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Additional Information

1 **Development of backcross generations and new interspecific hybrid combinations for**
2 **introgression breeding in eggplant (*Solanum melongena*)**

3

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17 ABSTRACT

18 Introgression breeding can contribute to broadening the genetic background of eggplant (*Solanum*
19 *melongena*). We used six eggplant varieties and 44 interspecific hybrids between these eggplant
20 accessions and 10 accessions of wild relatives from the primary genepool species *S. insanum* and
21 secondary genepool species *S. anguivi*, *S. dasyphyllum*, *S. incanum*, *S. lichtensteinii*, and *S.*
22 *tomentosum* to obtain first backcross generations to *S. melongena*. Pollen viability in cultivated and
23 wild parents and interspecific hybrids with *S. insanum* was high, while for interspecific hybrids with
24 secondary genepool species it was variable. First backcross generations to *S. melongena* were
25 obtained with interspecific hybrids of all the wild species, with the best results being obtained in
26 crosses with hybrids between *S. melongena* and *S. insanum*. However, ample differences were
27 observed among eggplant varieties in the success of the crosses. Additionally, the six eggplant
28 varieties were crossed with secondary genepool species *S. campylacanthum*, *S. lidii* and *S. vespertilio*
29 and with tertiary genepool species *S. bonariense*, *S. elaeagnifolium* and *S. sisymbriifolium* with the
30 aim of obtaining new interspecific hybrids. Successful interspecific hybridization was achieved with
31 the three new secondary genepool species tested and, using embryo rescue, with the tertiary genepool
32 species *S. elaeagnifolium*. The new backcross generations and interspecific hybrids obtained will
33 contribute to broadening the genetic background of the eggplant and to the genetic enhancement of
34 this crop.

35

36 *Keywords:* *Solanum melongena*, interspecific hybrids, backcrossing, introgression breeding

37

38 **1. Introduction**

39

40 Despite the large morphological diversity of cultivated eggplant (*Solanum melongena* L.)
41 (Portis et al., 2015; Kaushik et al., 2016) its genetic diversity is narrow, in particular in the modern
42 cultivars (Muñoz-Falcón et al., 2009). The low genetic diversity of eggplant, compared to its wild

43 relatives (Furini and Wunder, 2004; Vorontsova et al., 2013), is very likely a result of the genetic
44 bottleneck during domestication from its ancestor, the weedy *S. insanum*, in Southeast Asia and China
45 (Meyer et al., 2012). Although introgression from *S. insanum* has probably been common during the
46 evolution of the crop (Davidar et al., 2015), artificial selection coupled with limited genetic diversity
47 of *S. insanum* has contributed to maintaining a narrow genetic background of eggplant.

48 Depending on the phylogenetic relationships with eggplant and potential for hybridization
49 with eggplant, its crop wild relatives are assigned to the primary, secondary or tertiary genepools
50 (Syfert et al., 2016). *Solanum insanum* is the only species included in the primary genepool of
51 eggplant (Knapp et al., 2013). However, eggplant is also related to a large number of Old World
52 species of *Solanum* section *Leptostemonum*, most of which are native to Africa, where the greatest
53 diversity of wild relatives of eggplant can be found (Weese and Bohs, 2010; Vorontsova et al., 2013;
54 Aubriot et al., 2016). In this respect, around 50 wild species of the Anguivi, Melongena and Climbing
55 phylogenetic groups of the eggplant secondary genepool have been identified as potentially most
56 interesting as genetic resources for eggplant breeding (Syfert et al., 2016). Also, a number of other
57 *Solanum* species, some of them from the New World, are part of the tertiary genepool species of
58 eggplant (Daunay and Hazra, 2012; Syfert et al., 2016). Among them, *S. torvum* and *S.*
59 *sisymbriifolium* have been traditionally considered as very interesting for eggplant breeding due to
60 their multiple resistances to the most important eggplant fungal and bacterial diseases, like those
61 caused by *Fusarium oxysporum*, *Verticillium dahliae*, or *Ralstonia solanacearum*, as well as to
62 nematodes (Bletsos et al., 2003; Gousset et al., 2005; Daunay and Hazra, 2012). Although sexual and
63 somatic interspecific hybrids with *S. torvum* have been obtained, they are highly sterile and although
64 reciprocal crosses have been attempted, no backcross generations have been produced (Sihachakr et
65 al., 1989; Bletsos et al., 1998; Kumchai et al., 2013). Regarding *S. sisymbriifolium*, no viable sexual
66 interspecific hybrids have been obtained and tetraploid somatic hybrids have been sterile (Gleddie et
67 al., 1986; Bletsos et al., 1998). Other tertiary genepool species that have recently raised interest for
68 eggplant breeding are *S. bonariense*, which is a relative of *S. torvum* (Nurit-Silva et al., 2012) with

69 high vigour and that has largely been unexplored but that may share some of the useful characteristics
70 of *S. torvum* for eggplant breeding, and *S. elaeagnifolium* which is an invasive weed highly tolerant
71 to drought (Christodoulakis et al., 2009). To our knowledge, no interspecific hybrids have been
72 obtained between eggplant and either *S. bonariense* or *S. elaeagnifolium*. Although Plazas et al.
73 (2016) attempted crossing *S. melongena* with *S. elaeagnifolium*, no fruit set was obtained.

74 Contrarily to many other crops, in which wild species have been used for introgression
75 breeding (Hajjar and Hodgkin, 2007; Warschefsky et al., 2014), the use of wild species in eggplant
76 breeding has been very limited (Daunay and Hazra, 2012; Rotino et al., 2014). Although interspecific
77 hybrids, with different degrees of viability and fertility, between eggplant and wild relatives have
78 been obtained with some 25 species (Rotino et al., 2014; Devi et al., 2015; Plazas et al., 2016) most
79 of the studies have been for taxonomic purposes and preliminary breeding works and have not
80 undertaken the development of backcross generations. Nonetheless, in some instances introgression
81 materials have been obtained with eggplant relatives, including eggplant lines with introgression of
82 resistance to *Fusarium oxysporum* from *S. aethiopicum* (Toppino et al., 2008), or to *Verticillium*
83 *dahliae* from *S. linnaeanum* (Liu et al., 2015), or backcross generations with *S. aethiopicum* or *S.*
84 *incanum* (Prohens et al., 2012, 2013). Also, alloplasmic male-sterile *S. melongena* lines containing
85 the cytoplasm of wild relatives of eggplant have been obtained (Khan et al., 2015). However, no
86 systematic attempts have been undertaken to obtain multiple sets of eggplant materials containing
87 introgressions from different wild relatives of eggplant.

