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2 **Improvement of in vitro donor plant competence to increase de novo shoot organogenesis in rose genotypes**

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7

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11 **ABSTRACT**

12 A procedure was developed for *in vitro* propagation of *Rosa* genotypes along with an efficient *de novo* shoot
13 organogenesis (DNSO) method. We tested, on one genotype (hybrid of *Rosa wichurana*), the effects of MS basal
14 medium complemented with two growth regulators to achieve either shoot elongation or shoot multiplication of plants.
15 These media were complemented with carbohydrate concentrations from different sources. Then, the impacts of
16 various carbohydrates (fructose, glucose, maltose, sorbitol, sucrose) on the growth and development of several rose
17 genotypes during donor plant subculturing were studied on SMM. The results showed high variability in growth and
18 development between genotypes. Contrary to other members of the *Rosaceae* family, no correlation was found between
19 the shoot size and number when the amount of sorbitol was increased.

20 Murashige and Skoog medium supplemented with 3.0 mg.L⁻¹ BAP and containing fructose or glucose at 30 g.L⁻¹ was
21 chosen to induce leaf explants for the DNSO experiments. MS basal medium complemented with TDZ/IBA at three
22 ratios and the same range of carbohydrate sources were tested for DNSO. Significant genotypic variations with regard
23 to the percentage of regeneration was demonstrated with six genotypes. For two genotypes, a hybrid of *Rosa wichurana*
24 and *Rosa* 'White Pet', we defined the conditions required to obtain 100% DNSO. For *Rosa chinensis* 'Old blush' and
25 the rootstock genotype *Rosa* 'Natal Briar', we obtained 74 and 87.5% DNSO and only 56.67% and 37.5% for *Rosa*
26 GUY SAVOY® ('Delstrimen') and *Rosa* 'Félicité et Perpétue' respectively. This adventitious shoot regeneration
27 method may be used for large-scale shoot propagation and genetic engineering studies in *Rosa*.

28 **1. Introduction**

29 Rose is an important plant in the ornamental field but also in cosmetics and food industry. Rose is the most
30 important economically species with production of cut flowers, potted roses and garden roses. Thanks to its broad
31 diversity and high quality genome sequence, rose is increasingly seen as a model plant for woody ornamentals
32 (Hibrand-Saint Oyant *et al.*, 2018; Mujib *et al.*, 2013). Major ornamental traits such as scent, flowering and *in*
33 *vitro* production can be studied in rose.

34 Rose varieties are conventionally propagated by cuttings or grafting onto a rootstock. The history of *in vitro* rose
35 culture started in 1945 (Nobecourt and Kofler), and since then many reports have documented simple and rapid
36 rose micropropagation methods.

37 Biotechnology approaches are increasingly used to improve horticultural crop production (Chebet *et al.*, 2003), to
38 boost production by shortening the production time and generating healthy, disease-free plants. *In vitro*
39 multiplication could be used for rapid and mass propagation of cut flowers (Bao *et al.*, 2009) or pot plant
40 production (Fotopoulos and Sotiropoulos, 2004). Martin (1988) demonstrated that up to 400,000 plants could be
41 annually cloned, from a single rose using this technology. Despite the availability of successful micropropagation
42 techniques, *de novo* regeneration methods involving organogenesis (process of forming new organs) and somatic
43 embryogenesis (embryo derived from a somatic cell) remain challenging. Generally, two key pathways can lead
44 to the regeneration of new plants from *in vitro* cultured explants or single cells. Organogenesis and somatic
45 embryogenesis are essential and critical tools for plant multiplication, crop improvement, plant functional
46 genomics and genome editing.

47 The organogenesis system, also called *de novo* shoot organogenesis (DNSO) (Duclercq *et al.*, 2011), refers to the
48 capacity to regenerate a new tissue culture plant from somatic cells. The advantages of DNSO are a short callus
49 phase, which maintains uniformity, and a reduction in somaclonal variation, often derived from the callus,
50 suspension or protoplasts (for review, see Mujib *et al.* 2013). However, some somaclonal variations in terms of
51 habit and leaf shape have been observed on adventitious shoots from *Rosa persica* x *xanthina* explants (Lloyd *et*
52 *al.*, 1988).

