesis. Microspore cultures hold
51 high interest for research purposes, as they provide a large population of haploid cells in

52 suspension. Any modification made at the haploid level will be carried by the DH plant
53 regenerated, being especially useful for mutagenesis and genetic transformation (Eudes
54 et al. 2014). In addition, isolated microspore cultures provide a unique opportunity to
55 study diverse topics related to embryo development, such as cell identity and tissue
56 patterning (Soriano et al. 2014), change of cell fate, cell proliferation (Daghma et al.
57 2014), autophagy (Corral-Martínez et al. 2013), totipotency (Li et al. 2014), cell-to-cell
58 communication and diverse genetic and genomic studies (Ferrie and Möllers 2011).
59
60 The efficiency of microspore embryogenesis varies greatly among species, even among
61 genotypes within the same species (Ferrie et al. 1995). The determinant importance of
62 the genotype in the efficiency of *in vitro* production of haploids is known since the first
63 attempts of inducing it (Nitsch 1972). A poor embryogenic response limits the utility of
64 DH technology in breeding programs and in both basic and applied experimental
65 research, so the identification of highly embryogenic genotypes is a fundamental step
66 for progressing in the practical application of this technology. Therefore, countless
67 studies have screened for high androgenic response (reviewed in Ferrie and Caswell
68 2011). Highly embryogenic genotypes have been identified or developed in some
69 species, and they are widely used for doubled haploidy research. For example, but not
70 only, *Brassica napus* cv. Topas line DH4079 (Ferrie and Keller 1995), barley cv. Igri
71 (Hoekstra et al. 1992), or wheat cv. Chris, cv. Pavon 70 and cv. Bob White (Kasha et al.
72 2003).
73
74 Common eggplant (*Solanum melongena*) is one of the most important vegetable crops
75 worldwide and important breeding efforts have been made to improve its traits and
76 performance. In terms of production, it was ranked in the sixth position in 2013, with

77 almost 50 million of tons produced (FAOSTAT 2016). Eggplant DH embryos and
78 plants can successfully be obtained through anther culture, and adaptations of the
79 method developed by Dumas de Vaulx and Chambonnet (1982) are commonly used to
80 obtain DHs useful for breeding programmes (Seguí-Simarro 2016). They are also used
81 as a tool to assist research, for example to assess gene exchanges (Toppino et al. 2008)
82 and to reduce the ploidy of tetraploid somatic hybrids between common eggplant and
83 wild relatives (Rizza et al. 2002; Rotino et al. 2005). However, eggplant is still
84 considered recalcitrant to microspore culture because, although microspore-derived
85 embryos are initially formed, they do not complete embryogenesis and transform into
86 calli (Corral-Martínez and Seguí-Simarro 2012), making it mandatory additional steps
87 to regenerate DH plants from these calli through organogenesis (Rivas-Sendra et al.
88 2015). This is why the very scarce studies published about eggplant microspore culture
89 are focused on the improvement of not only induction, but also regeneration rates
90 (Corral-Martínez and Seguí-Simarro 2012; 2014; Miyoshi 1996; Rivas-Sendra et al.
91 2015), and no basic research has been done yet using eggplant microspore cultures as
92 platform.
93
94 In the present work we developed an eggplant DH segregating population, characterized
95 its phenotypic variation, and selected lines with high embryogenic response and
96 heritable and stable performance, which hopefully will pave the way for a more
97 intensive utilization of eggplant microspore cultures both in basic and applied research.
98
99 **Materials and methods**
100
101 *Plant material*

102 In this study, we used eggplant donor plants of cv. Bandera, a commercial F1 hybrid
103 (Seminis Vegetable Seeds Ibérica, S.A., Spain) that showed good androgenic response
104 in previous studies (Corral-Martínez and Seguí-Simarro 2012; Salas et al. 2011).
105 Bandera was used as a control reference at each stage of the experiments. We also used
106 a population of *in vitro* obtained DHs derived from Bandera, and a second generation of
107 Bandera-derived DH plants (DHS1) obtained after selfing of the first DH population.
108 Plants were grown in 30 cm pots under natural light at the greenhouses of Universitat
109 Politècnica de València (Spain). Experiments were performed during five consecutive
110 years. Twenty plants of Bandera were grown from March to July of the first year. One
111 plant of each genotype of the DH population was grown from September of the second
112 year to August of the third year. Their androgenic capacity was evaluated through
113 anther culture from January to August of the third year. Twenty plants of each selected
114 selfed DH genotype (S1 lines) were grown from March to August of the fourth year and
115 from February to May of the fifth year. Their androgenic capacity was evaluated
116 through microspore culture during July and August of the fourth year and during April
117 and May of the fifth year.
118
119 *Generation of the DH population*
120 Isolated microspore culture was performed according to Corral-Martínez and Seguí-
121 Simarro (2014). Anthers containing mostly vacuolate microspores (Salas et al. 2012)
122 were dissected from the bud, surface sterilized with 70% ethanol for 30 s and with 4 g/l
123 sodium hypochlorite for 5 min, and rinsed three times in sterile distilled water. Anthers
124 were crushed in a small beaker with sterile distilled water using a syringe piston, and
125 the microspores contained in the locules were isolated by filtration through a 41 µm
126 nylon mesh (Millipore), followed by three centrifugation steps at 100 *g* for 4 min each.