88 We have recently reported the development of interspecific hybrids with 10 different wild
89 species (Plazas et al., 2016), which is a first step for developing materials of eggplant with
90 introgressions from several wild species. Many of the hybrids obtained have been morphologically
91 characterized (Kaushik et al., 2016), which has provided relevant information for breeders on the
92 potential interest of the different wild species. Backcrossing of interspecific hybrids to the cultivated
93 eggplant is the next natural step in an introgression breeding programme in this species (Toppino et
94 al., 2008; Daunay and Hazra, 2012; Liu et al., 2015), but its success depends on the fertility of the

95 hybrids, which may preclude or difficult the development of backcross generations, and the direction
96 of the cross (Daunay and Hazra, 2012; Rotino et al., 2014). Also, developing new interspecific
97 hybrids with other potentially interesting wild species from the secondary and tertiary gene pools may
98 also contribute to the final objective of broadening the genetic base of eggplant with introgressions
99 from wild species. In this respect, when obtaining interspecific hybrids, the use of *S. melongena* as
100 female parent is frequently preferred, as it has several advantages, like allowing the 100% recovery
101 of the cytoplasm of the cultivated parent, easier manipulation of flowers, more regular fruit set, greater
102 number of seeds per fruit and absence of dormancy in the seeds (Lester and Kang, 1998; Behera and
103 Singh, 2002; Devi et al., 2016; Plazas et al., 2016).

104 In this work we evaluate the fertility of multiple interspecific hybrids of several eggplant
105 varieties with accessions (i.e., distinct, uniquely identifiable sample of seeds representing a
106 population, which is maintained in storage for conservation and use) corresponding to six different
107 wild species, perform crosses between these interspecific hybrids and their respective *S. melongena*
108 parents in order to develop backcross generations. In addition, we perform new interspecific
109 hybridizations with different accessions of six other wild species of the secondary and tertiary
110 gene pools in order to develop new interspecific hybrids. Our aim is to provide relevant information
111 and new backcross generations and interspecific hybrids with the ultimate objective of broadening
112 the genetic background of eggplant.

113

114 **2. Material and methods**

115

116 *2.1. Plant material*

117

118 2.1.1. First backcross generations

119

120 The plant material used for obtaining first backcrosses to cultivated eggplant consisted of: a)
121 six eggplant cultivars used as recurrent parents originating from Ivory Coast (MEL1 to MEL3) and
122 Sri Lanka (MEL4 to MEL6); b) 10 accessions of wild eggplant relatives corresponding to three
123 accessions of the only primary genepool species *S. insanum* (INS1-INS3), and seven to the secondary
124 genepool species *S. anguivi* (2 accessions, ANG1-ANG2), *S. dasyphyllum* (1 accession, DAS1, *S.*
125 *incanum* (1 accession, INC1), *S. lichtensteinii* (2 accessions, LIC1-LIC2), and *S. tomentosum* (2
126 accessions, TOM1-TOM2); and, c) 44 interspecific hybrids between the six eggplant accessions and
127 the 10 wild species accessions, totaling 18 hybrids with *S. insanum*, 10 with *S. anguivi*, four with *S.*
128 *dasyphyllum*, four with *S. incanum*, six *S. lichtensteinii*, and two with *S. tomentosum*). The codes of
129 the hybrids used and their respective wild and cultivated parents are presented in Table 1. Further
130 information on the origin of these cultivated and wild accessions can be found in Plazas et al. (2016).
131 Five plants of each of the six *S. melongena* recurrent parents, 10 wild parental accessions and, as
132 available, of the 44 interspecific hybrids were used. Only the *S. melongena* accessions and
133 interspecific hybrids were used for making the crosses to obtain the backcrosses to *S. melongena*. All
134 accessions were used for determining pollen viability.

135

136 2.1.2. New interspecific hybrids

137

138 Twelve accessions from three other species of the secondary genepool (*S. campylacanthum*,
139 four accessions; *S. lidii*, one accession; *S. vespertilio*, two accessions) and three species of the tertiary
140 genepool (*S. bonariense*, one accession; *S. eleagnifolium*, two accessions; *S. sisymbriifolium*, two
141 accessions) (Table 2) were used for interspecific hybridization with the same six *S. melongena*
142 accessions (MEL1-MEL6) used for obtaining the backcrosses. Five plants of each of the *S. melongena*
143 parents and of the wild parental accessions were used for interspecific hybridization.

144 **Table 1**

145 Materials used for obtaining first backcross combinations to *S. melongena*. They correspond to
 146 accessions of *S. melongena* (MEL1 to MEL6) and wild species of the primary and secondary
 147 gene pools, and the respective interspecific hybrids between *S. melongena* and wild species.

<i>S. melongena</i>						
Accession	MEL1	MEL2	MEL3	MEL4	MEL5	MEL6
Primary gene pool						
<i>S. insanum</i>						
INS1	MEL1×INS1	MEL2×INS1	MEL3×INS1	MEL4×INS1	INS1×MEL5	MEL6×INS1
INS2	MEL1×INS2	MEL2×INS2	MEL3×INS2	MEL4×INS2	MEL5×INS2	MEL6×INS2
INS3	INS3×MEL1	INS3×MEL2	INS3×MEL3	INS3×MEL4	MEL5×INS3	INS3×MEL6
Secondary gene pool						
<i>S. anguivi</i>						
ANG1	---	MEL2×ANG1	MEL3×ANG1	MEL4×ANG1	MEL5×ANG1	---
ANG2	MEL1×ANG2	MEL2×ANG2	ANG2×MEL3	ANG2×MEL4	MEL5×ANG2	ANG2×MEL6
<i>S. dasyphyllum</i>						
DAS1	MEL1×DAS1	MEL2×DAS1	MEL3×DAS1	---	MEL5×DAS1	---
<i>S. incanum</i>						
INC1	INC1×MEL1	---	MEL3×INC1	---	MEL5×INC1	MEL6×INC1
<i>S. lichtensteinii</i>						
LIC1	MEL1×LIC1	---	---	---	MEL5×LIC1	MEL6×LIC1
LIC2	MEL1×LIC2	---	MEL3×LIC2	MEL4×LIC2	---	---
<i>S. tomentosum</i>						
TOM1	---	MEL2×TOM1	TOM1×MEL3	---	---	---

148

149

150

151 **Table 2**

152 Twelve wild species of the secondary and tertiary gene pools used for obtaining new interspecific
 153 hybrids with cultivated eggplant (*S. melongena*), including the germplasm accession code and the
 154 FAO germplasm bank of origin of each accession.

