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Additional Information

A refined method for ovule culture in sugar beet (*Beta vulgaris* L.)

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17 **Abstract**

18 Induction of gynogenesis through ovule culture is a valuable tool to produce haploid and doubled
19 haploid plants in sugar beet (*Beta vulgaris* L.). However, there is still large room for refining the
20 method. In this study we investigated the gynogenic response of cultured ovules of three sugar
21 beet genotypes, the effect of the application to inflorescences of different pretreatments with
22 mannitol at 4ºC and with 5-azacytidine and 2,4-D, and the effect of the use of different basal
23 culture media and sucrose concentrations. The response was evaluated in terms of percentages of
24 induction of gynogenesis, embryogenesis and callogenesis, as well as of regenerated plants. We
25 showed that a pretreatment with 0.5 M mannitol at 4°C for 4 days, and with 50 μM 5-AzaC for 1
26 hour, notably improved the percentage of embryogenesis and plant regeneration. Besides, the use
27 of MS basal medium and 60 g/L sucrose was also found beneficial. This study provides new
28 ways to improve the efficiency of haploid induction and plant regeneration through ovule culture
29 in sugar beet, and is potentially applicable to ovule culture in other crops.

30

31 **Keywords:** 2,4-D, 5-azacytidine, doubled haploid, gynogenesis, haploid, mannitol, ovule
32 culture,

33

34 **Introduction**

35

36 Sugar beet (*Beta vulgaris* L.) is an allogamous, biennial and self-incompatible plant (Pazuki et
37 al. 2017, 2018a,b,c) belonging to the chenopodiaceous family. It is also one of the most
38 important industrial and economic crops (Pazuki et al. 2018c; Pazuki et al. 2017) with a great
39 weight in the global sugar markets (Hassani et al. 2018). This is why extensive efforts have been
40 devoted to create new, improved sugar beet cultivars. In this context, inbred lines play a very
41 important role in sugar beet plant breeding (Nagl et al. 2004). Doubled haploid (DH) production
42 is a fast method useful to produce new series of homozygous lines that can subsequently be used
43 for artificial hybridization. Completely homozygous lines can typically be obtained only in just
44 one *in vitro* generation through DH technology, which reduces considerably the time and
45 resources needed to create pure lines, as compared with conventional breeding methods based on
46 several generations of selfing and selection (reviewed in Seguí-Simarro 2016). Another
47 advantage of DHs relies in their full homozygosity, which makes the phenotypic selection for
48 qualitative and quantitative traits much easier. In contrast, conventional methods are much more
49 time-consuming, especially in biennial crops, and complete homozygosity for allogamous
50 species such as sugar beet is not guaranteed (Klimek-Chodacka and Baranski 2013).
51 Although the most commonly used and successful DH technology is induction of androgenesis,
52 this approach has not yet been efficiently implemented in sugar beet. For this reason, induction
53 of gynogenesis is the method most commonly used in this crop (Nagl et al. 2004; Aflaki et al.
54 2017). In sugar beet, as in many other species, induction of gynogenesis through ovule culture is
55 strongly influenced by many genetic and environmental factors, including the genotype and
56 growth conditions of donor plants, the type and duration of the pretreatment applied to flower

57 buds, and the composition of the *in vitro* culture medium, among others (reviewed in Aflaki et al.
58 2017; Bohanec 2009; Chen et al. 2011). However, as opposed to other DH techniques, induction
59 of gynogenesis through ovule culture may require two or more consecutive *in vitro* culture
60 phases, with potentially different nutritional and hormonal needs, which must be properly
61 determined in advance. In the literature there are examples of the use of different organic
62 nitrogen sources, carbohydrates as carbon sources, and growth regulators (Chen et al. 2011).
63 Another main factor affecting the induction of gynogenic embryos from ovule cultures is the
64 type and level of stress used to induce the switch from the gametophytic pathway towards a
65 sporophytic one. Some physical treatments, including high and low temperatures, starvation and
66 high osmotic pressure (mannitol-containing solid medium), applied either to donor plants or to
67 cultured explants, have shown significant effects on embryogenesis induction (Chen et al. 2011).
68 Among them, the application of a cold stress has been the most successfully used up to now to
69 induce gynogenesis in sugar beet cultured ovules (Bohanec 2009; Aflaki et al. 2017; Pazuki et al.
70 2018a,b).
71 However, the efficiency of this method is still low and there is still much room to improve the
72 method, since there are a number of factors, proved useful when used in the context of other DH
73 techniques, that have not yet been tested in ovary cultures. This is the case of the application of
74 mannitol as an osmotic agent, either alone or combined with cold, or the use of 5-azacytidine
75 (AzaC), a DNA-demethylating agent known to increase the rate of embryogenesis induction in
76 somatic and microspore embryogenesis of a number of species (Grzybkowska et al. 2018;
77 Leljak-Levanić et al. 2004; Osorio-Montalvo et al. 2018; Testillano 2019). The auxin 2,4-D has
78 typically been used as a growth regulator in plant tissue culture, and in the particular case of *in*
79 *vitro* embryogenesis, it has also been used at high concentrations, alone or combined with 5-