127 Microspores were suspended in sterile distilled water at a final cell density of 500,000
128 microspores/ml, plated and incubated at 35ºC in darkness for 3 days to induce
129 embryogenesis. After induction treatment, microspores were collected by
130 centrifugation, resuspended at the same density in NLN medium (Nitsch and Nitsch
131 1967) supplemented with 20 g/l sucrose, 0.5 mg/l 1-naphthaleneacetic acid and 0.5 mg/l
132 6-benzilaminopurine, and incubated at 25ºC in darkness.
133
134 After 30 days of culture, new individuals were regenerated according to Rivas-Sendra et
135 al. (2015). Calli larger than 1 mm were placed in MS medium supplemented with 20 g/l
136 sucrose, 8 g/l plant-agar, 0.2 mg/l indole-3-acetic acid and 4 mg/l zeatin (Miyoshi
137 1996). Shoots showing a visible meristem surrounded by leaf primordia were
138 transferred to MS medium with 8 g/l plant-agar. Shoots that did not develop roots
139 before the stem elongated were cut and subcultured in fresh basal MS medium with 8
140 g/l plant-agar to induce rooting. Rooted shoots were finally transferred to pots with
141 substrate and acclimated in a growing chamber. The ploidy of all the new individuals
142 was checked by flow cytometry. DH individuals were transferred to the greenhouse and
143 constituted the DH population used in this study.
144
145 *Flow cytometry*
146 Small pieces of young leaves taken from *in vitro*-produced plantlets at the moment of
147 acclimatization (when at least 4 true leaves were formed) were chopped with a razor
148 blade in 0.5 ml of nuclei extraction buffer from CyStain UV Precise P kit (Partec).
149 Extracted nuclei was filtered through 30 μm CellTricks filters (Partec), 1.5 ml of DAPI-
150 based staining buffer from CyStain UV Precise P kit (Partec) was added and samples

151 were incubated for 2 min. Samples were immediately analysed using a Partec CyFlow
152 Ploidy Analyzer flow cytometer.
153
154 *Anther culture of DH plants*
155 Anther culture was performed according to Salas et al. (2011). Flower buds were
156 surface sterilized with 70% ethanol for 30 s and with 4 g/l sodium hypochlorite for 5
157 min, and rinsed three times in sterile distilled water. Microspore stage from one anther
158 of each bud was assessed under a light microscope, and only anthers containing young
159 and mid microspores were used for anther culture. As demonstrated by Salas et al.
160 (2012), the unusual thickness of eggplant anthers makes that the best way to ensure that
161 microspores are at the right stages (vacuolate microspores and young bicellular pollen)
162 when medium components reach the anther locule, is to select anthers at previous stages
163 (with young and mid microspores). According to Dumas de Vaulx et al. (1981), anthers
164 were plated in C medium supplemented with 120 g/l sucrose, 8 g/l Bacto-agar, 5 mg/l
165 kinetin and 5 mg/l 2,4-D, and incubated at 35ºC in darkness for 8 days, followed by 4
166 more days at 25ºC in 12/12 photoperiod. Then, they were transferred to R medium
167 supplemented with 30 g/l sucrose, 8% Bacto-agar and 0.1 mg/l kinetin, and incubated in
168 the same conditions. When embryos emerging from anthers were visible, the total
169 number of embryos was recorded for each donor genotype.
170
171 *Morphological characterization of the DH population*
172 Morphological traits were recorded from individual plants of the original DH population
173 and the parental hybrid Bandera using 10 primary descriptors developed by EGGNET
174 (van der Weerden and Barendse 2007). These descriptors included leaf, flower and fruit
175 characteristics. Leaf prickles, leaf surface shape, corolla color, fruit predominant color,

176 fruit additional color, fruit additional color distribution, fruit cross section, fruit color
177 intensity under calix and fruit calix prickles were measured in a scale with
178 predetermined values corresponding to the EGGNET descriptors. Since this was a
179 characterization of individual DH plants, no replicates could be made. The number of
180 flowers per inflorescence was counted in five inflorescences per plant and expressed as
181 a quantitative trait. Besides these primary descriptors, fruit production, seed production,
182 and germination rate of the DHS1 were also recorded (Table 1). To assess the
183 germination rate, 20 seeds of each line obtained after selfing were sterilized in 4 g/l
184 sodium hypochlorite solution for 5 min, rinsed three times for 5 min each in sterile
185 distilled water and plated in MS medium supplemented with 1.5% sucrose and 0.7%
186 plant agar. Dishes with seeds were incubated at 25ºC with a 12/12 photoperiod and
187 germination rate was recorded after 2 weeks.
188

189 *Data collection and statistical analysis*

190 In order to evaluate the androgenic ability of the DH genotypes in anther culture,
191 between 15 and 90 anthers of each genotype were cultured, except for the Bandera
192 hybrid, where more plants were available and 135 anthers could be used. The number of
193 embryos produced after 3 months was recorded and the efficiency was expressed as
194 number of embryos produced/100 anthers. In order to evaluate the androgenic ability of
195 the DH genotypes in isolated microspore culture, between 15 and 60 buds of each
196 genotype were used. The number of calli produced was recorded after 30 days of culture
197 and the efficiency was expressed as number of calli/ml of culture. Anther and
198 microspore culture assays were repeated thrice. An ANOVA test ($p \leq 0.05$) was
199 performed to assess global significant differences, and then a Fisher's least significant
200 difference (LSD) test for multiple comparisons was performed in order to group the