53 The prospect of developing a reliable protocol has fostered a tremendous amount of work geared towards
54 identifying the key factors influencing regeneration (more than 3500 papers since 1975 with about 60 on rose).
55 Plant growth regulators (PGR) have been extensively studied, particularly auxin and cytokinin. The aim has not
56 yet been fulfilled, particularly in some so-called 'recalcitrant' species.

57 In general, plant regeneration in rose is obtained by the embryogenesis pathway rather than organogenesis,
58 although the first report of the regeneration process indicated it was obtained by organogenesis (Hill, 1967). The
59 first report on embryogenesis in rose was published in 1995 by Roberts *et al.* Then several teams developed this
60 regeneration technique, focused on the cultivated species, often tetraploid ones (Dohm *et al.*, 2002; Estabrooks *et*
61 *al.*, 2007; Kintzios *et al.*, 1999; Xing *et al.*, 2014). In parallel, several studies on organogenesis have been
62 conducted that were focused on the kind of explants, such as leaves or leaflets, roots, internodes and petioles
63 (Arene *et al.*, 1993; Estabrooks *et al.*, 2007; Lloyd *et al.*, 1988; Pati *et al.*, 2004). The organogenesis process in
64 rose was developed either after an induction phase on a cytokinin-rich medium (Dubois *et al.*, 2000; Pati *et al.*,
65 2004) or on regeneration medium containing cytokinins and auxins (Dubois *et al.*, 2000; Pourhosseini *et al.*, 2013),
66 or with cytokinin alone (Arene *et al.*, 1993; Ibrahim and Debergh, 2001; Lloyd *et al.*, 1988). In all of these studies,
67 regeneration was obtained in the dark or under low light conditions. The roses used in these studies were
68 predominantly cultivated ones, even though some wild species such as *Rosa persica*, *Rosa wichurana*, *Rosa*
69 *laevigata* (Lloyd *et al.*, 1988), *Rosa chinensis* (Li *et al.*, 2002) and *Rosa damascena* (Pati *et al.*, 2004) have also
70 been used.

71 Few studies have been conducted to document the strong involvement of sugars in metabolic and developmental
72 processes (for review, see Yaseen *et al.* 2013). Indeed, sugars play a trophic role to sustain the high metabolic
73 activity of heterotrophic organs during growth and act as a signal to control diverse developmental processes
74 (Bolouri Moghaddam and Van den Ende, 2013; Lastdrager *et al.*, 2014; Matsoukas, 2014; Barbier *et al.*, 2015;
75 Xiong *et al.*, 2013).

76 To further improve the *de novo* shoot organogenesis process in rose genotypes, it is essential to control the
77 physiological state of donor plants in order to prepare explant tissues and increase their regeneration potential. No
78 comparative experiments have been published on the kind of sugars involved in rose mother-plant production or
79 in the regeneration medium.

80 *In vitro* plant cells, tissues and organ cultures are mainly heterotrophic structures that are unable to produce their
81 own sugars. Additional exogenous carbohydrates are thus required in the artificial culture media to meet the high
82 energy requirements of developmental processes such as *in vitro* rooting, shoot proliferation and plant regeneration
83 (Barbier *et al.*, 2015; Matsoukas *et al.*, 2013; Yaseen *et al.*, 2013). Many types of carbohydrate, including sucrose,
84 sucrose-derivative hexoses (glucose and fructose) and polyols (sorbitol and mannitol) have been tested in terms of
85 the morphogenesis potential of *in vitro* cultured tissues (for review, see Yaseen *et al.* 2013). Mainly sucrose and
86 sorbitol have been tested as exogenous carbon sources since they are photosynthesis products and phloem-