80 AzaC, as a stress treatment to induce microspore and somatic embryogenesis (Abdollahi and
81 Rashidi 2018; Ardebili et al. 2011; Leljak-Levanić et al. 2004; Nowaczyk et al. 2015). However,
82 the usefulness of these treatments in the context of ovule culture still awaits to be tested. In this
83 work, we developed an optimization of the protocol for sugar beet ovule culture in three different
84 genotypes, focusing on the study of the different response of each genotype, the effect of the type
85 of culture medium, the combined application of mannitol together with cold temperature, and of
86 AzaC in combination with 2, 4-D, as pretreatments to inflorescences previous to ovule culture,
87 and the use of different concentrations of sucrose in the culture medium for production of sugar
88 beet haploid and DH plants. Our results constitute a step forward towards the development of a
89 refined protocol for sugar beet ovule culture.
90
91 **Materials and Methods**
92
93 *Plant materials and growth conditions*
94 Three sugar beet genotypes, namely 7233, Posada and Monatunno, were used as donor plants.
95 7233 is a multigerm, bolting-sensitive traditional cultivar, Posada and Monatunno are
96 monogerm, bolting-resistant F1 hybrids. They all were provided from the Sugar Beet Seed
97 Institute (Karaj, Iran) and the Agriculture and Natural Resources Research and Education Center
98 of Hamedan, Hamedan, Iran. Donor plants were first vernalized (4-8°C for 100 days) and. Then,
99 well developed plants with 8-10 leaves were grown from December 2018 to September 2019 in a
100 plant growth chamber set to 22°C during the day and to 18°C at night, with a photoperiod of 16/8
101 h (light/darkness) and a light intensity of 480–500 μmol.m^{-2}.s^{-1}.
102

103 *Initial conditions for ovule culture*

104 Inflorescences of 5-7 cm in length were collected from donor plants and transferred to the
105 laboratory at 0°C in a box with ice and water. The inflorescences were then placed in the
106 refrigerator at 4°C for 5 days in darkness. For surface disinfection of inflorescences, they were
107 immersed in 70% ethanol for 30 s followed by 2.5% sodium hypochlorite for 13 min and finally
108 three washes with cold sterile distilled water (5 min each). The ovules (1 mm in length) were
109 removed from the flower buds using two syringes under a stereomicroscope and placed on 100 ×
110 15 mm plastic petri dishes containing 20 mL of sterile culture medium. The culture medium for
111 gynogenesis induction was prepared according to Baranski (1996). Briefly, Murashige and
112 Skoog (MS; Murashige and Skoog, 1962) culture medium was supplemented with 0.2 mg/L
113 benzyl amino purine (BAP), 0.5 mg/L naphthalene acetic acid (NAA) and 30 g/L sucrose, pH
114 5.8. For the different experiments, the induction medium was solidified with 8 g/L agar or 2.8
115 g/L Phytagel. After ovule plating, culture dishes were sealed with Parafilm and transferred to a
116 growth chamber with 16/8 h light/darkness photoperiod at 25±2°C and a light intensity of 40
117 $\mu mol.m^{-2}.s^{-1}$. For plant regeneration, MS culture medium without hormones was used.

118

119 *Ploidy analysis*

120 Ploidy of donor plants and ovule culture-derived plants was estimated in parallel by two
121 methods, chromosome counting and measurement of the length and width of stomata guard cells.
122 For chromosome counting, initially, the plant materials were pretreated at 4°C in the dark for 24
123 h. The samples were then immersed in a 3:1 solution of absolute alcohol and glacial acetic acid
124 for 20 h. Hydrolysis was performed with 1M HCl at 60°C for 30 min, and then stopped by
125 immersion of the samples in ice water 3 times (5 min each). Samples were then stained with 1%

126 acetocarmine solution for 24 h and crushed on glass slides. Chromosomes were observed and
127 counted under a light microscope at 100x magnification. To measure the length and width of
128 stomatal guard cell of the epidermal layer from the abaxial leaf surface, leaf fragments of donor
129 plants (diploid) and ovule culture-derived plantlets were immersed in nail polish and then stuck
130 to adhesive tape. After a few minutes, the leaf fragments were quickly separated from the
131 adhesive tape so that a thin epidermal layer remained on the adhesive tape. Finally, the adhesive
132 tape was placed on a glass slide, observed under a light microscope at 65x magnification, and
133 imaged with a digital CCD camera to perform measurements. At least three cells of each leaf
134 fragment were measured.
135
136 *Medium modifications*
137 Using the culture medium above described as the starting point, we made a series of
138 modifications of several culture components in order to evaluate the effect of such modifications
139 in the response of ovule cultures. The modifications performed included:
140 1) The replacement of the MS basal medium by the PGoB medium (De Greef and Jacobs 1979),
141 solidified in both cases with 8 g/L agar.
142 2) The application to inflorescences of a pretreatment consisting in their incubation at 4ºC in
143 liquid MS medium with different concentrations of mannitol (0, 0.5 and 1 M), during 4 or 7
144 days. After the pretreatment, inflorescences were then removed from the medium and
145 prepared for ovule culture. After each pretreatment, ovules were isolated and cultured in MS
146 medium with 2.8 g/L Phytagel, keeping the rest of conditions unchanged.
147 3) The application to inflorescences of a pretreatment consisting in their incubation for 1 h with
148 solutions of 5-AzaC (0, 50 and 100 μM) and 2,4-D (0, 100 and 200 μM). After the incubation,