Wild species	Accessions	Germplasm accession code ^a	FAO germplasm bank code	Country of origin
Secondary gene pool				
<i>S. campylacanthum</i>	CAM5	MM680	FRA030	Tanzania
	CAM6	MM700	FRA030	Tanzania
	CAM7	MM1414	FRA030	Kenya
	CAM8	MM1426	FRA030	Kenya
<i>S. liddii</i>	LID1	4788	ESP026	Spain
<i>S. vespertilio</i>	VES1	4601A	ESP026	Spain
	VES2	BGV-3213	ESP003	Spain
Tertiary gene pool				
<i>S. bonariense</i>	BON1	BON1	ESP026	Spain
<i>S. eleagnifolium</i>	ELE1	MM1627	FRA030	Senegal
	ELE2	ELE2	ESP026	Greece
<i>S. sisymbriifolium</i>	SIS1	SOLN-78	ESP026	Unknown
	SIS2	1180	ESP026	Unknown

155 ^aAccessions with MM codes originate from the INRA (Avignon, France) germplasm bank, while
 156 the rest of the accessions belong to the COMAV (Valencia, Spain) germplasm collection.

157

158

159 2.2. *Cultivation conditions*

160

161 Seeds of all materials were germinated according to the protocol of Ranil et al. (2015).
162 Germinated seeds were transferred to seedling trays and kept in a climatic chamber with a under a
163 photoperiod and temperature regime of 16 h light (25 °C):8 h dark (18 °C). Seedlings were
164 transplanted in April 2015 to a pollinator-free glasshouse at the campus of the Universitat Politècnica
165 de València Spain (greenhouse GPS coordinates: 39°29'01" N, 0°20'27" W). Plants were grown in
166 25 L pots filled with commercial growing substrate and irrigated and fertilized using a drip irrigation
167 system. Plants were pruned and trained with vertical strings. Phytosanitary treatments against spider
168 mites were performed when necessary.

169

170 2.2. *Pollen viability*

171

172 Pollen viability of the 44 interspecific hybrids and their parents was evaluated in a mixture of
173 pollen extracted from several mature fully-opened flowers using an enzymatic method. Enzymatic-
174 based pollen viability was assessed using the fluorescein diacetate (FDA) fluorochromatic reaction
175 modified from Heslop-Harrison et al., (1984). Pollen samples were incubated in the dark with a
176 solution of FDA (10 mg·L⁻¹) in water for 10 min, and then observed at 200 x under a Zeiss Axiovert
177 40 CFL (Carl Zeiss AG, Oberkochen, Germany) inverted microscope equipped for fluorescence
178 microscopy, using a 494 nm excitation filter and a 510 nm emission filter to excite/visualize FDA
179 emission. Viable pollen grains were considered as those displaying intense fluorescence, while those
180 that displayed no or weak fluorescence were considered as non-viable.

181 In an attempt to correlate the FDA-based viability estimation with a faster, cheaper, easy-to-
182 measure and therefore more convenient pollen morphological analysis, we performed a parallel
183 morphological study. Using the same inverted microscope, pollen grains were observed under phase
184 contrast at 200 x. Pollen grains with normal shape and size, and with a filled and turgent appearance

185 were considered as viable, while those appearing dwarf, wrinkled or empty were considered as non-
186 viable. For each parental or interspecific hybrid, between 400 and 800 pollen grains were counted
187 from at least three microscope fields for both FDA-based viability estimation and for morphological
188 assesment.

189

190 *2.4. Backcrossing and interspecific hybridization*

191

192 In order to obtain first backcross generations, reciprocal crosses were performed between *S.*
193 *melongena* accessions and interspecific hybrids, although priority was given to hybridizations in
194 which the interspecific hybrids were used as female parents, due to their lower pollen fertility.
195 *Solanum melongena* was used to cross with new wild species as female parent, their flowers were
196 emasculated before flower opening, then pollen deposited on the stigma by gently rubbing a glass
197 slide covered with pollen of the male parent. Pollinated flowers were tagged and a record was kept
198 for calculating the percentage of fruit set.

199

200 *2.5. Seed extraction and embryo rescue*

201

202 Fruits that had set and developed corresponding to both first backcrosses and new interspecific
203 hybrids with secondary genepool species were left on the plant until they reached full physiological
204 maturity. Seeds from these fruits were extracted manually at the lab and the seed was placed on filter
205 paper and left to dry at room temperature. Seeds obtained for each fruit were subsequently counted.
206 For fruits corresponding to interspecific hybridizations of *S. melongena* with tertiary genepool
207 species, the immature fruits were harvested between 20 and 30 days after pollination and brought to
208 the laboratory where immature seeds, when present, were extracted under sterile conditions and
209 embryos cultivated in vitro using the protocol indicated in Plazas et al. (2016).