87 translocated to different sink organs where they are metabolized (Maurel *et al.* 2004; Gao *et al.* 2003), particularly
88 in the *Rosaceae* family. Yaseen *et al.* (2009) reported that both sucrose and sorbitol play a central role in *in vitro*
89 shoot proliferation in M9 and M26 apple rootstocks. Likewise, sucrose modulates *in vitro* shoot development in
90 cork oak (Romano *et al.*, 1995) and *Eclipta alba* (Baskaran and Jayabalan, 2005). In *Stevia rebaudiana*, shoot
91 proliferation was found to be sensitive to sucrose and fructose (Preethi *et al.*, 2011), while shoot proliferation in
92 *Prunus mume* was more sensitive to glucose (Harada and Murai, 1996). Sucrose is the most widely used
93 carbohydrate source for plant regeneration (Fatima *et al.*, 2015) while mannitol is considered to be a metabolically
94 inert carbohydrate, except in a few species (Conde *et al.*, 2007; Noiraud *et al.*, 2001) and it had very little effect
95 on *in vitro* shoot development and even led to cell death in soybean explants (Sairam *et al.*, 2003). Taken together,
96 these findings indicate that the choice of carbohydrates and/or their concentration could be a powerful lever to
97 successfully manage the morphogenic competence of plant tissue cultures. Furthermore, sugars are increasingly
98 considered as signal entities that are perceived by diverse sensors and regulate many fundamental plant biology
99 processes through a complex regulatory network integrating endogenous (hormones) and exogenous
100 (environment) cues (Broeckx *et al.*, 2016; Moore *et al.*, 2003; Robaglia *et al.*, 2012; Rolland *et al.*, 2006, Sakr *et*
101 *al.*, 2018). It seems clear that the morphogenic potential of plant tissues could be a complex mechanism and may
102 result from interlinked processes involving sugars, hormones and environmental factors.

103 The aim of the present research was to develop a strategy to improve the totipotency and organogenic potential,
104 especially the DNSO process, in seven rose genotypes by adjusting the micropropagation method and medium.
105 To determine the specific physiological state that explants should be excised after the clonal cycle in donor plants,
106 we studied the type and amount of carbohydrate source at various regeneration stages, including mother plant
107 production.

108 **2. Materials and Methods**

109 *2.1. Plant material and culture conditions*

110 Four diploid cultivars, differing in their growth habit and recurrent blooming capacity, were used in this
111 study. A hybrid of *Rosa wichurana* (Rw) obtained from a rose garden in the “Jardin de Bagatelle” (Paris, France)
112 is a once-flowering genotype with indeterminate vegetative shoots and a ground-cover habit. *Rosa chinensis* ‘Old
113 Blush’ (OB) is a continuous-flowering genotype with a terminal inflorescence and bush habit. The cultivated roses
114 *Rosa* ‘Félicité et Perpétue’ (FP), a once-flowering genotype with indeterminate vegetative shoots and climbing
115 habit, and *Rosa* ‘White Pet’ (WP), historically named ‘Little White Pet’, a sport of FP with a continuous-flowering
116 behaviour (terminal inflorescences and bush habit, Iwata *et al.* 2012). The plants were maintained in a greenhouse
117 in the general following conditions: minimum air temperature maintained at 18°C, with an aeration at 20°C;
118 relative humidity maintained at 70% and no complementary lighting.

119 The methodology was validated on three other rose genotypes obtained as bare-rooted plants from the “Société
120 Nouvelle des pépinières Georges Delbard” (Malicorne, France). These plants were multiplied in the greenhouse
121 before their *in vitro* introduction.

122 These varieties are two continuous-flowering genotypes and tetraploid cultivated garden roses
123 ($2x=4n=28$) (*Rosa* PIMPRENELLE® (‘Deldog’) and *Rosa* GUY SAVOY® (‘Delstrimen’)) and one diploid
124 rootstock variety (*Rosa* ‘Natal Briar’).