149 inflorescences were washed with sterile water for 5 min. For each pretreatment, ovules were
150 then isolated and cultured in MS medium with 8 g/L agar, keeping the rest of conditions
151 unchanged.
152 4) The use of different concentrations of sucrose (30, 60 and 90 g/L) in MS medium solidified
153 with 2.8 g/L Phytagel and keeping the rest of conditions unchanged.
154 The effects of these modifications were measured by the calculation of the following parameters:
155 - The percentage of gynogenesis induction (%G), defined as the percentage of cultured ovules
156 that undergo any type of development leading to haploid or DH plantlet formation.
157 - The percentage of embryogenesis induction (%E), defined as the percentage of cultured ovules
158 that undergo embryogenesis.
159 - The percentage of callus induction (%C), defined as the percentage of cultured ovules that
160 develop as callus masses instead of as embryos.
161 - The percentage of plant regeneration (%PR), defined as the percentage of cultured ovules that
162 are eventually able to produce haploid or DH plantlets.
163
164 *Experimental Design and Statistical Analysis*
165 For the experiments involving the modification of only one parameter (the response of the
166 different genotypes, the type of culture medium and the use of different sucrose concentrations),
167 a completely randomized design was used. Those involving variation of two parameters
168 (mannitol + cold and 5-AzaC + 2,4-D) were conducted as factorial experiments based on a
169 completely randomized design. Each experiment consisted in three replications, considering as a
170 replication one culture dish containing 10 ovaries, for a total of 30 ovules used in each
171 experiment. For each treatment, the four parameters above described (%G, %E, %C and %PR)

172 were calculated. Data were analyzed by ANOVA. A square root transformation ($\sqrt{x+0.5}$) was
173 applied to percentage data. A multiple comparison of means was performed using the Duncan's
174 Multiple Range Test ($\alpha = 0.05$) using SPSS 16 software.
175
176 **Results**
177 Inflorescences harvested at the appropriate stage (5-7 cm long; Fig. 1A) were used to isolate
178 sugar beet ovules (Figs. 1B). Over the first 2-3 weeks from culture initiation, most of the
179 cultured ovules turned brown, and after about four weeks, some of them formed embryo-like
180 structures (Fig. 1C). After about two further weeks, these structures elongated, making visible
181 the first signs of organ differentiation (Fig. 1D). These embryos progressively grew and
182 transformed into plantlets where greening cotyledons began to be clearly visible (Fig. 1E). In
183 parallel to embryogenesis, we also found that after about four weeks from culture onset, some
184 ovules of 'Posada' developed into undifferentiated calli (Fig. 1F). Different organs were formed
185 on their surface after approximately two additional weeks (Figs. 1G, H). In both pathways (direct
186 embryogenesis and callogenesis), '7233' and 'Posada' ovules eventually produced entire, fully
187 functional plantlets (Figs. 1I, J). However, the speed of plant growth and regeneration was
188 different. Plant regeneration in '7233' cultivar was observed after 20-25 days from culture
189 initiation, while in 'Posada' this process took about 2 months. To determine the ploidy level of
190 ovule-derived plants, chromosome counting was performed in the root tip cells of diploid donor
191 plants to have a reference with which to compare cells of ovule-derived plants. Diploid cells of
192 donor plants presented 18 chromosomes ($2n=2x=18$), whereas ten out of the twelve plantlets
193 regenerated from ovule culture experiments presented 9 chromosomes ($n=x=9$) and were
194 considered as haploid, and the other two showed 18 chromosomes and were considered as