201 different genotypes in groups of homogeneity, considering significant differences when
202 p-value was <0.05.
203
204 **Results and discussion**
205
206 A population of 80 DH individuals was developed from Bandera microspore cultures.
207 Plantlets were regenerated through indirect organogenesis, acclimatized and hardened in
208 growth chambers, and then grown to flowering in the greenhouse. Their ploidy was
209 checked by flow cytometry, confirming their doubled haploidy. To ensure that each
210 individual of the DH population had a different genotype, only one DH plantlet from
211 each callus was selected and regenerated for this study. Their characterization in terms
212 of morphological traits, reproductive fitness and androgenic competence is described
213 and discussed next.
214
215 **The eggplant DH population presented moderate morphological variability**
216
217 Our population of regenerated DH plants showed variability in morphology and
218 performance, as revealed by the different values of the descriptors used. Table 2 shows
219 the values of Bandera, of a chosen DH line (DH36), and the average and ranges of the
220 values of the entire DH population. Two of the descriptors measured for the fruit, Fr-
221 Section and Fr-UnderC, showed no variability among the DH individuals or compared
222 with the parental hybrid. Fruits always presented a circular section and no grooves
223 (value 1), and the intensity of color under the calix was always the same as in the rest of
224 the fruit (value 9). For the descriptors that presented variability (Figure 1), the most
225 frequent group always corresponded to the value of cv. Bandera, except in the case of

226 Fr-Prickles. This character was highly variable, ranging from less than 3 to more than
227 30, but as a group, the DH individuals presented notably fewer prickles in the fruit calix
228 than the parental hybrid. Leaf related characters presented variability in a short range of
229 values. Most of the genotypes did not have prickles on the leaf, and the bullae of the
230 leaf surface were slightly variable. Flower color (Figures 2A-C) presented variability
231 from almost white (value 4) to strong pinkish-violet (value 8). The number of flowers
232 per inflorescence was highly variable among DH genotypes, ranging from 2 to 7. At
233 commercial ripeness, skin color of Bandera fruits (Figure 2D), which belong to the
234 varietal type '*Listada*' (Nuez and Llácer 2001), was characterized as purple with white
235 stripes (Table 2). In our derived DH population, primary and secondary fruit colors
236 presented an interesting variability, being either white or with different purple shades
237 from lilac grey (value 6) to black (value 9). Color distribution was mostly stripped, but
238 4% of the genotypes presented uniformly colored fruits. For an easier comparison, fruit
239 color patterns were grouped in 3 categories: white with dark stripes (Figures 2E, F),
240 dark with white stripes (Figures 2G, H) and uniformly dark (Figures 2I, J).
241
242 All the variability above described was restricted within the ranges of predetermined
243 values of the primary morphological descriptors used. However, we also found some
244 characters not included in these descriptors or values. While the standard number of
245 petals in eggplant flowers is usually five (Frary et al. 2007), DHs frequently presented a
246 higher number of whorl pieces, with anther and petal numbers ranging from six to eight
247 and with different degrees of cohesion, as shown in Figures 2A-C. This feature is
248 commonly found in eggplant varieties with globose and round fruit types (Frary et al.
249 2007). Abnormalities were also observed on the leaf surface. While the parental hybrid
250 Bandera had no prickles on the leaf surface (Figure 3A), 2.6% of the individuals

251 showed prickles on the midrib and secondary leaf veins (Figure 3B). However, the most
252 striking abnormality was the presence, in several DH individuals, of small leaf blades
253 growing on the midrib, perpendicular to the main leaf blade (Figures 3C, D). These
254 ectopic leaf-like structures were only observed in young plants cultured in growing
255 chambers, being absent in adult plants, once transferred to the greenhouse. The transient
256 nature of this trait suggests that it might be related to the different environmental
257 conditions of growth chambers and greenhouses, being light intensity and duration the
258 most likely influencing parameters. Indeed, eggplant is known to be highly dependent
259 on light conditions (Uzun 2007). Abnormal morphologic traits were also found in other
260 studies dealing with DH populations. For example, Malik et al. (2008) found that some
261 highly responding *B. napus* DH lines presented epinastic leaves, non-abscised petals
262 and pale flower color. Nevertheless, these unusual traits appear irrelevant as long as
263 they do not compromise biological performance or agronomical usefulness.
264
265 This variability generated in gametic cells cultured *in vitro* is referred to as
266 gametoclonal variation (Veilleux 1998) and may be caused by meiotic recombination,
267 by spontaneous mutations or by the process of *in vitro* culture itself (Kaeppler et al.
268 2000; Malik et al. 2008). One advantage of doubled haploidy is the possibility to
269 unmask, fixing them in homozygosity, many of these events that might otherwise
270 remain silent due to dominance effects in heterozygous individuals. Indeed, previous
271 studies in eggplant demonstrated the convenience of DH lines as a powerful tool to
272 reveal variability in diverse important agronomical traits, including fruit number and
273 yield (Rotino et al. 1991), even in cases when donor plants had a high level of
274 homozygosity (Sanguineti et al. 1990). It is known that the impact of gametoclonal
275 variation differs among species from null to high (Snape et al. 1988). Our study,