125

126 *2.2. Meristems and in vitro culture conditions*

127 In order to work with healthy bacteria-free materials, and since *in vitro* mother-plant material is often
128 internally contaminated by bacteria if introduced *in vitro* via node culture (data not shown), meristem cultures
129 were conducted to obtain bacteria-free *in vitro* plants. From the plants maintained in the greenhouse, shoot stems
130 were harvested, surface-sterilized by rapid immersion in 70% ethanol, and meristems (axillary buds) were prepared
131 as described by Lebras *et al.* (2014). Meristems were grown in a growth chamber at $20 \pm 2^\circ\text{C}$ under a 16 h
132 photoperiod (light intensity of $40 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ provided by fluorescent tube lights).

133

134 *2.3. Clone maintenance and micropropagation*

135 Meristem-derived *in vitro* plants were multiplied and maintained in clonal cycles by subculturing nodes, with
136 each containing two axillary buds. In a first step, explants were cultured on Murashige et Skoog (MS) basal
137 medium complemented with 0.05 g.L^{-1} Fe-EDDHA, 0.1 mg.L^{-1} GA₃ and 0.5 mg.L^{-1} BAP (6-benzylaminopurine),

138 sucrose 30 g.L⁻¹ and solidified with 3 g.L⁻¹ Phytigel™ (Sigma) (shoot elongation medium, SEM). The medium
139 pH was adjusted to 5.7 before sterilization (113°C, 20 min). The cultures were conducted under a 16 h photoperiod
140 with a photosynthetic flux (PAR) of 56.4 μmol m⁻² s⁻¹ (generated by a combination of two Sylvania Luxline
141 F58W/840 fluorescent lamps and one Osram Biolux L 58W/72-965 fluorescent lamp). The temperature regime
142 was 23 ± 0.5 °C during the 16 h light period and 19 ± 0.5 °C during the 8 h dark period.

143

144 Individual Rw shoots were subcultured every month on SEM or SMM (corresponding to an SEM cytokinin
145 enriched medium: 3 mg.L⁻¹ BAP without GA₃), while only SMM was used for the other genotypes. Moreover, on
146 each medium (SEM and SMM), two replicates of a sugar comparison were carried out using various carbohydrate
147 types and concentrations, corresponding to sucrose, glucose, fructose, maltose or sorbitol at 10, 15, 30 or 40 g.L⁻¹
148 ¹. To assess and compare the impact of the medium hormonal and sugar content on plant development, different
149 phenotypic traits were measured after one month on the medium, such as: shoot size (height), shoot number, a
150 scale of the presence or absence of callus (0=absence and 1-2-3-4-5=scale of size callus), the number of new roots,
151 node number and ramification (axillary bud burst).

152 *In vitro* plants maintained in a clonal cycle were used as explant sources for the induction of shoot regeneration
153 via organogenesis.

154

155 2.4. *De novo* shoot organogenesis

156 For the Rw genotype, donor plants were subcultured on SEM and SMM, while only SMM was used for the other
157 genotypes.

158 Explants were excised from 6 week-old *in vitro* plants cultivated on SMM medium with glucose or fructose at 30
159 g.L⁻¹. The clonal cycle included 1 week in darkness. Explants, consisting of the two proximal leaves and
160 corresponding to the three terminal leaflets with subtending petiolules, were incubated on induction medium and
161 the impact of sugar was compared using 5 types of carbohydrate source (fructose, glucose, maltose, sorbitol and
162 sucrose) at different concentrations (10, 15, 30, 40 g.L⁻¹).

163 For each condition, two replicates of 10-12 wounded leaf explants were placed with their abaxial side on petri
164 dishes containing solid regeneration MS medium according to Ibrahim and Debergh (2001). Two or three scratches
165 (depending of the leaf maturity and size) were made on the abaxial of each leaflet with a scalpel perpendicular to
166 the midrib. Shoot regeneration medium (SRM) containing macro- and micro-elements, MS vitamins, was
167 complemented with 0.56 mM myo-inositol and solidified with 3 g.L⁻¹ Phytigel. The effects of growth regulator

168 combinations were evaluated by supplementing the medium with 2.3, 4.6 or 9.2 μM of TDZ (Thidiazuron) (SRM
169 1, 3 and 6, respectively) and with 0.23 μM (SRM 1 and 3) or 0.46 μM (SRM 6) of IBA using compartmentalized
170 petri dishes, as shown in the Figure 4b. The medium pH was adjusted to 5.7 before sterilization. Cultures were
171 maintained in a tissue culture room under the above described temperature regime.