195 diploid. To confirm these estimations, we measured the length and width of stomatal guard cells
196 of the plants previously determined as haploid and diploid. Stomatal guard cells of all diploid
197 plantlets showed average lengths (20.6±0.46 μm) and widths (15.7±0.51 μm) higher than those
198 of haploid leaves, which were 16.9±0.40 μm long and 13.8±0.40 μm wide. In summary, ten out
199 to 12 plants (83.3%) were haploid, and 2 (16.7%) were diploid.
200
201 **Characterization of the response to ovule culture in the three genotypes**
202 The three genotypes tested showed significant differences for the four parameters studied. The
203 highest percentages of gynogenesis induction and direct embryogenesis (~27%) were observed
204 in 'Monatunno', while the lowest ones (~10-15%) occurred in '7233' (Figs. 2A, B). The highest
205 percentage of callus induction (3.33%) was observed in 'Posada', whereas it was null in the other
206 two genotypes (Fig. 2C). The high percentages of induction of gynogenesis and of direct
207 embryogenesis of 'Monatunno' were not reflected in the percentage of regenerated plants, which
208 was null (Fig. 2D). On the contrary, '7233' and 'Posada' showed a ~3% of regeneration, which
209 made us choose them for further assays.
210
211 **Effect of different basal culture media**
212 For this assay, we used 'Posada' since its resistance to bolting makes it more valuable, and it was
213 the genotype showing the best overall performance in the previous assay. We found significant
214 differences between the use of MS and PGoB as basal culture media in terms of percentages of
215 induction of gynogenesis (Fig. 3A) and of embryogenesis (Fig. 3B), which were higher with the
216 use of MS. However, no significant differences were found for the percentages of callus

217 formation (Fig. 3C) and plant regeneration (Fig. 3D). Overall, the use of MS provided the best
218 results. For this reason, we decided to use it for the rest of experiments.
219

220 **Effect of different pretreatmens with mannitol combined with cold temperature**

221 Although our first idea was to use 'Posada' for the rest of assays, we found that flowering
222 induction in 'Posada' was difficult, and long times were needed for this cultivar to flower. In
223 order to expedite the work, for the next experiments we decided to change to '7233', which
224 showed a similar performance in the initial conditions, and in addition, exhibited faster plant
225 regeneration. When applied as a pretreatment to inflorescences, different concentrations of
226 mannitol combined with cold temperature (7ºC) for 4 or 7 days showed significant differences
227 for the percentages of gynogenesis and embryogenesis induction, callus formation and plant
228 regeneration (Table 1). The maximum frequency of induction of gynogenesis and of direct
229 embryogenesis (23.33%) was obtained using 0.5 M mannitol combined with a cold pretreatment
230 of 4°C for 4 days. However, only 1M mannitol + 4°C for 4 days was able to induce callus
231 formation. Interestingly, this was the only combination unable to promote embryogenesis. These
232 two concentrations of mannitol (0.5 and 1 M combined with 4°C for 4 days were the only
233 pretreatments promoting plant regeneration.
234

235 **Effect of different concentrations of 5-AzaC and cold 2,4-D**

236 In this experiment, the combined effects of different 5-AzaC and 2, 4-D pretreatments of the
237 inflorescence were analyzed. There were significant differences for the percentages of
238 gynogenesis and embryogenesis induction (Table 2). The highest rate of gynogenesis (26.67%)
239 and embryogenesis induction (23.33%) were achieved using 50 µM 5-AzaC pretreatment

240 without 2,4-D. Although no significant differences were found for the percentages of callus
241 formation and plant regeneration, this was also the only combination capable of regenerating
242 plantlets.
243
244 **Effect of different sucrose concentrations**
245 Finally, we tested the effect of using 30 (control condition), 60 and 90 g/L of sucrose in the
246 culture medium. We found significant differences in the percentage of gynogenesis induction
247 (Fig. 4A), being 60 g/L the best performing concentration (16.67%). This concentration also
248 yielded the best results in terms of embryogenesis induction (Fig. 4B), callus formation (Fig. 4C)
249 and plant regeneration (Fig. 4D). 90 g/L appeared toxic for ovules, since it produced only few
250 embryos, none of which was able to regenerate into plantlets.
251
252 **Discussion**
253
254 **Both embryogenesis and callus formation can be induced from cultured sugar beet ovules**
255 It is well known that the genotype plays a crucial role in many different types of *in vitro*
256 morphogenic processes in general, and in particular in haploid and DH production through
257 microspore embryogenesis (Salas et al. 2011; Parra-Vega et al. 2013), as well as through ovule
258 culture (Aflaki et al. 2017; Lux et al. 1990; Pazuki et al. 2018a, b). We evaluated the gynogenic
259 response of three different sugar beet genotypes. The highest percentage of gynogenesis
260 induction and embryogenesis was observed in 'Monatunno', whereas 'Posada' was the best
261 performing in terms of callus formation. This indicates that it is possible to induce both
262 morphogenic ways from sugar beet ovules, as it happens in other *in vitro* processes, although

263 with different efficiencies (Salas et al. 2011; Parra-Vega et al. 2013). It is possible that at first,
264 only haploid embryos are produced from haploid cells of the embryo sac, most likely the egg
265 cell, but the conditions are not well optimized for a proper completion of embryogenesis, which
266 ends up in the best cases with the formation of true, viable embryos with some anatomical
267 malformations that do not interfere with germination, as those shown in Fig. 1E. However, other
268 conditions (for example a pretreatment with 1 M mannitol during 4 days at 4ºC) may be
269 insufficient to ensure embryo progression, ending up with the formation of undifferentiated
270 callus masses such as those of Fig. 1F, in coherence with what happens in other DH systems
271 such as eggplant microspore cultures (Corral-Martínez and Seguí-Simarro 2012, 2014). Although
272 haploid and DH plants can be obtained by both ways, embryos are always desirable, which
273 stresses the need for tailored culture protocols for each genotype, in order to promote all its
274 embryogenic potential.
275 'Monatunno' was unable to regenerate plantlets, so in terms of plant regeneration, which at last
276 is the most relevant parameter, the best genotypes were '7233' and 'Posada'. There are only
277 speculations about how the genotype determines the gynogenesis-responsive phenotype. It was
278 proposed that the genotype dependence of this trait is related to the allogamous nature of this
279 crop (Pazuki et al 2018b), and that this could possibly be a quantitative trait, with a dominance
280 towards low production (Bohanec 2009). It seems clear that there must be a genetic control of
281 this process, but much more work is still need to elucidate it.
282