276 together with others (Rotino et al. 1991), showed that in the particular case of eggplant,
277 gametoclonal variation exists and may be used for breeding purposes, but it is not
278 dramatic.
279

280 **The eggplant DH population presented a slightly reduced reproductive ability**
281

282 All individuals of the DH population were selfed to produce a second DH generation
283 (DHS1) through which we evaluated the reproductive ability of the DH population. As
284 seen in Table 2, 85% of the DHs were able to set fruits. From them, 83% produced
285 seeds. The germination rate was higher than 70% in 87% of the lines, and only 7%
286 produced seeds unable to germinate in our conditions (two weeks at 25ºC and 12/12
287 photoperiod). In summary, 63% of the individuals of the total DH population could be
288 reproduced by seed. These results indicate that the reproductive traits of our DH
289 population are slightly affected. This is not surprising, since DH lines fully express
290 deleterious recessive genes that may be masked by dominance in the heterozygous
291 parental. The strong selection pressure during the *in vitro* regeneration eliminates
292 genotypes with major lethal genes, but this selection is only effective on genes related to
293 vegetative growth, while no selection pressure is exerted on reproductive traits
294 (Bohanec 2002). Other possible causes of the reduced reproductive ability include
295 increased seed dormancy, low pollen fertility due to high frequency of meiotic
296 irregularities such as formation of univalents, trivalents, or unequal chromosome
297 segregation, known to occur as a consequence of *in vitro* culture (Doğramacı-Altuntepe
298 et al. 2001; Immonen and Robinson 2000), or due to the particularities of the species
299 used (Oleszczuk et al. 2011). Alternatively, inbreeding depression may also be a source
300 of reduction in reproductive ability, which makes reasonable to expect the moderate

301 signs of reduced reproductive ability we found. However, eggplant is generally
302 considered a self-pollinating species (Frary et al. 2007), so it is expected to be able to
303 bear high or complete homozygosis without important detrimental effects.
304
305 **The DH population showed a wide range of androgenic competence**
306
307 The androgenic competence of the 66 surviving DHs (the rest up to 80 aged or died
308 before this assay started) and the donor hybrid Bandera was compared by culturing
309 anthers of each single plant. Although microspore culture is the method of choice due to
310 its higher efficiency, this method requires a minimum number of buds at the right stage
311 for microspore isolation. This is possible when working with homogeneous populations
312 (e.g. hybrids or DH lines), but when analyzing individual plants, the amount of
313 available buds at a given time is insufficient. This is why we opted for anther culture.
314 Bandera yielded 146.5 embryos/100 anthers, a value that was set as the reference. The
315 response of DHs was variable among lines, ranging from 0 to 237.5 embryos/100
316 anthers (Table 3). 29 DHs did not produce any embryo (omitted in Table 3). A Fisher's
317 least significant difference (LSD) test for multiple comparisons grouped the
318 embryogenic yield of these DH lines in 5 categories of homogeneity. The individuals
319 with highest yield, belonging to the higher group (e in Table 3), included DH36, DH40,
320 DH15, DH39, DH72, DH41 and DH34, together with the parental hybrid Bandera. Five
321 of them produced more embryos than Bandera (up to 1.6x). However, these increased
322 yields were not significantly different from Bandera. The DH individual with the
323 highest yield, DH36, only appeared in the highest category, being absent from the
324 others.
325

DH plants were selfed and DHS1 seed was collected separately from each line. To further assess their androgenic competence, our goal was to evaluate to what extent androgenic competence was inherited in the next seed generation. We aimed to use DHS1 plants from the seven genotypes with the highest yield as donor plants for isolated microspore cultures, and then compare their efficiency with the parental hybrid. However, DH39 did not produce any fruit and DH34 and DH72 produced very few seeds with a low germination rate (less than 60%), so they were discarded. The androgenic ability in isolated microspore culture of the remaining four DHS1 lines was higher than Bandera, which yielded 65.1 calli/ml (Table 4). The differences in yield between the DH lines and 'Bandera' were higher than for anther culture, ranging from 1.2 to 4.1-fold. DH36 was also in this case the line with the highest callus yield (compare Figures 4A and B).

When evaluated in anther culture, the DH lines showed a high variability in their androgenic response, as shown by the high values of SD in Table 3. This variability was notably reduced when androgenic response was evaluated in microspore culture, as shown in Table 4. A high number of factors, difficult to control, influence the efficiency of microspore embryogenesis, including growing conditions of donor plants, seasonal effects, and *in vitro* culture conditions (Rotino 1996). Among the *in vitro* conditions, anther tissues have a prominent role in the excretion of different substances that may promote or inhibit embryo development (Seguí-Simarro et al. 2011), making the exact composition of the culture medium more unpredictable than in microspore cultures. This is why it is not surprising to find higher variability in the response of microspores to anther culture than to microspore culture. Microspore culture is a more technically demanding procedure, but once optimized it allows for a higher control of culture

351 conditions, which in turn provides more stable efficiency (Forster et al. 2007), as it was
352 the case in our experiments.
353

