

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

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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  
24 with 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.*  
29 *vespertilio* and with tertiary genepool species *S. bonariense*, *S. elaeagnifolium* and *S.*  
30 *sisymbriifolium* with the aim of obtaining new interspecific hybrids. Successful interspecific  
31 hybridization was achieved with the three new secondary genepool species tested and, using  
32 embryo rescue, with the tertiary genepool species *S. elaeagnifolium*. The new backcross generations  
33 and interspecific hybrids obtained will contribute to broadening the genetic background of the  
34 eggplant and to the genetic enhancement of 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  
45 China (Meyer et al., 2012). Although introgression from *S. insanum* has probably been common  
46 during the evolution of the crop (Davidar et al., 2015), artificial selection coupled with limited  
47 genetic diversity of *S. insanum* has contributed to maintaining a narrow genetic background of  
48 eggplant.

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

69 tertiary genepool species that have recently raised interest for eggplant breeding are *S. bonariense*,  
70 which is a relative of *S. torvum* (Nurit-Silva et al., 2012) with high vigour and that has largely been  
71 unexplored but that may share some of the useful characteristics of *S. torvum* for eggplant breeding,  
72 and *S. elaeagnifolium* which is an invasive weed highly tolerant to drought (Christodoulakis et al.,  
73 2009). To our knowledge, no interspecific hybrids have been obtained between eggplant and either  
74 *S. bonariense* or *S. elaeagnifolium*. Although Plazas et al. (2016) attempted crossing *S. melongena*  
75 with *S. elaeagnifolium*, no fruit set was obtained.

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

90 We have recently reported the development of interspecific hybrids with 10 different wild  
91 species (Plazas et al., 2016), which is a first step for developing materials of eggplant with  
92 introgressions from several wild species. Many of the hybrids obtained have been morphologically  
93 characterized (Kaushik et al., 2016), which has provided relevant information for breeders on the  
94 potential interest of the different wild species. Backcrossing of interspecific hybrids to the

95 cultivated eggplant is the next natural step in an introgression breeding programme in this species  
96 (Toppino et al., 2008; Daunay and Hazra, 2012; Liu et al., 2015), but its success depends on the  
97 fertility of the hybrids, which may preclude or difficult the development of backcross generations,  
98 and the direction of the cross (Daunay and Hazra, 2012; Rotino et al., 2014). Also, developing new  
99 interspecific hybrids with other potentially interesting wild species from the secondary and tertiary  
100 gene pools may also contribute to the final objective of broadening the genetic base of eggplant with  
101 introgressions from wild species. In this respect, when obtaining interspecific hybrids, the use of *S.*  
102 *melongena* as female parent is frequently preferred, as it has several advantages, like allowing the  
103 100% recovery of the cytoplasm of the cultivated parent, easier manipulation of flowers, more  
104 regular fruit set, greater number of seeds per fruit and absence of dormancy in the seeds (Lester and  
105 Kang, 1998; Behera and Singh, 2002; Devi et al., 2016; Plazas et al., 2016).

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

115

## 116 **2. Material and methods**

117

### 118 *2.1. Plant material*

119

#### 120 *2.1.1. First backcross generations*

121

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

137

#### 138 2.1.2. New interspecific hybrids

139

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

147 **Table 1**

148 Materials used for obtaining first backcross combinations to *S. melongena*. They correspond to  
 149 accessions of *S. melongena* (MEL1 to MEL6) and wild species of the primary and secondary  
 150 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	---	---	---

151

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153

154 **Table 2**

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

Wild species	Accessions	Germplasm accession code <sup>a</sup>	FAO germplasm bank code	Country of origin
<b>Secondary gene pool</b>				
<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
<b>Tertiary gene pool</b>				
<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

158 <sup>a</sup>Accessions with MM codes originate from the INRA (Avignon, France) germplasm bank, while  
 159 the rest of the accessions belong to the COMAV (Valencia, Spain) germplasm collection.