283 **Basal media with high inorganic NO_3:NH_4 ratios favor induction of gynogenesis**

284 The composition of the culture medium plays a major role in inducing gynogenesis responses
285 (Chen et al. 2011). Our results confirmed this notion. Although the two basal media tested

286 provided the same percentage of regenerated plantlets, MS was clearly superior to PGoB in
287 inducing embryogenesis, which indicates that MS should be combined with other factors to
288 promote a proper development of the induced embryos. In the literature, three main basal media
289 have been used for sugar beet ovule culture, namely N_6, PGoB and MS, being the latter two the
290 most commonly used (Baranski 1996; De Greef and Jacobs 1979). We compared MS and PGoB.
291 MS has higher concentrations of NH_4NO_3 and KH_2PO_4, whereas PGoB is enriched in KCl,
292 $NaH_2PO_4.2H_2O$ and $(NH_4)_2SO_4$. Thus, it seems that the higher percentage of embryogenesis
293 induction in MS is related to its higher inorganic NO_3:NH_4 ratio. In line with this, the higher
294 response of red beet cultured ovules in N_6 medium compared to MS medium was attributed to
295 the higher rate on inorganic NO_3:NH_4 in the former, together with reduced nitrogen content
296 (Baranski 1996). Thus, it seems that basal media with high inorganic NO_3:NH_4 ratios favor
297 gynogenesis induction.

298

299 **Sucrose promotes growth, whereas mannitol determines the type of morphogenic process**
300 **induced**

301 Cold temperature and osmotic stress are known to be inducers of *in vitro* embryogenic processes
302 such as microspore embryogenesis in several species (reviewed in Shariatpanahi et al. 2006). To
303 induce these processes, metabolizable carbohydrates such as sugars can be used, being sucrose
304 the most widely used (Yaseen et al. 2013). Indeed, different sucrose concentrations have
305 previously been tested in sugar beet ovule cultures, with different results (Baranski 1996; Gurel
306 et al. 2000; Gurel et al. 2003; Lux et al. 1990; Pazuki et al. 2018a, b). However, since soluble
307 sugars also serve as carbon sources, the osmotic potential progressively decreases as they are
308 being used up (Lipavska and Vreugdenhil 1996; Yaseen et al. 2013). To avoid this, non-

309 metabolizable osmotic agents such as mannitol have been used to create stable osmotic
310 pressures, alone or combined with cold, in order to induce different morphogenic processes,
311 including gynogenesis in sugar beet (Aflaki et al. 2017; Gurel et al. 2000, 2003; Lux et al. 1990;
312 Pazuki et al. 2018a, b). In this work, we tested the effects of using different concentrations of
313 two osmotic agents, a metabolizable (sucrose) and a non-metabolizable one (mannitol), at
314 different culture stages.
315 For sucrose, the maximum percentages of all parameters studied were obtained using 60 g/L.
316 This indicates that this particular system requires a specific sucrose concentration for optimal
317 growth and that beyond that level, sucrose may have detrimental effects. However, the most
318 important observation was that the percentages of both embryo and callus formation increased,
319 and also the percentage of plantlet regeneration. This highlights that an optimal concentration of
320 sucrose is positive for growth and development in general, as described for other *in vitro*
321 processes (Lipavska and Vreugdenhil 1996). In other words, 60 g/L sucrose would be the
322 optimal concentration to promote cellular growth in general, regardless of the morphogenic
323 pathway undergone by the haploid embryo.
324 We applied to our lines different combinations of mannitol and cold as pretreatments to
325 inflorescences, prior to ovule isolation and culture, and found that the best results were obtained
326 with 0.5 M mannitol for 4 days at 4°C, since this combination yielded the highest percentages of
327 embryogenesis and also of plant regeneration. Similar plant regeneration was obtained with 1 M
328 mannitol for 4 days, but these conditions promoted only callus formation. As mentioned before,
329 when possible, embryogenesis is preferred. Aside of this, our results point to the notion that
330 shorter treatments work better, and most importantly, that different mannitol concentrations lead
331 to different morphogenic responses, since lower concentrations favor embryo versus callus

332 production and higher concentration have the opposite effect. Most likely, high mannitol
333 concentrations would be toxic for the developing embryo, having a detrimental effect that leads
334 to its transformation into undifferentiated callus.
335