354 Our results clearly showed that androgenic response is highly variable in the
355 segregating DH population. Since plants were cultured together under the same
356 conditions, such variability suggests that androgenic competence is a genetically
357 controlled trait, and not all DH regenerants in our population carry the most favorable
358 allele combinations. Genetic control of androgenic responsiveness has already been
359 studied in several species, including rice (Miah et al. 1985; Quimio and Zapata
360 1990)(Yamagishi et al. 1996), *Brassica napus* (Zhang and Takahata 2001) and even
361 eggplant (Salas et al. 2011). In short, the conclusion of these studies was that this is a
362 recessive trait controlled by a few recessive nuclear genes, with strong additive effects.
363 Due to this, efforts have been made to breed for improved androgenic response. In
364 maize, Petolino et al. (1988) showed it possible, and suggested that anther culture *per se*
365 allows for the selection of genes favoring an increased response. In their experiments, a
366 single cycle of selection resulted in more than a six-fold increase. Similarly, Malik et al.
367 (2008) obtained dramatic increases from initially recalcitrant genotypes in *B. napus*,
368 The four-fold increase in microspore culture after a single *in vitro* DH generation adds
369 to these evidences, including eggplant in the list of species where breeding for improved
370 androgenic response is possible.
371

372 **DH36 is a DH line with stably high embryogenic competence**

373

374 Our DH36 line had an average yield of 237.5 embryos per 100 anthers cultured, and
375 267.36 calli/ml in isolated microspore culture. Previous reports on eggplant

376 androgenesis with different genotypes found results far behind the yield of DH36. In
377 anther culture experiments, the response of the commercial hybrids Ecavi (Salas et al.
378 2011) and Cristal (Salas et al. 2012) was reported to be 60.9 and 53 embryos/100
379 anthers, respectively. Other studies reported maximal responses of 14.2 embryos (Başay
380 et al. 2011) and 3.67 embryos/100 anthers with the most responsive of the genotypes
381 used (Alpsoy and Seniz 2007). In microspore culture, Corral-Martínez and Seguí-
382 Simarro (2012) reported 5 calli/ml for Ecavi and 2 calli/ml for Cristal. The yield of the
383 DH36 line obtained in our study greatly surpassed all other genotypes previously tested,
384 including Bandera donors. In addition, DH36 calli showed a good organogenic and
385 regenerative performance (Figure 4C). In general, it is thought that genotypes with
386 highly androgenic response use to be recalcitrant to organogenesis and regeneration,
387 which makes them difficult to use for genetic transformation (Malik et al. 2008). Our
388 observations showed that this seems not the case for DH36. In addition, as seen in Table
389 2, the values of morphological descriptors for DH36 were identical to Bandera, with the
390 exception of some fruit traits including predominant (1 vs 7) and additional color (7 vs
391 1) and the presence of prickles (9 vs 7). In terms of reproductive ability, the only
392 difference (minor) was a seed germination rate of 95% vs 100% in Bandera. In
393 conclusion, DH36 could be considered a high response line, phenotypically and
394 agronomically similar to the donor hybrid (Bandera), genetically stable and self-
395 perpetuating, and therefore useful for basic and applied research in eggplant microspore
396 embryogenesis.

397

398 **Concluding remarks**

399

400 We developed here an eggplant DH population from a commercial hybrid which
401 showed variability in morphological and reproductive traits, as well as in androgenic
402 competence. This population can be perpetuated by seed without further segregation,
403 which makes it useful for genetic analysis and mapping of segregating characters. It
404 may also be used to facilitate the study of the genetic control of androgenic competence
405 in eggplant. We also developed the, to the best of our knowledge, first eggplant DH line
406 with high androgenic response. The use of this line will provide material to be used both
407 for basic research about morphogenesis, and for applied research of *in vitro* DH
408 production. It could even be used to explore the possibility of using crosses to transfer
409 its androgenic competence to recalcitrant genotypes of agronomic interest. Hopefully,
410 this high response line will encourage the use of eggplant as a research platform for the
411 study of this fascinating experimental phenomenon.

412

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419

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564

565

Tables

Table 1. Morphological and reproductive traits evaluated and their description

Trait	Code	Units/description
Leaf prickles	L-Prickles	0 = none
		1 = very few (1-2)
		3 = few (3-5)
		5 = intermediate (6-10)
		7 = many (11-20)
		9 = very many (>20)
Leaf surface shape	L-Surface	1 = flat
		5 = intermediate
		9 = very convex or bullate
Number of flowers per inflorescence	Fl-Number	Quantitative trait
Corolla color	Fl-Color	1 = greenish white
		3 = white
		5 = pale violet
		7 = light violet
		9 = bluish violet
Fruit production	Fr-Prod	Yes/No
Fruit predominant color	Fr-MainC	1 = milk white
		2 = yellowish
		3 = green
		4 = unknown
		5 =reddish
		6 = lilac grey
		7 = purple
		8 = purple black
		9 = black
Fruit additional color	Fr-AddC	1 = milk white
		2 = yellowish
		3 = green
		4 = unknown
		5 =reddish
		6 = lilac grey
		7 = purple
		8 = purple black
		9 = black
Fruit additional color distribution	Fr-CDistr	1 = uniform
		3= mottled
		5 = netted
		7 = striped
		9 = other
Fruit cross section	Fr-Section	1=circular, no grooves
		3 = elliptic
		5 = few grooves (~4)
		7 = many grooves (~8)
		9 = very irregular
Fruit color intensity under calix	Fr-UnderC	0 = none
		1 = very weak
		3 = weak
		5 = medium
		7 = strong
		9 = very strong
Fruit calix prickles	Fr-Prickles	0 = none
		1 = very few (<3)
		3 = few (~5)