160

161



162 2.2. *Cultivation conditions*

163

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

174

175 2.2. *Pollen viability*

176

177 Pollen viability of the 44 interspecific hybrids and their parents was evaluated in a mixture  
178 of pollen extracted from several mature fully-opened flowers using an enzymatic method.  
179 Enzymatic-based pollen viability was assessed using the fluorescein diacetate (FDA)  
180 fluorochromatic reaction modified from Heslop-Harrison et al., (1984). Pollen samples were  
181 incubated in the dark with a solution of FDA (10 mg·L<sup>-1</sup>) in water for 10 min, and then observed at  
182 200 x under a Zeiss Axiovert 40 CFL (Carl Zeiss AG, Oberkochen, Germany) inverted microscope  
183 equipped for fluorescence microscopy, using a 494 nm excitation filter and a 510 nm emission filter  
184 to excite/visualize FDA emission. Viable pollen grains were considered as those displaying intense  
185 fluorescence, while those that displayed no or weak fluorescence were considered as non-viable.

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

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

194

#### 195 *2.4. Backcrossing and interspecific hybridization*

196

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

204

#### 205 *2.5. Seed extraction and embryo rescue*

206

207 Fruits that had set and developed corresponding to both first backcrosses and new  
208 interspecific hybrids with secondary genepool species were left on the plant until they reached full  
209 physiological maturity. Seeds from these fruits were extracted manually at the lab and the seed was  
210 placed on filter paper and left to dry at room temperature. Seeds obtained for each fruit were  
211 subsequently counted. For fruits corresponding to interspecific hybridizations of *S. melongena* with  
212 tertiary genepool species, the immature fruits were harvested between 20 and 30 days after  
213 pollination and brought to the laboratory where immature seeds, when present, were extracted under  
214 sterile conditions and embryos cultivated in vitro using the protocol indicated in Plazas et al.  
215 (2016).

216

## 217 2.6. Data analysis

218

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

235

## 236 3. Results

237

### 238 3.1. Pollen viability

239

240 Pollen grains of the cultivated *S. melongena* and the wild species of the primary and  
241 secondary gene pools and the hybrids between *S. melongena* and the only primary gene pool species

242 *S. insanum* were mostly of normal shape and size, with average values of morphologically normal  
243 grains above 88% and non-significant ( $P>0.05$ ) differences among them (Table 3; Figure 1). When  
244 considering the hybrids with secondary genepool species, the hybrids between *S. melongena* and *S.*  
245 *incanum* displayed pollen grains which were mostly filled, turgent and normally shaped, with an  
246 average value of morphologically normal pollen of 77.4%, being non-significantly different from its  
247 parents; however, the rest of hybrids displayed a significantly lower frequency of morphologically  
248 normal pollen (Table 3; Figure 1), with average values ranging between 9.6% for the hybrids of *S.*  
249 *melongena* with *S. tomentosum* to 31.5% for the hybrids between *S. melongena* and *S. lichtensteinii*  
250 (Table 3).  
251

252 **Table 3**

253 Average values (based on accession means) for morphologically normal pollen and FDA-based  
 254 pollen viability of cultivated eggplant (*S. melongena*), wild species from the primary and secondary  
 255 gene pools, and of interspecific hybrids between *S. melongena* and wild species. Between 400 and  
 256 800 pollen grains were counted from at least three microscope fields for each accession or hybrid.

Species / hybrid	Accessions / hybrids	Morphologically normal pollen (%) <sup>a</sup>	FDA-based viability (%) <sup>a</sup>
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 <sup>b</sup>		<0.0001	<0.0001

257 <sup>a</sup>Means within columns separated by different letters are significantly different at  $P < 0.05$ , according  
258 to the Student-Newman-Keuls test using arcsine transformed data (arcsine of the squared root of  
259 percentage/100).