210

211 2.6. Data analysis

212

213 For pollen viability percentage values, the average of each of the cultivated and wild
214 accessions and of interspecific hybrids were calculated from the replicates for each of the
215 accessions or hybrids. The percentage data were transformed using the arcsine transformation,
216 which consists in obtaining the arcsine of the squared root of percentage/100 (Little and Hills,
217 1978), and subjected to ANOVA factorial analysis using the species or interspecific hybrid parental
218 species combination as factor. Significant differences among species or interspecific hybrid parental
219 species combinations were detected using the Student-Newman-Keuls (SNK) multiple range test.
220 The mean percentage value for each accession or hybrid for morphologically normal pollen and
221 pollen viability estimated with FDA was used to calculate the linear coefficient of correlation
222 between these two pollen characters. For comparison of fruit set percentage in the crosses between:
223 a) *S. melongena* parentals and interspecific hybrids for obtaining first backcross generations, or b)
224 *S. melongena* parentals and wild species for obtaining new interspecific hybrids, we used the
225 Marascuilo's post hoc multiple comparison procedure, which allows the comparison among several
226 proportions or percentages (Marascuilo and McSweeney, 1977), at $P < 0.05$. The standard error of
227 the mean (SE) was also calculated for the fruit set percentage of each of the *S. melongena*
228 accessions and from the number of seeds per seeded fruit.

229

230 3. Results

231

232 3.1. Pollen viability

233

234 Pollen grains of the cultivated *S. melongena* and the wild species of the primary and secondary
235 gene pools and the hybrids between *S. melongena* and the only primary gene pool species *S. insanum*
236 were mostly of normal shape and size, with average values of morphologically normal grains above

237 88% and non-significant ($P>0.05$) differences among them (Table 3; Figure 1). When considering the
238 hybrids with secondary genepool species, the hybrids between *S. melongena* and *S. incanum*
239 displayed pollen grains which were mostly filled, turgent and normally shaped, with an average value
240 of morphologically normal pollen of 77.4%, being non-significantly different from its parents;
241 however, the rest of hybrids displayed a significantly lower frequency of morphologically normal
242 pollen (Table 3; Figure 1), with average values ranging between 9.6% for the hybrids of *S. melongena*
243 with *S. tomentosum* to 31.5% for the hybrids between *S. melongena* and *S. lichtensteinii* (Table 3).
244

245 **Table 3**

246 Average values (based on accession means) for morphologically normal pollen and FDA-based
 247 pollen viability of cultivated eggplant (*S. melongena*), wild species from the primary and secondary
 248 gene pools, and of interspecific hybrids between *S. melongena* and wild species. Between 400 and
 249 800 pollen grains were counted from at least three microscope fields for each accession or hybrid.

Species / hybrid	Accessions / hybrids	Morphologically normal pollen (%) ^a	FDA-based viability (%) ^a
Cultivated eggplant			
<i>S. melongena</i>	6	95.0 c	68.9 cd
Primary gene pool			
<i>S. insanum</i>	3	96.9 c	67.7 cd
Secondary gene pool			
<i>S. anguivi</i>	2	93.1 c	87.6 d
<i>S. dasyphyllum</i>	1	88.8 c	54.7 c
<i>S. incanum</i>	1	92.3 c	67.3 cd
<i>S. lichtensteinii</i>	2	92.8 c	59.6 c
<i>S. tomentosum</i>	1	88.4 c	66.1 cd
Hybrids with primary gene pool species			
<i>S. melongena</i> x <i>S. insanum</i>	18	94.9 c	62.2 c
Hybrids with secondary gene pool species			
<i>S. melongena</i> x <i>S. anguivi</i>	10	24.6 ab	18.1 ab
<i>S. melongena</i> x <i>S. dasyphyllum</i>	4	25.5 ab	14.9 ab
<i>S. melongena</i> x <i>S. incanum</i>	4	77.4 c	61.0 c
<i>S. melongena</i> x <i>S. lichtensteinii</i>	6	31.5 b	25.7 b
<i>S. melongena</i> x <i>S. tomentosum</i>	2	9.6 a	5.5 a
Probability of F ^b		<0.0001	<0.0001

250 ^aMeans within columns separated by different letters are significantly different at $P < 0.05$, according
251 to the Student-Newman-Keuls test using arcsine transformed data (arcsine of the squared root of
252 percentage/100).

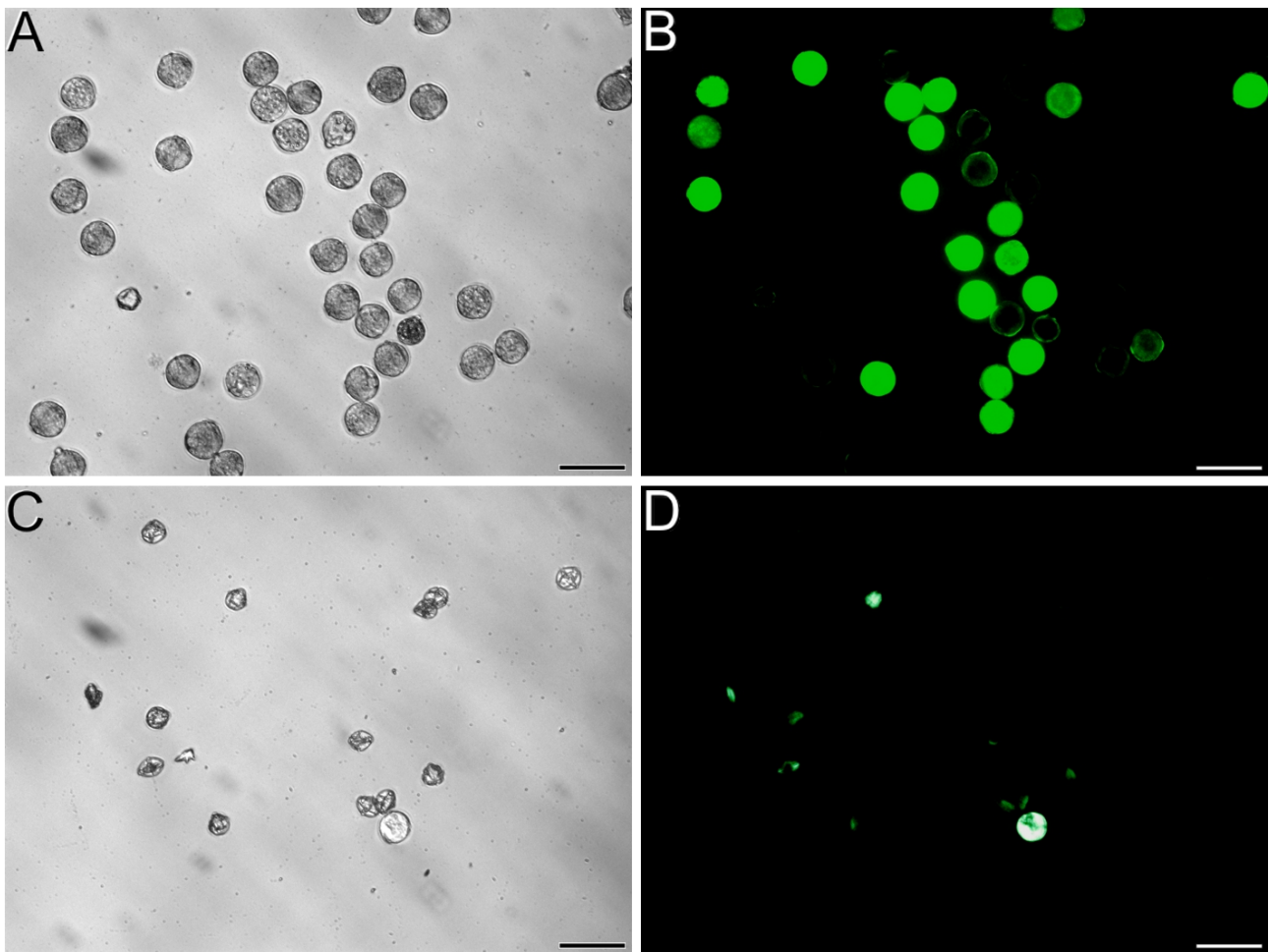
253 ^bBased on arcsine transformed data (arcsine of the squared root of percentage/100).