336 **A pretreatment of inflorescences with 5-AzaC is a promising way to reprogram unfertilized**
337 **ovules**

338 To the best of our knowledge, 5-AzaC has not yet been applied to ovule culture, neither as a
339 pretreatment nor in the culture medium. However, there are data from somatic embryogenesis,
340 where the combined use of 5-AzaC and 2,4-D has been proven useful or useless depending on
341 the mode of application. For example, it increased induction in *Acca sellowiana* when applied as
342 a pretreatment to inflorescences (Fraga et al. 2012). However, when applied to the ovule culture
343 medium, results were not different from controls in carrot and pumpkin (Leljak-Levanić et al.
344 2004; Yamamoto et al. 2005). This is why we used 5-AzaC at different concentrations as a
345 pretreatment to inflorescences, both alone and together with 2,4-D. The use of 2, 4-D as a growth
346 regulator in a variety of plant tissue culture protocols is well known. In addition, at high
347 concentrations, it was proposed as a novel source of stress to induce microspore embryogenesis
348 (Abdollahi and Rashidi 2018; Ardebili et al. 2011; Nowaczyk et al. 2015; Shariatpanahi et al.
349 2006). In light of our results, it is evident that for sugar beet ovule cultures, the application of
350 different concentrations of 2,4-D as a pretreatment, either alone or together with 5-AzaC, was
351 not better than its absence.

352 On the contrary, the highest percentages of induction of gynogenesis, embryogenesis and plant
353 regeneration were obtained with 50 µM 5-AzaC alone, without 2,4-D. This combination,
354 together with 100 µM 5-AzaC+ 200 µM 2,4-D, were the only ones producing calli, but at much

355 less percentage than embryos. Thus, it appears that a pretreatment with 50 μM 5-AzaC is
356 beneficial to promote the development of unfertilized sugar beet haploid embryos. According to
357 the role of 5-AzaC, a cytidine analogue unable of being methylated, it is likely that this
358 beneficial effect is due to the generation of hypomethylated DNA upon incorporation to
359 replicating DNA strands. Hypomethylated DNA is more prone to be expressed, and this has been
360 exploited to promote reprogramming, totipotency acquisition and the subsequent expression of
361 certain developmental programs in cells not initially programmed to express such programs. For
362 example, the induction of somatic embryogenesis in pumpkin and cocoa, or the induction of
363 microspore embryogenesis in rapeseed and barley (Grzybkowska et al. 2018; Leljak-Levanić et
364 al. 2004; Osorio-Montalvo et al. 2018; Testillano 2019). This promising line could be exploited
365 in the future with the use of other epigenetic modifiers such as Trichostatin A, know to modify
366 the methylation status of DNA through histone deacetylation (Li et al. 2014).

367

368 **Concluding remarks**

369 In this work, we tried different factors in an attempt to improve the efficiency of DH production
370 in sugar beet culture ovules. Some of them were found insufficient for such goal, whereas others
371 proved to improve the embryo and plantlet regeneration yield. However, the results are still
372 improvable. Perhaps, a promising way could be the use of other hypomethylating drugs, as
373 mentioned above. However, the main bottleneck we found was the percentage of DHs produced,
374 which was as low as 16.7%. Other studies showed that there is a percentage of haploid sugar beet
375 plantlets that undergo spontaneous chromosome doubling while they grow (Tomaszewska-Sowa
376 2010). However, the reported final rate of DHs was still low. The present work was not intended
377 to optimize this stage of the process of DH production, but for the future, application of

378 chromosome doubling agents such as colchicine or other antitimitotics such as trifluralin,
379 oryzalin or amiprophos-methyl should be considered in order to generate a full protocol.

380

381 **Author Contribution**

382 MRA conceived and designed the experiments. SS performed the experiments. SS, MRA, AMA,
383 HEK and MA prepared reagents, materials and analytic tools. MRA, SS and JMSS analyzed the
384 data and wrote the manuscript. All authors read and approved the final manuscript.

385

386 **Conflicts of Interest:** The authors declare no conflict of interest.

387

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481

482 **Tables**

483 **Table 1:** Effect of pretreatments of inflorescences with different concentrations of mannitol
484 combined with exposure to cold temperature for different durations in '7233', expressed as
485 percentages of induction of gynogenesis (%G), embryogenesis (%E), callus formation (%C) and
486 plant regeneration (%PR). Different letters indicate significant differences ($p \leq 0.05$).

487

Mannitol	Cold pretreatment	%G	%E	%C	%PR
0 M	4°C (4 days)	10 b	10 b	0 b	0 b
	4°C (7 days)	3.3 b	3.3 bc	0 b	0 b
0.5 M	4°C (4 days)	23.3 a	23.3 a	0 b	6.7 a
	4°C (7 days)	6.7 b	6.7 bc	0 b	0 b
1 M	4°C (4 days)	6.7 b	0 c	6.7 a	6.7 a
	4°C (7 days)	3.3 b	3.3 bc	0 b	0 b

488

489 **Table 2:** Combined effects of 5-AzaC and 2,4-D treatments in '7233' expressed as percentages
490 of induction of gynogenesis (%G), embryogenesis (%E), callus formation (%C) and plant
491 regeneration (%PR). Different letters indicate significant differences ($p \leq 0.05$).