172 All cultures were initially incubated in the dark at 23°C for 16 h at 19°C for 8 h for 1 or 2 weeks depending on
173 the time to achieve DNSO.

174 After 6 weeks, the following explant characteristics were measured: relative size of callus (0 to 5 scale,
175 corresponding to the absence (0) or presence of callus (1 to 5) and depending on the callus diameter (in cm) 1
176 corresponding to <0.5; 2 is 0.5 to 1; 3 is 1 to 1.5; 4 is 1.5 to 2 and 5 is >2), presence or absence of roots, shoot
177 number per explant and shoot size.

178 The regenerated plants were rooted on MS basal medium supplemented with 0.1 mg. L⁻¹ AIB and 0.5 mg.L⁻¹ NAA
179 at pH 6.

180

181 *2.5. Statistical analyses*

182 All data were statistically analyzed with the R software package, version 2.11.1 (<http://www.r-project.org/>),
183 including ANOVA, HSD tests for multiple comparisons and logistic analysis. Quantitative data (shoot size, shoot
184 number) were analyzed with ANOVA, followed by a Tukey's HSD post-hoc test. Bimodal DNSO data (i.e. 1 for
185 DNSO and 0 for no DNSO) were fitted in logistic models. 95% confidence intervals were then calculated for each
186 LOD-odds ratio. Log odds ratios were then converted back into DNSO probabilities. The confidence interval was
187 not calculated when no DNSO was observed.

188 **3. Results**

189 To define the best conditions for DNSO on various genotypes, we first tested two mediums and different sugars
190 on the genotype Rw (Fig. 5). The best medium was defined for its potential to induce young tissue. This medium
191 was then used on other genotypes (Fig. 5) and DNSO was conducted on explants which were taken on in vitro
192 plants produced on this best medium.

193

194 *3.1. Clonal cycle of the hybrid of Rosa wichurana (Rw)*

195 A clonal cycle was performed to multiply *in vitro* mother plants and produce explants able to induce *de novo* shoot
196 organogenesis.

197 *In vitro* culture of Rw was achieved via meristem culture. The effects of combined growth regulators (SEM and
198 SMM) and carbohydrates (different types and concentrations) on shoot and root development and on callus
199 formation during the clonal cycle were evaluated (illustrated in Table 1 and Fig. 1).

200 For fructose, glucose and sucrose, we observed an effect-relationship between the increased sugar concentration
201 and the callus size, irrespective of the media (SEM or SMM). Roots developed only on SMM medium with an
202 increased number of shoot correlating with the increase concentration of fructose, glucose and sucrose (Table 1b).
203 In this respect, no significant relationship was noted for maltose and sorbitol (Table 1a).

204 Node formation (NoNode) and ramifications were only observed on SEM. With fructose, glucose or sucrose, we
205 observed an increase in the node number and branching until 30 g.L⁻¹ of these sugars, followed by a decrease at
206 higher concentration (40 g.L⁻¹). For maltose and sorbitol, the relationship was less clear but a trend revealed a
207 maximum node number with the minimum quantity of these two sugars (i.e. 10 g.L⁻¹) (Table 1a).

208 Except for sorbitol, the shoot size and shoot number increased with the increased sugar concentration until 30 g.L⁻¹
209 (Table 1). For the high concentration (40 g.L⁻¹), we noted a negative effect on the shoot number and shoot size,
210 except for maltose and sorbitol.

211 No statistically significant difference in shoot size was observed for fructose, glucose and sucrose at 30 g.L⁻¹
212 regardless of the medium, and for sucrose at 40 g.L⁻¹ with SEM (Table 1). On SEM, the highest shoot number (but
213 without any significant difference) was observed regardless of the sugar and concentration variation, except for
214 maltose at 10 g.L⁻¹, sorbitol at 10-15-30 g.L⁻¹ and sucrose at 30 g.L⁻¹. On SMM, the highest shoot number was
215 observed for fructose 15-30 g.L⁻¹, glucose 15-30 g.L⁻¹, maltose 30-40 g.L⁻¹ and sucrose 30 g.L⁻¹ (Table 1 and Table
216 S1).