254

255

256

257



258

259 **Figure 1.** Pollen grains of *S. melongena* (A, B) and of a hybrid between *S. melongena* and secondary
260 gene pool *S. tomentosum* (C, D) stained with FDA and observed under phase contrast (A, C) and
261 fluorescent light (B, D). Bars: 50 μm.

262

263

264 The assessment of pollen viability with the FDA enzymatic reaction generally resulted in
265 estimates of viability lower (~20%) than percentages of morphologically normal pollen (Table 3;
266 Figure 1). However, a high degree of correlation was observed between morphologically normal
267 pollen and pollen viability estimated with FDA was observed, with a correlation coefficient among
268 mean values for accessions and hybrids of $r=0.904$ ($P<0.0001$). When considering the *S. melongena*
269 parents and wild species, the range of variation was much higher than with the morphological
270 assessment, and significant differences ($P<0.05$) were observed between the highest value, which was
271 found in *S. anguivi* (87.6%), and the lowest values of *S. dasyphyllum* (54.7%) and *S. lichtensteinii*
272 (59.6%) (Table 3). The hybrids between *S. melongena* and the primary genepool species *S. insanum*
273 and the secondary genepool species *S. incanum* displayed average values (62.2% and 61.0%,
274 respectively) that were not significantly different to those of any of its parents. Regarding the
275 interspecific hybrids of *S. melongena* with other wild species of the secondary genepool, they
276 displayed significantly lower viability than its cultivated and wild parents, with the extreme values
277 being the hybrids with *S. tomentosum* and *S. lichtensteinii*, which presented significant differences
278 among them, with average values of 5.5% and 25.7%, respectively (Table 3).

279

280 3.2. Development of backcross generations

281

282 A total of 1052 crosses between the six cultivated *S. melongena* accessions and the 44
283 interspecific hybrids between *S. melongena* and wild relatives were performed, of which 615 were
284 made using *S. melongena* as male parent and 437 as female parent (Table 4). Crosses of *S. melongena*
285 with interspecific hybrids with the primary genepool species *S. insanum* had a high degree of success,
286 with a 38.4% and 43.2% of fruit set on the crosses made when using *S. melongena* as male or female
287 parent, respectively (Table 4). Also, all fruits of the backcross with the hybrid with *S. insanum* were
288 seeded, with a mean over 500 seeds/fruit in both directions, significantly higher than in the rest of
289 backcrosses with secondary genepool species (Table 4). The results of crossings of *S. melongena* with

290 hybrids with secondary genepool species were variable depending on the interspecific hybrid parental
291 wild species and the direction of the cross. When using *S. melongena* as a male parent, fruit set in
292 hybrids with *S. incanum* was significantly higher than in the rest of hybrids with secondary genepool
293 species (Table 4). Fruits of backcrosses were generally seeded, with the exception of a certain
294 percentage of fruits in the crosses with hybrids *S. melongena* × *S. dasyphyllum* (25.0%) and *S.*
295 *melongena* × *S. tomentosum* (42.9%), which were parthenocarpic. The number of seeds per fruit was
296 very variable, with significantly higher values, of over 200 seeds/fruit, in the backcrosses between *S.*
297 *melongena* and hybrids with *S. incanum* or *S. lichtensteinii*, than in the interspecific hybrids with the
298 three other species (*S. anguivi*, *S. dasyphyllum*, and *S. tomentosum*), which had less than 20 seeds per
299 fruit. When using *S. melongena* as a female parent, the fruit set percentage of backcrosses was much
300 lower, and no fruit set was obtained when using hybrids with *S. lichtensteinii* or *S. tomentosum* as
301 male parents, and the percentage was very low when using hybrids with *S. anguivi* (1.4%) or *S.*
302 *dasyphyllum* (4.3%). Furthermore in the former case, all fruits obtained were parthenocarpic, while
303 in the latter most of them were parthenocarpic (Table 4). Also, in the backcrosses with the hybrid
304 with *S. incanum* the fruit percentage was much lower than when using it as a female parent and the
305 number of seeds was also lower, with less than 100 seeds/fruit (Table 4). No significant differences
306 were observed among the different hybrids of the same parental combination in the success of
307 crossing when using them as male parent in the crossings with *S. melongena*.

308 Important differences were observed among *S. melongena* parents in the success of the
309 crosses. When using *S. melongena* as a male parent it was observed that some accessions, like MEL1,
310 MEL5 and MEL6 displayed a greater degree of success in the crosses with interspecific hybrids of *S.*
311 *melongena* with both primary and secondary genepool species (Figure 2). When using *S. melongena*
312 as a female parent, again important differences were observed among accessions, with MEL1, MEL5
313 and MEL6 having a greater degree of success when used as female parents than the rest of accessions
314 in the crosses with hybrids with primary genepool species. When crossing with secondary genepool

315 species, the two only accessions for which fruit set was obtained was with MEL1 and MEL6, with
316 very low fruit set values (Figure 2).