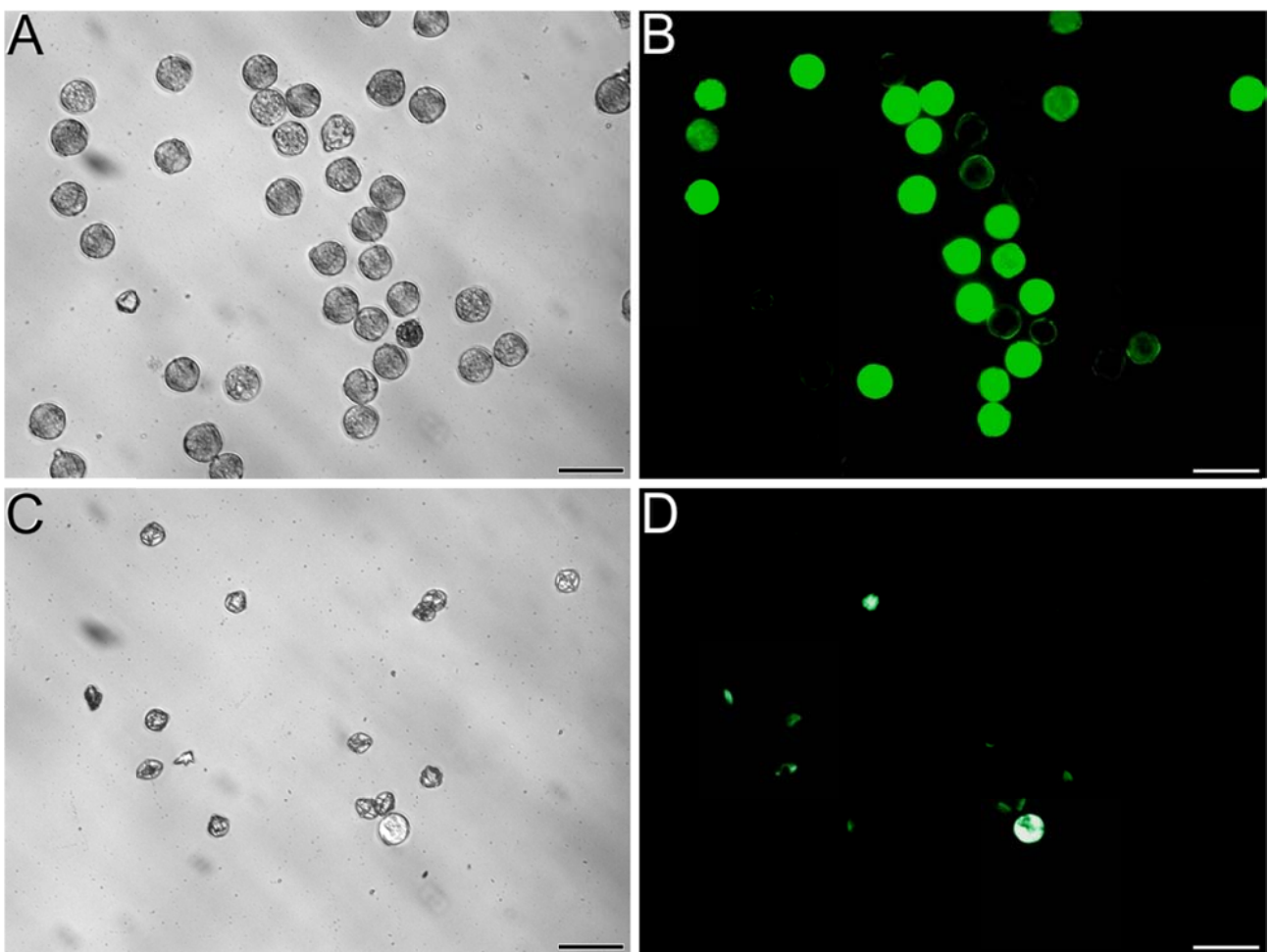
260 <sup>b</sup>Based on arcsine transformed data (arcsine of the squared root of percentage/100).

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264



265

266 **Figure 1.** Pollen grains of *S. melongena* (A, B) and of a hybrid between *S. melongena* and  
267 secondary gene pool *S. tomentosum* (C, D) stained with FDA and observed under phase contrast (A,  
268 C) and fluorescent light (B, D). Bars: 50  $\mu\text{m}$ .

269

270

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

286

### 287 3.2. Development of backcross generations

288

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

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

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



323 secondary gene pool species, the two only accessions for which fruit set was obtained was with  
324 MEL1 and MEL6, with very low fruit set values (Figure 2).