5-AzaC	2,4-D	%G	%E	%C	%PR
0 µM	0 µM	6.7 bc	6.7 bc	0	0
	100 µM	10 bc	10 bc	0	0
	200 µM	10 bc	10 bc	0	0
50 µM	0 µM	26.7 a	23.3 a	3.33	3.33
	100 µM	16.7 ab	16.7 ab	0	0
	200 µM	3.3 c	3.3 c	0	0
100 µM	0 µM	10 bc	10 bc	0	0
	100 µM	3.3 c	3.3 c	0	0
	200 µM	13.3 bc	10 bc	3.33	0

492

493 **Figure legends**

494

495 **Fig 1.** Plant regeneration in sugar beet unfertilized ovule culture. A: 5-7 cm long inflorescences,
496 at the right stage for ovule culture. B: Unfertilized ovule at the appropriate stage for ovule
497 culture. C: Embryo-like structure developing from the ovule, four weeks after ovule culture
498 initiation. D: Development of embryo six weeks after culture initiation. Note the occurrence of
499 root hairs at the distal part of the embryo. E: Regenerating plantlet, eight weeks after culture
500 initiation. F: Callus induction from ovule, four weeks after culture initiation. G: Development of
501 green organogenic nodes on the callus surface of callus, six weeks after culture initiation. H, I, J:
502 Different stages in the rooting and regeneration of ovule-derived plantlets. Bars: 1 mm.

503

504 **Fig 2:** Response of '7233', 'Posada' and 'Monatunno' expressed as percentages of induction of
505 gynogenesis (%G, A), embryogenesis (%E, B), callus formation (%C, C) and plant regeneration
506 (%PR, D). Different letters indicate statistically significant differences ($p \leq 0.05$).

507

508 **Fig 3:** Effect of the type of basal medium (MS or PGoB) in 'Posada' expressed as percentages of
509 induction of gynogenesis (%G, A), embryogenesis (%E, B), callus formation (%C, C) and plant
510 regeneration (%PR, D). Different letters indicate statistically significant differences ($p \leq 0.05$).

511

512 **Fig 4:** Effect of different concentrations of sucrose (30, 60 and 90 g/L) in ovule culture in '7233'
513 expressed as percentages of induction of gynogenesis (%G, A), embryogenesis (%E, B), callus
514 formation (%C, C) and plant regeneration (%PR, D). Different letters indicate statistically
515 significant differences ($p \leq 0.05$).