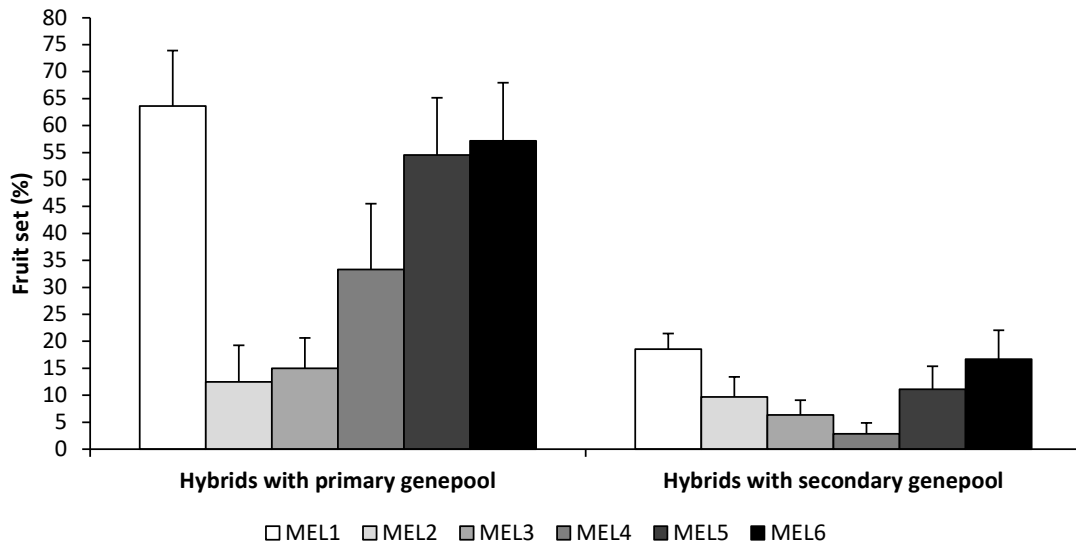
317 **Table 4**

318 Number of crosses, fruit set, seedless fruits, seeds per seeded fruit (mean \pm SE) in first backcrosses of interspecific hybrids between cultivated eggplant
 319 (*Solanum melongena*) and wild relatives from the primary and secondary gene pools, according to the direction of the cross.

Interspecific hybrids used as parents	Hybrids (n)	<i>S. melongena</i> used as male parent				<i>S. melongena</i> used as female parent			
		Crosses (n)	Fruit set (%) ^a	Seedless fruits (%)	Seeds/seeded fruit (n) ^a	Crosses (n)	Fruit set (%) ^a	Seedless fruits (%)	Seeds/seeded fruit (n)
Hybrids with primary gene pool species									
<i>S. melongena</i> x <i>S. insanum</i>	18	146	38.4 b	0.0	701.8 \pm 36.6	37	43.2 b	0.0	551.5 \pm 49.5
Hybrids with secondary gene pool species									
<i>S. melongena</i> x <i>S. anguivi</i>	10	200	11.5 a	0.0	16.0 \pm 3.9	142	1.4 a	100.0	---
<i>S. melongena</i> x <i>S. dasyphyllum</i>	4	103	7.8 a	25.0	17.3 \pm 4.8	185	4.3 a	62.5	25.5 \pm 14.7
<i>S. melongena</i> x <i>S. incanum</i>	4	36	41.7 b	0.0	205.0 \pm 12.2	13	15.3 b	0.0	83.5 \pm 18.5
<i>S. melongena</i> x <i>S. lichtensteinii</i>	6	85	11.8 a	0.0	221.8 \pm 20.5	24	0.0 a	---	---
<i>S. melongena</i> x <i>S. tomentosum</i>	2	45	15.6 a	42.9	7.7 \pm 0.4	36	0.0 a	---	---

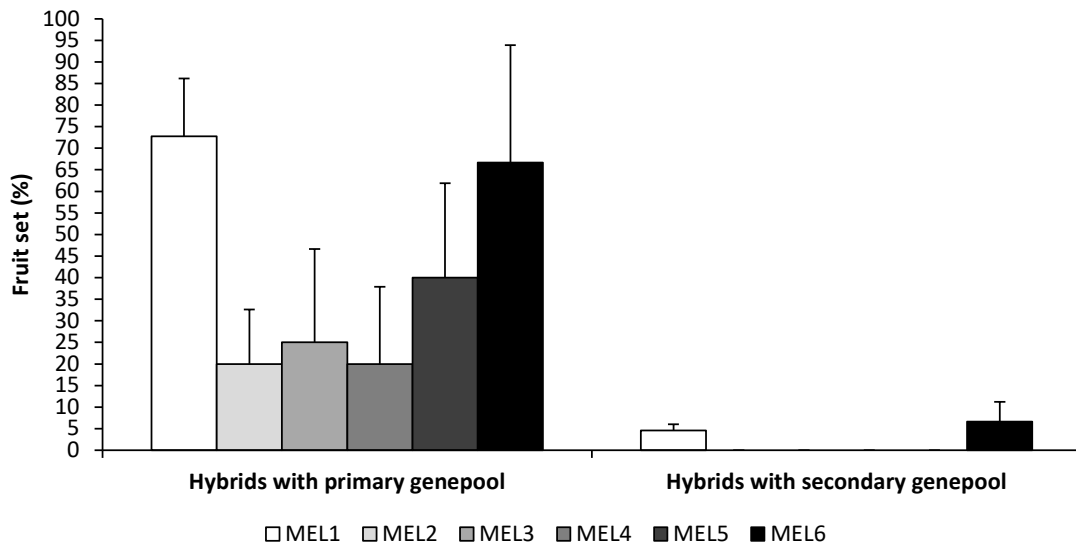
320 ^aMeans within columns separated by different letters are significantly different at P<0.05, according to the Marascuilo's post-hoc multiple comparison
 321 procedure for proportions.