		5 = intermediate (~10) 7 = many (~20) 9 = very many (>30)
Seed production	S-Prod	Yes/No
Germination rate	S-Germ	%

569

570 **Table 2.** Results of the evaluation of morphological and reproductive traits of the donor
571 Bandera hybrid, the DH population and a DH line (DH36). Data for Bandera and DH36
572 genotypes are values of each parameter. Data for the DH population are expressed as
573 the value average and range (bracketed) of each parameter.

	Bandera Value	**DH population Mean [range]**	**DH36 Value**
L-Prickles	0	0.1 [0-7]	0
L-Surface	1	1.5 [1-3]	1
Fl-Number	3-5	3.9 [2-7]	4-5
Fl-Color	7	6.9 [4-8]	7
Fr-Prod	Yes	85% Yes – 15% No	Yes
Fr-MainC	7	4.8 [1-9]	1
Fr-AddC	1	3.5 [1-7]	7
Fr-CDistr	7	6.9 [1-7]	7
Fr-Section	1	1.0 [1-1]	1
Fr-UnderC	9	9.0 [9-9]	9
Fr-Prickles	7	5.2 [1-9]	9
S-Prod	Yes	83% Yes - 17% No	Yes
S-Germ	100%	84.9 [0-100]	95%

574

575 **Table 3.** Androgenic competence in anther culture of DH individuals and the donor
576 hybrid (Bandera). Fisher's least significant difference (LSD) test found statistically
577 significant differences among the androgenic competence of DH individuals, as well as
578 compared to Bandera. Means are placed in five homogeneity groups (a-e). Note that one
579 DH individual can belong to more than one homogeneity group, and means followed
580 with at least a common letter are not statistically different at p<0.05. All the non-
581 responsive genotypes (not shown in this table) belonged to group a.

Genotype	**Anthers plated**	**Responding anthers**	**Embryos/100 anthers (mean ± SD)**	**LSD**				
				G1	**G2**	**G3**	**G4**	**G5**
DH47	86	1	1,19 ±1,68	a				
DH68	48	1	2,63 ±3,72	a	b			
DH50	29	1	3,33 ±4,71	a	b			
DH52	46	2	4,76 ±6,73	a	b			
DH76	32	1	7,14 ±10,10	a	b			
DH13	71	4	7,69 ±10,88	a	b			
DH46	87	4	8,54 ±12,07	a	b			
DH33	28	1	8,82 ±12,48	a	b			
DH22	37	1	9,09 ±12,86	a	b			
DH14	28	1	10,00 ±14,14	a	b			
DH20	23	1	10,00 ±14,14	a	b			
DH75	38	1	10,00 ±14,14	a	b			
DH64	19	1	10,71 ±15,15	a	b			

DH21	23	1	11,76	±16,64	a	b			
DH66	24	2	12,22	±1,57	a	b			
DH60	36	2	14,29	±20,20	a	b			
DH63	32	1	14,29	±20,20	a	b			
DH19	37	2	14,58	±20,62	a	b			
DH53	27	2	14,71	±20,80	a	b			
DH70	40	3	14,79	±6,03	a	b			
DH37	62	2	20,45	±28,93	a	b	c		
DH43	33	2	22,22	±31,43	a	b	c		
DH42	23	2	22,35	±19,82	a	b	c		
DH31	43	1	23,08	±32,64	a	b	c		
DH29	60	2	27,47	±11,91	a	b	c		
DH65	29	2	30,28	±35,75	a	b	c		
DH30	85	1	37,21	±52,62	a	b	c		
DH16	30	3	38,24	±54,07	a	b	c		
DH17	35	2	47,37	±66,99	a	b	c		
DH74	33	5	68,33	±73,07	a	b	c		
DH34	47	2	72,22	±102,14	a	b	c	d	e
DH41	54	5	74,24	±104,99	a	b	c	d	e
Bandera	135	24	146,46	±137,45		b	c	d	e
DH72	18	3	157,69	±201,25	a	b	c	d	e
DH39	29	6	162,50	±229,81	a	b	c	d	e
DH15	31	3	185,00	±261,63			c	d	e
DH40	23	5	231,82	±327,84				d	e
DH36	22	1	237,50	±335,88					e

Table 4. Androgenic competence in isolated microspore culture of DHS1 lines derived from DH individuals and the donor hybrid (Bandera). Fisher's least significant difference (LSD) test found statistically significant differences among the androgenic competence of DHS1 lines and Bandera. Means are placed in 4 homogeneity groups (a-d).