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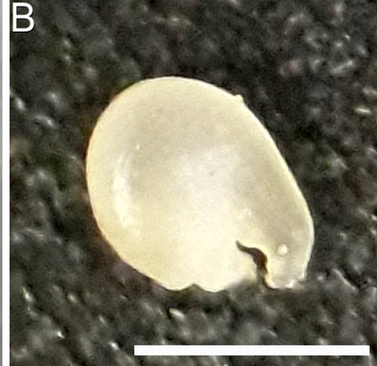
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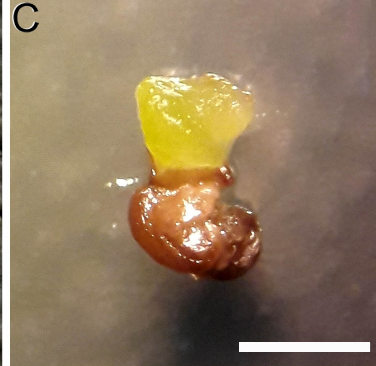
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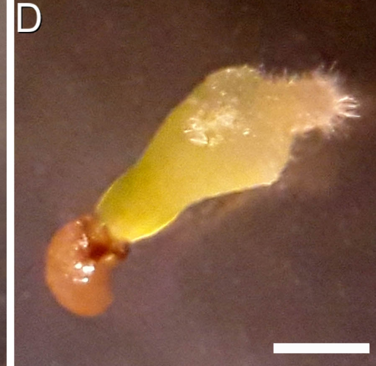
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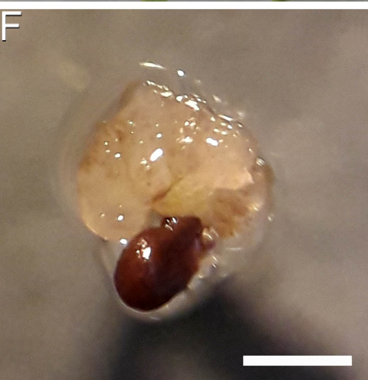
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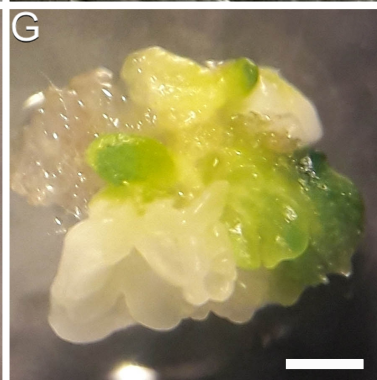
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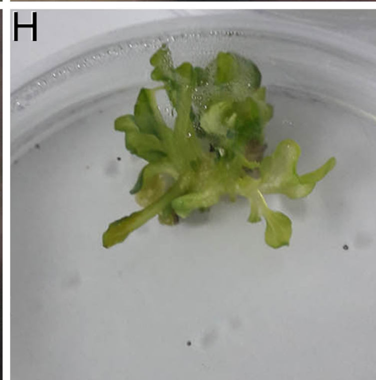
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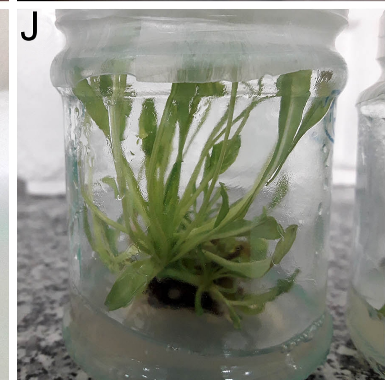
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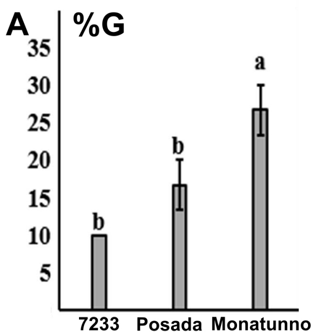
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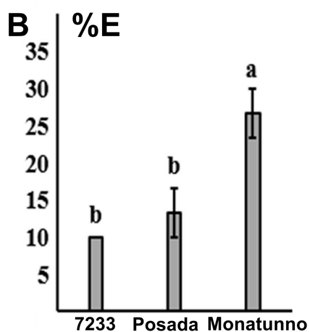
7233
Posada
Monatunno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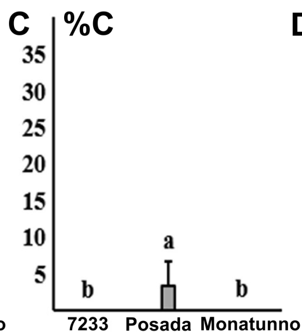
Monatunno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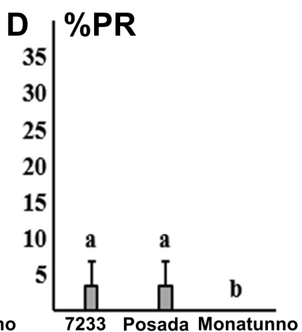
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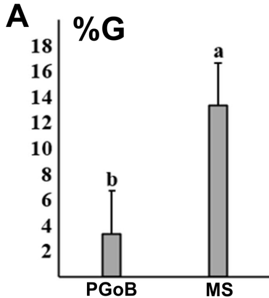
Monatunno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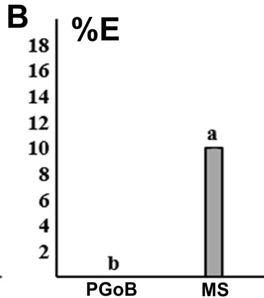
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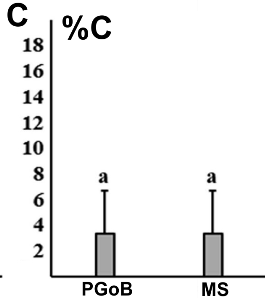
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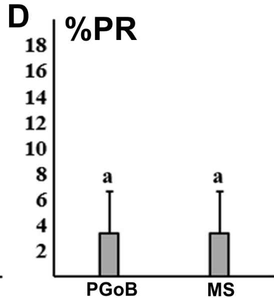
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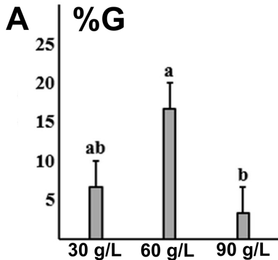
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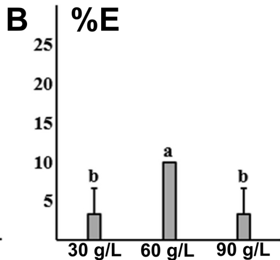
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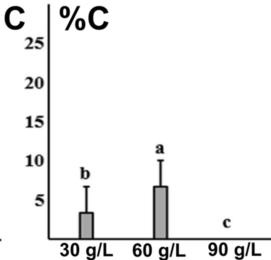
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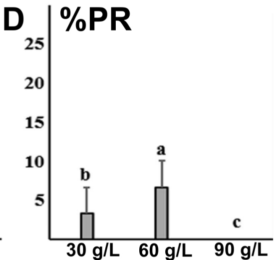
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