S. melongena (♂)



322

S. melongena (♀)



323

324 **Figure 2**

325 Fruit set percentage (\pm SE) of the first backcrosses between six *S. melongena* accessions (MEL1 to

326 MEL 6) and hybrids of *S. melongena* with wild species of the primary and secondary genepools when

327 using *S. melongena* as a male (above) or female (below) parent.

328

329 3.3. Development of new hybrids

330

331 A total of 263 crosses were made between *S. melongena* (as female parent) and the three
332 secondary genepool wild species *S. campylacanthum*, *S. lidii* and *S. vespertilio* (Table 5). The
333 percentage of fruit set in the interspecific crosses with *S. melongena* varied between 2.6% for crosses
334 with *S. lidii* to 24.5% with *S. campylacanthum*, which significant differences in fruit set among these
335 two latter species (Table 5). All fruits obtained after crossing *S. melongena* with *S. lidii* and *S.*
336 *vespertilio* were seeded and in the case of crosses with *S. vespertilio* had a large number of seeds
337 (over 500 seeds per fruit). In the crosses with *S. campylacanthum*, despite the larger fruit set almost
338 two thirds of the fruits were parthenocarpic and the number of seeds was much lower than in the
339 hybrids with *S. vespertilio* and similar to the only fruit obtained with *S. lidii*. When considering
340 crosses with tertiary genepool species *S. bonariense*, *S. eleagnifolium* and *S. sisymbriifolium*, a total
341 of 173 crosses were made. Considerable differences were found in fruit set, with significantly higher
342 values in *S. eleagnifolium* (26.8%) than in *S. sisymbriifolium* (4.0%) and *S. bonariense* (0.0%).
343 However, all fruits obtained after crossing with tertiary genepool species were parthenocarpic or
344 presented embryos aborted at an early stage, with the exception of a single fruit in which 11 embryos
345 could be rescued and cultivated *in vitro* to produce viable hybrid plants (Table 5). As a result of the
346 interspecific hybridizations with secondary and tertiary genepool species performed 12 new
347 interspecific hybrid combinations were obtained, of which 11 were with secondary genepool
348 accessions and 1 with the tertiary genepool species *S. eleagnifolium*.

349

350 **Table 5**

351 Number of crosses, fruit set, seedless fruits, seeds per fruit (mean \pm SE) in crosses between cultivated
 352 eggplant (*Solanum melongena*; as female parent) and wild relatives from the secondary and tertiary
 353 genepools (as male parents).

Wild species used as parentals	<i>S. melongena</i> (female parent)				
	Accessions (n)	Crosses (n)	Fruit set (%) ^a	Seedless fruits (%)	Seeds/seeded fruit (n)
Secondary genepool					
<i>S. campylacanthum</i>	4	184	24.5 b	62.2	133.3 \pm 40.7
<i>S. lidii</i>	1	39	2.6 a	0.0	103 ^b
<i>S. vespertilio</i>	2	40	10.0 ab	0.0	562.5 \pm 104.2
Tertiary genepool					
<i>S. bonariense</i>	1	65	0.0 a	---	---
<i>S. eleagnifolium</i>	2	56	26.8 b	93.3	11 ^{b,c}
<i>S. sisymbriifolium</i>	2	52	4.0 a	100.0	---

354 ^aMeans within column separated by different letters are significantly different at P<0.05, according
 355 to the Marascuilo's post-hoc multiple comparison procedure for proportions.

356 ^bNo SE is given, as only one fruit was obtained.

357 ^cEmbryos rescued from immature seeds.

358

359

360 **4. Discussion**

361

362 Broadening the genetic base of crops may help in coping with the challenges posed by the
 363 upcoming climate change (Dempewolf et al., 2014; Warschefsky et al., 2014). This is particularly
 364 true in crops with a narrow genetic base, like eggplant (Mace et al., 1999; Furini and Wunder, 2004;

365 Muñoz-Falcón et al., 2009; Weese and Bohs, 2010; Vorontsova et al., 2013). Broadening the genetic
366 background of eggplant can help not only to produce new varieties with traits introgressed from wild
367 species, but also to improving the potential for obtaining hybrids heterotic for yield by crossing
368 genetically distant parent lines (Rodríguez-Burruezo et al., 2008).

369 Although interspecific hybridization of eggplant with related species has been reported with
370 different degrees of success (Bletsos et al., 1998; Lester and Kang, 1998; Behera and Singh, 2002;
371 Daunay and Hazra, 2012; Kumchai et al., 2013; Rotino et al., 2014; Devi et al., 2016; Plazas et al.,
372 2016), successful introgression requires backcrossing of the hybrids to the cultivated parent. This step
373 many times represents the most important challenge for introgression breeding (Rieseberg and
374 Carney, 1998), due to sterility and/or lack of fruit set in some hybrid combinations. In previous works,
375 backcrosses have been obtained between eggplant and several wild species (Rotino et al., 2008;
376 Mennella et al., 2010; Prohens et al., 2012; 2013; Liu et al., 2015); however, our work represents the
377 first systematic attempt to obtain backcrosses between several varieties of cultivated eggplant a
378 number of wild species accessions from the primary and secondary gene pools.

379 The high pollen viability observed in the parental cultivated and wild accessions indicates that
380 the conditions in which the plants developed were appropriate for a normal pollen formation process
381 (Giorno et al., 2013), which has allowed us to make an adequate assessment of the potential fertility
382 of interspecific hybrids. The high pollen viability, similar to their parents, of the hybrids between *S.*
383 *melongena* and *S. insanum* is a confirmation that both species are closely related (Weese and Bohs,
384 2010; Meyer et al., 2012; Knapp et al., 2013). Viability in the hybrids with secondary gene pool
385 species has been very variable. Viability has been higher in hybrids with *S. incanum* and *S.*
386 *lichtensteinii*, which are wild species phylogenetically close to eggplant (Weese and Bohs, 2010;
387 Knapp et al., 2013; Vorontsova et al., 2013; Aubriot et al., 2016), than in the hybrids with the other
388 phylogenetically more distant species. In any case, viable pollen, has been found in all the
389 interspecific hybrids, indicating that potential exists for obtaining backcrosses with all the
390 interspecific hybrids evaluated. The comparatively higher values estimated observed by us using

391 morphology with respect to the FDA-based estimation was expected, as some normal-shaped pollen
392 grains may not be able to germinate and fertilize the egg cells (Dafni and Firmage, 2000). However,
393 we also showed that both methods are proportional. Thus, we propose that the study of pollen
394 morphology may be useful for rapid estimations of viability in pollen of eggplant cultivated and wild
395 accessions when high precision is not mandatory, or for situations where a fluorescence microscope
396 is not available.