Genotype	Calli/ml (mean ± SD)		LSD[a]
Bandera	65,08	±54,11	a
DH15	76,85	±68,56	a
DH41	92,00	±27,65	b
DH40	149,11	±79,49	c
DH36	267,36	±33,96	d

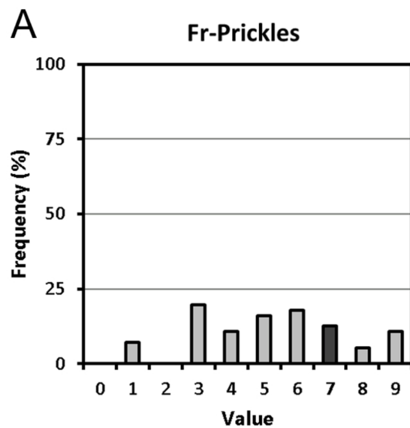
Figure legends

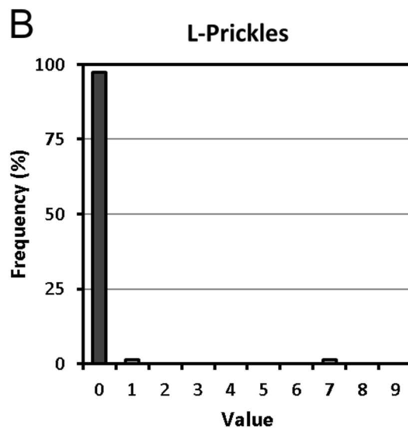
Figure 1. Graphical representation of the variability shown by the DH population. Each chart corresponds to each of the morphological descriptors used. The light grey columns correspond to the values of DH lines, whereas the dark grey columns correspond to the values of cv. Bandera which, with the exception of Fr-Prickles, were always the most frequent values in the DH population.

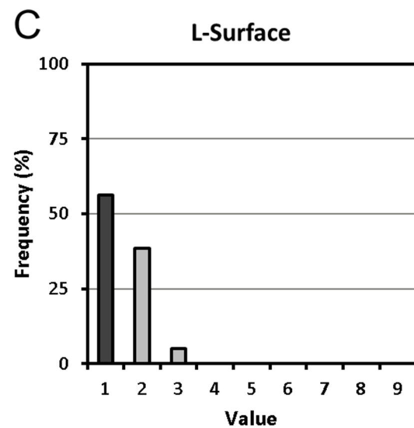
Figure 2. Morphological variability of the DH population. A-C: Flowers with different petal color including white (A), pinkish (B) and purple (C). These flowers present unusual piece numbers (6-7) and different levels of cohesion in some of their petals. D: Bandera fruits showing, as expected, a remarkable phenotypical homogeneity. E, F: DH fruits with white background and different levels of dark stripes. G, H: DH fruits with different levels of dark background and white stripes. I, J: DH fruits with different levels of uniformly dark background.

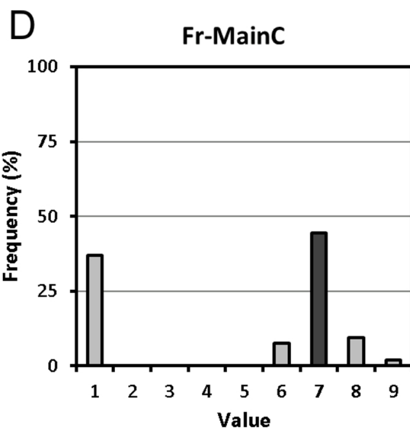
Figure 3. Examples of leaf surfaces of the Bandera hybrid (A) with no prickles, and of some DH individuals with prickles (white arrows) on the midrib and secondary leaf veins (B), and with large (C) and small (D) leaf blades (black arrows) on the midrib.

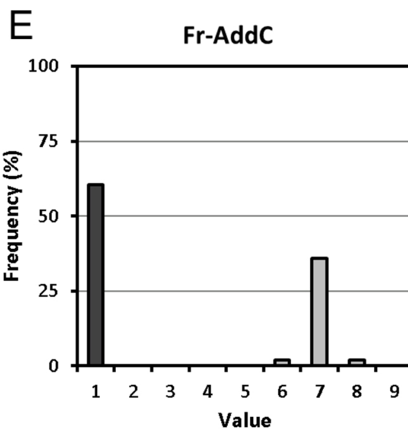
Figure 4. Isolated microspore culture dishes of Bandera (A) and DH36 (B). Note the difference between genotypes in terms of androgenic response. C shows a microspore-derived callus with shoot organogenic nodules (arrows) developing on its surface.