217 Thus, the shoot height (Shoot size) was clearly higher on SEM compared to SMM, while the opposite trend was
218 observed for basal multiplication (Noshoot) (Fig. 1 and Table 1), especially for SMM containing fructose as
219 carbohydrate source. Overall, we observed better Rw shoot development on media containing fructose, glucose or
220 sucrose compared to those containing maltose and sorbitol. The least favorable medium for shoot development
221 (shoot number and size) was that containing 3 mg.L⁻¹ of BAP (SMM) with sorbitol or maltose as carbohydrate
222 source (Fig. 2). In this genotype, the best medium for the shoot number was SMM supplemented with fructose at
223 30 g.L⁻¹, while the best shoot growth was obtained with SEM supplemented with glucose at 30 g.L⁻¹.

224 **As the aim of this study was to obtain young tissue for DNSO and since SMM was the best medium for new**
225 **young shoot induction (Table 1), this medium was chosen for subsequent analyses.**

226

227 3.2. Clonal cycle of five rose genotypes

228 We then opted to evaluate SMM (BAP 3 mg.L⁻¹) on six other genotypes: *Rosa* GUY SAVOY® ('Delstrimen')
229 (GS), *Rosa* PIMPRENELLE® ('Deldog') (P), *Rosa chinensis* 'Old Blush' (OB), *Rosa* 'Félicité et Perpétue' (FP),
230 *Rosa* 'Natal Briar' (NB) and *Rosa* 'White Pet' (WP).

231 GS appeared to be the most productive genotype in terms of growth (Shoot size of 2,75) and new shoot formation
232 (shoot number = NoShoot) (Table 2) up to 6.4 shoots using fructose at 15 g.L⁻¹. For GS and P, in contrast to Rw,
233 we observed a trend towards a negative impact of the sugar concentration (fructose, sorbitol or sucrose) on the
234 shoot size, i.e. the shoot size decreased as the sugar concentration increased. For FP, there were few significant
235 differences in the shoot size and number relative to the sugar type and concentration. Overall, except for GS,
236 sorbitol, regardless of the concentration and genotype, was less effective in promoting shoot growth and stem
237 multiplication (Table 2). Whatever the genotype, the best outcomes, in terms of multiplication level and *in vitro*
238 growth, were obtained with fructose and glucose at concentrations between 10 and 30 g.L⁻¹ (except WP).

239 Some preliminary analyses were performed on the DNSO potential of explants from the two media and in various
240 sugar types and concentrations (data not shown). The results showed that no DNSO was obtained in explants from
241 SEM and that sucrose and fructose were the most favorable sugars.

242 **Therefore, for further DNSO experiments, we decided to select sucrose at 30 g.L⁻¹ or fructose at 30 g.L⁻¹ as**
243 **the carbon source and SMM for explant production.**

244

245 3.3. De novo shoot organogenesis of *Rosa wichurana*

246 We used 6 week-old Rw *in vitro* plants (cultivated 5 weeks under light conditions then one week under dark
247 conditions). Explants (leaves) from SMM sucrose at 30 g.L⁻¹ or SMM fructose at 30 g.L⁻¹ were placed on SRM1,
248 3 or 6. On these three media, the same range of sugars (type and concentration) as those used in the clonal cycle
249 were tested. All regeneration was observed 6 weeks after limited callus formation on tissue, indicating an indirect
250 regeneration process.

251 As shown in Fig. 4a, we first observed callus induction on the petiole or petiolule and on the leaf scratches. Calli
252 were creamy and light brown in color. We observed the first shoot organogenesis right after this callus induction
253 between 3 to 6 weeks (Fig. 4c).