397 In general, the success of the backcrossing of the interspecific hybrids to *S. melongena* has
398 matched the results obtained for interspecific hybridization between *S. melongena* and the respective
399 wild species (Plazas et al., 2016). In this respect, backcrosses of the interspecific hybrids of *S.*
400 *melongena* with *S. insanum* to the cultivated *S. melongena* parent have been highly efficient, with a
401 high fruit set percentage and many seeds per fruit when the cross is made in both directions. These
402 results are in agreement with the high success, comparable to that of intraspecific crosses in *S.*
403 *melongena*, of interspecific hybridization between *S. melongena* and *S. insanum* (Davidar et al., 2015;
404 Plazas et al., 2016). This is additional evidence supporting the claim that *S. insanum* is the wild
405 ancestor of *S. melongena* (Meyer et al., 2012, Knapp et al., 2013). The most successful backcrosses
406 with secondary gene pool species have been obtained with *S. incanum*, which was also the secondary
407 gene pool species with higher pollen viability. *Solanum incanum* is also very close to *S. melongena*
408 (Furini and Wunder, 2004; Knapp et al., 2013; Vorontosva et al., 2013). Also, the backcrosses of the
409 interspecific hybrid with *S. lichtensteinii*, the other wild species used belonging to the Eggplant clade
410 (Knapp et al., 2013; Syfert et al., 2016), have been relatively successful. The backcrosses with the
411 hybrids with the three other species, which belong to the Anguivi phylogenetic group (Syfert et al.,
412 2016) have been less successful, and the lower rate of success has been obtained with the hybrids
413 with *S. tomentosum*, which presented low fertility, as estimated by pollen viability. In any case,
414 backcrosses with interspecific hybrids of all species could be obtained, which will facilitate
415 introgression breeding with all the species, especially taking into account fertility generally recovers
416 in backcross generations, compared to the interspecific hybrids (Rieseberg and Carney, 1998). Our

417 results also indicate that using the interspecific hybrids as female parents may be a better strategy for
418 obtaining backcross generations, particularly when the pollen viability of the interspecific hybrid is
419 low. Considerable differences have been found among the *S. melongena* genotypes in the success of
420 backcrossing. According to the results obtained, we consider that best *S. melongena* accessions
421 among those tested for successful backcrossing are MEL1 and MEL6, followed by MEL5. The rest
422 of accessions in general had a poorer performance.

423 New interspecific hybrids with secondary genepool species *S. campylacanthum*, *S. lidii* and
424 *S. vespertilio*, which may be used for subsequent backcrossing, have been obtained. Hybrids between
425 eggplant and this three wild species have been reported previously (Lester and Kang, 1998; Daunay
426 and Hazra, 2012; Rotino et al., 2014). *Solanum campylacanthum* is phylogenetically one of the closest
427 relatives of eggplant (Mace et al., 1999; Knapp et al., 2013; Vorontsova et al., 2013; Syfert et al.,
428 2016), has a wide range of distribution habitats and is extremely variable, which suggests that traits
429 of interest for eggplant breeding for adaptation to climate change may be found in this species.
430 *Solanum lidii* and *S. vespertilio* are endemic species from the Canary Islands (Spain) and are
431 characterized by heterandrous and zygomorphic corolla, which in the case of *S. vespertilio* are also
432 tetramerous (instead of the typical pentamerous corollas of *Solanum*) (Prohens et al., 2007). Apart
433 from their potential interest for eggplant breeding, interspecific hybrids and backcross generations
434 may also be an experimental material of great value for understanding the genetic basis of the odd
435 flower morphology of these two species.

436 Interspecific hybridization with tertiary genepool species was unsuccessful with *S. bonariense*
437 and *S. sisymbriifolium*. Although to our knowledge this is the first attempt for interspecific
438 hybridization with *S. bonariense*, several previous attempts have been performed at crossing *S.*
439 *melongena* and *S. sisymbriifolium* (Bletsos et al., 1998; Plazas et al., 2016) due to the interest of the
440 latter as source of resistance to several diseases (Bletsos et al., 1998; Daunay and Hazra, 2012), but
441 have always been unsuccessful at obtaining sexual interspecific hybrids. Amazingly, we have been
442 able to obtain interspecific hybrids between *S. melongena* and *S. elaeagnifolium* through embryo

443 rescue. To our knowledge, this is the first report of hybrids between these two species and represents
444 a first step for introgressing the extreme tolerance to drought of *S. elaeagnifolium* (Christodoulakis
445 et al., 2009) into the genetic background of *S. melongena*. However, the feasibility of using *S.*
446 *elaegnifolium* as a source of variation for eggplant breeding will depend on the fertility of the
447 interspecific hybrids and the development of backcross generations.

448

449 **5. Conclusions**

450

451 Backcrosses to *S. melongena* have been obtained with the hybrids of eggplant with six wild
452 relatives from the primary and secondary gene pools, confirming that eggplant is amenable to
453 introgression breeding from wild relatives. Fruit set percentage and number of seeds per fruit have
454 been highest in backcrosses with the only primary gene pool species, *S. insanum*. Although pollen
455 viability in eggplant is better estimated with FDA-based methods, the measurement of morphological
456 parameters may be used as an alternative, easier although less precise method. Despite low pollen
457 viability in some hybrids with some secondary gene pool species, backcross generations have been
458 obtained when using the hybrids as female parents. In addition, new interspecific hybrids have been
459 obtained with three secondary gene pool species, and with the tertiary gene pool species *S.*
460 *elaegnifolium*, which is highly tolerant to drought. The information obtained and plant material
461 developed will contribute to the development of a new generation of eggplant cultivars with a broader
462 genetic background.

463

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465

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478

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480

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