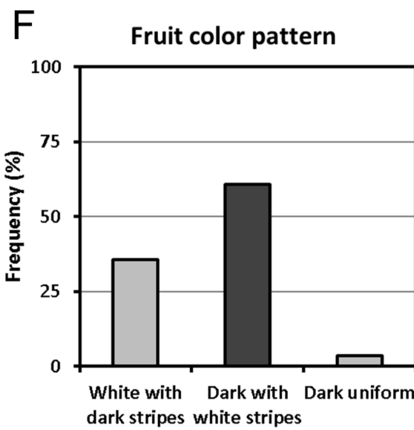

A
Fr-Prickles
100
75
50
25
0
Frequency (%)
0 1 2 3 4 5 6 7 8 9
Value

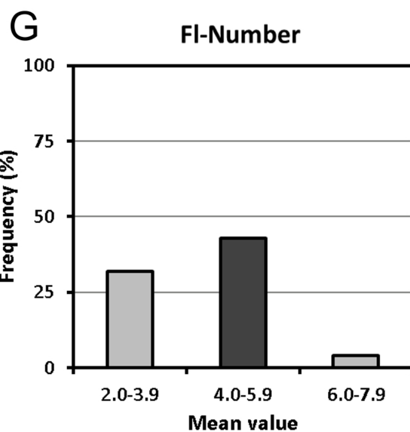

B
L-Prickles
100
75
50
25
0
Frequency (%)
0 1 2 3 4 5 6 7 8 9
Value

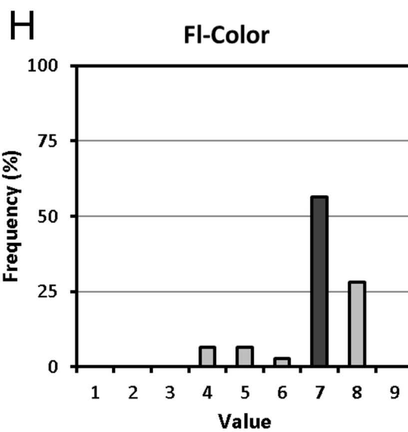

C
L-Surface
100
75
50
25
0
Frequency (%)
1 2 3 4 5 6 7 8 9
Value

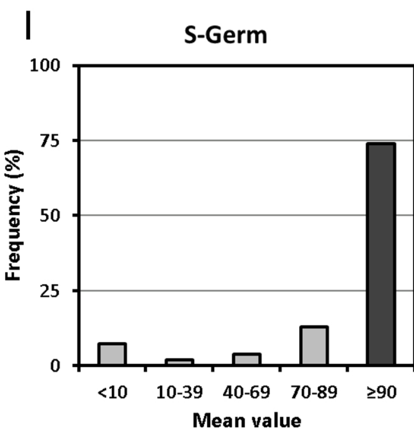

D
Fr-MainC
100
75
50
25
0
Frequency (%)
1 2 3 4 5 6 7 8 9
Value


E
Fr-AddC
100
75
50
25
0
Frequency (%)
1 2 3 4 5 6 7 8 9
Value

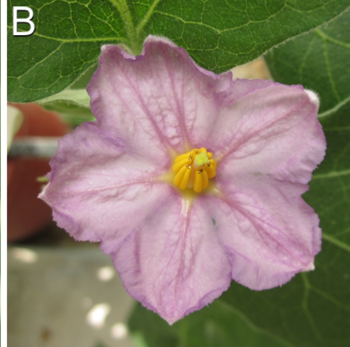

F
Fruit color pattern
100
75
50
25
0
Frequency (%)
White with dark stripes
Dark with white stripes
Dark uniform


G
Fl-Number
100
75
50
25
0
Frequency (%)
2.0-3.9
4.0-5.9
6.0-7.9
Mean value


H
Fl-Color
100
75
50
25
0
Frequency (%)
1 2 3 4 5 6 7 8 9
Value


I
S-Germ
100
75
50
25
0
Frequency (%)
<10
10-39
40-69
70-89
≥90
Mean value

A


B

C

D

E

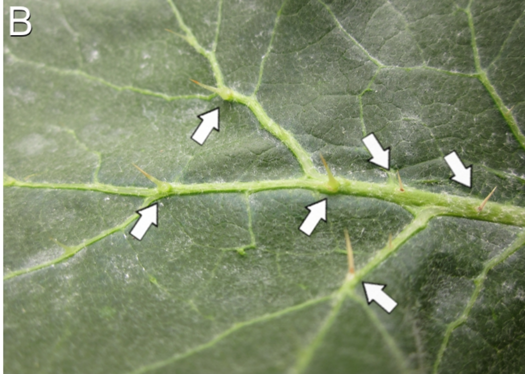
F

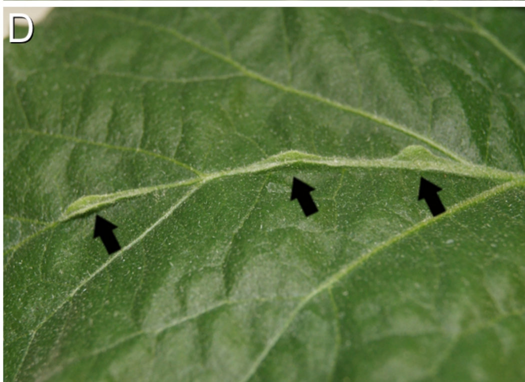
G

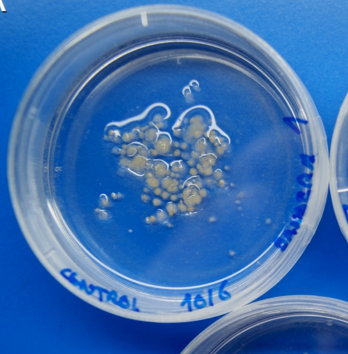
H

I

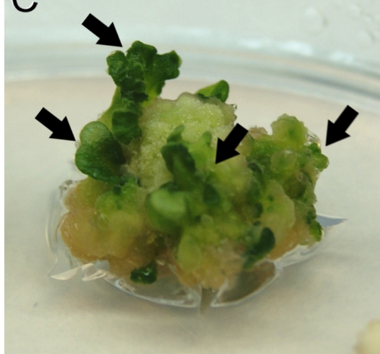
J

A

B

C

D


A

B


C