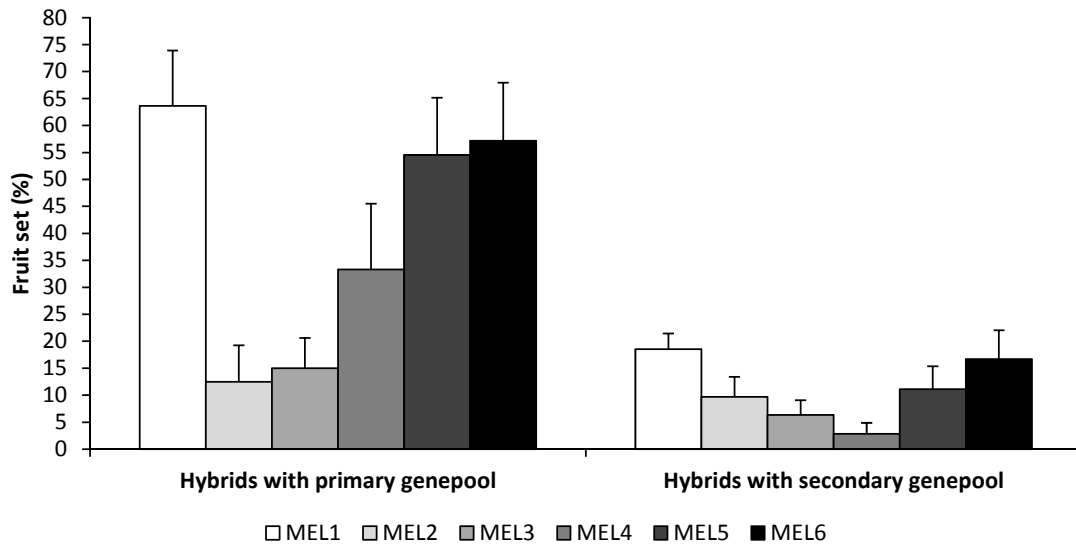
325 **Table 4**

326 Number of crosses, fruit set, seedless fruits, seeds per seeded fruit (mean  $\pm$  SE) in first backcrosses of interspecific hybrids between cultivated eggplant  
 327 (*Solanum melongena*) and wild relatives from the primary and secondary genepools, 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 (%) <sup>a</sup>	Seedless fruits (%)	Seeds/seeded fruit (n) <sup>a</sup>	Crosses (n)	Fruit set (%) <sup>a</sup>	Seedless fruits (%)	Seeds/seeded fruit (n)
Hybrids with primary genepool 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 genepool 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	---	---

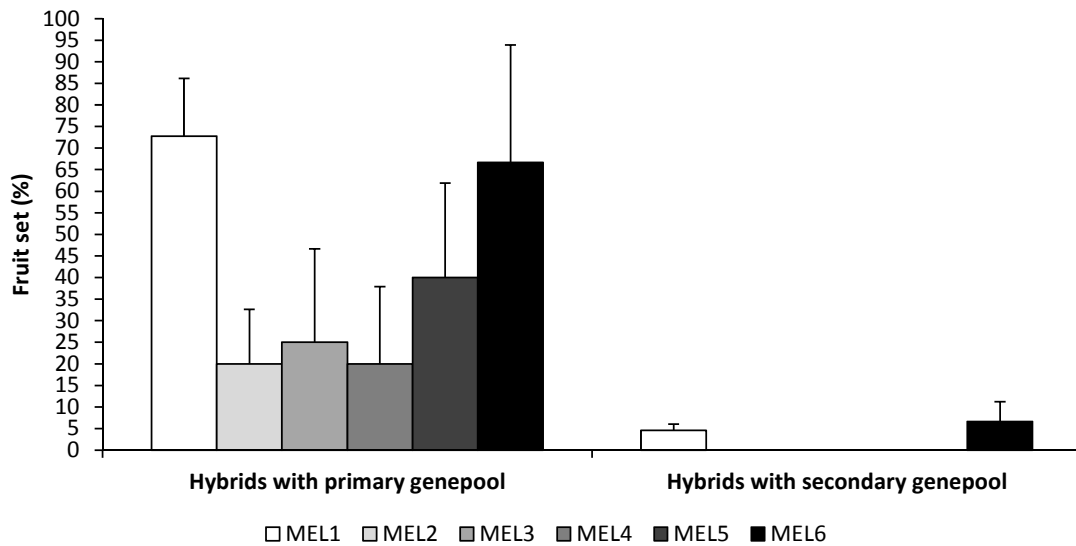
328 <sup>a</sup>Means within columns separated by different letters are significantly different at P<0.05, according to the Marascuilo's post-hoc multiple comparison  
 329 procedure for proportions.

*S. melongena* (♂)



330

*S. melongena* (♀)



331

332 **Figure 2**

333 Fruit set percentage ( $\pm$ SE) of the first backcrosses between six *S. melongena* accessions (MEL1 to  
 334 MEL 6) and hybrids of *S. melongena* with wild species of the primary and secondary genepools  
 335 when using *S. melongena* as a male (above) or female (below) parent.

336

### 337 3.3. Development of new hybrids

338

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

357

358 **Table 5**

359 Number of crosses, fruit set, seedless fruits, seeds per fruit (mean  $\pm$  SE) in crosses between  
 360 cultivated eggplant (*Solanum melongena*; as female parent) and wild relatives from the secondary  
 361 and tertiary gene pools (as male parents).

Wild species used as parentals	<i>S. melongena</i> (female parent)				
	Accessions (n)	Crosses (n)	Fruit set (%) <sup>a</sup>	Seedless fruits (%)	Seeds/seeded fruit (n)
Secondary gene pool					
<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 <sup>b</sup>
<i>S. vespertilio</i>	2	40	10.0 ab	0.0	562.5 $\pm$ 104.2
Tertiary gene pool					
<i>S. bonariense</i>	1	65	0.0 a	---	---
<i>S. eleagnifolium</i>	2	56	26.8 b	93.3	11 <sup>b,c</sup>
<i>S. sisymbriifolium</i>	2	52	4.0 a	100.0	---

362 <sup>a</sup>Means within column separated by different letters are significantly different at P<0.05, according  
 363 to the Marascuilo's post-hoc multiple comparison procedure for proportions.

364 <sup>b</sup>No SE is given, as only one fruit was obtained.

365 <sup>c</sup>Embryos rescued from immature seeds.

366

367

368 **4. Discussion**

369

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

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

377         Although interspecific hybridization of eggplant with related species has been reported with  
378 different degrees of success (Bletsos et al., 1998; Lester and Kang, 1998; Behera and Singh, 2002;  
379 Daunay and Hazra, 2012; Kumchai et al., 2013; Rotino et al., 2014; Devi et al., 2016; Plazas et al.,  
380 2016), successful introgression requires backcrossing of the hybrids to the cultivated parent. This  
381 step many times represents the most important challenge for introgression breeding (Rieseberg and  
382 Carney, 1998), due to sterility and/or lack of fruit set in some hybrid combinations. In previous  
383 works, backcrosses have been obtained between eggplant and several wild species (Rotino et al.,  
384 2008; Mennella et al., 2010; Prohens et al., 2012; 2013; Liu et al., 2015); however, our work  
385 represents the first systematic attempt to obtain backcrosses between several varieties of cultivated  
386 eggplant a number of wild species accessions from the primary and secondary gene pools.

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

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

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

425 Carney, 1998). Our results also indicate that using the interspecific hybrids as female parents may  
426 be a better strategy for obtaining backcross generations, particularly when the pollen viability of the  
427 interspecific hybrid is low. Considerable differences have been found among the *S. melongena*  
428 genotypes in the success of backcrossing. According to the results obtained, we consider that best *S.*  
429 *melongena* accessions among those tested for successful backcrossing are MEL1 and MEL6,  
430 followed by MEL5. The rest of accessions in general had a poorer performance.

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

444 Interspecific hybridization with tertiary gene pool species was unsuccessful with *S.*  
445 *bonariense* and *S. sisymbriifolium*. Although to our knowledge this is the first attempt for  
446 interspecific hybridization with *S. bonariense*, several previous attempts have been performed at  
447 crossing *S. melongena* and *S. sisymbriifolium* (Bletsos et al., 1998; Plazas et al., 2016) due to the  
448 interest of the latter as source of resistance to several diseases (Bletsos et al., 1998; Daunay and  
449 Hazra, 2012), but have always been unsuccessful at obtaining sexual interspecific hybrids.  
450 Amazingly, we have been able to obtain interspecific hybrids between *S. melongena* and *S.*



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

457

## 458 **5. Conclusions**

459

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

472

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474

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487

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