254 No differences in DNSO process were observed between the sugar types (sucrose or fructose) (data not shown).
255 The effect of the medium and sugar on the mean DNSO percentage is shown in Fig. 2. No significant differences
256 were observed among the tested media (SRM1-SRM3-SRM6) for this genotype, although SRM6 seemed to
257 display a slightly lower DNSO percentage, showing that the increase in auxin (IBA) and cytokinin (TDZ) could
258 be detrimental to the DNSO process for this genotype, regardless of a comparable growth regulator ratio between
259 SRM3 and SRM6. Despite the rise in cytokinin concentration between SRM1 and SRM3, no significant difference
260 in the DNSO percentage was observed.

261 The maximum DNSO percentage (100%) was observed using sucrose at 30 g.L⁻¹ on SMM during the clonal cycle,
262 then various SRM and sugars in the DNSO medium (Table 3). Fig. 2b shows that higher DNSO percentages were
263 obtained for fructose (85 to 92%) and glucose (75 to 88%) regardless of the concentration, and for glucose at 30
264 or 40 g.L⁻¹. The shoot regeneration frequency decreased from a mean of 77% to 40% when maltose was increased
265 up to 4%, while no or very low (9%) regeneration was recorded with sorbitol. Regenerated plants were rooted (Fig
266 4i-j), acclimated (Fig. 4k-l) and observed in the greenhouse (Fig. 4m). There were no discernable differences
267 between mother and regenerated plants.

268

269 *3.4. Application to other genotypes*

270 DNSO was improved on the five other corresponding genotypes: GS, OB, FP, NB and WP. All genotypes
271 originated from meristem cultures, were subcultured on fructose or sucrose at 30 g.L⁻¹ in SMM culture for 6 weeks
272 under light/dark conditions, then 1 week under dark conditions (Fig. 3 and Table S3) before DNSO assays. Under
273 our conditions, we observed an indirect DNSO process with a short callus phase for all of these genotypes, as
274 already described for Rw.

275 **For GS**, only fructose, glucose and sucrose on SRM media were tested. In all tested conditions (media or sugars),
276 the DNSO percentage was low, with no significant differences (Fig. 3). The highest percentage, whichever the
277 medium, was noted with sucrose at 30 and 40 g.L⁻¹, with a mean of 27% and 26%, respectively, and then 19% for
278 fructose at 30 g.L⁻¹ and glucose at 30 and 40 g.L⁻¹ (Fig. 3). The best conditions for achieving 57% DNSO for this
279 genotype involved producing plants on SMM medium containing sucrose at 30 g.L⁻¹ and used the SRM3 medium
280 containing fructose at 30 g.L⁻¹.

281 We observed high intra-genotype variability for **OB**, for which the best medium seemed to be SRM3, but the
282 differences were not significant (Fig. 3). The best DNSO levels were observed with fructose at 30 g.L⁻¹ (46%) and
283 40 g.L⁻¹ (40%). No regeneration was achieved with maltose or sorbitol. The maximum DNSO (74%) was obtained
284 for this genotype using fructose at 30 g.L⁻¹ to produce explants and the SRM3 containing fructose at 40 g.L⁻¹.

285 The lowest DNSO percentages were obtained with the **FP** genotype, for which the highest DNSO level was 37.5%
286 (Table S3) when using sucrose at 30 g.L⁻¹, then SRM1 with fructose at 30 g.L⁻¹. We also observed high variability
287 for this genotype. No regeneration was obtained with sorbitol (Fig. 3).

288 **For NB**, like GS, only fructose, glucose and sucrose were tested. The mean DNSO percentage for any given media
289 ranged from 10.5% for sucrose at 15 g.L⁻¹ to 37% for fructose at 40 g.L⁻¹. We observed a slight increase in the
290 DNSO percentage correlated with the increased sugar concentration (Fig.3). Therefore, the highest DNSO
291 percentages were obtained with fructose (62.5%) and sucrose (55.6%) at 40 g.L⁻¹ in SRM6 and SRM1, respectively
292 (Tables 3 and S2).

293 WP had a different behaviour than that of the genotype from which it was derived (FP), which had a low DNSO
294 capacity. Indeed, for WP, we observed 100% DNSO with glucose at 15 and 30 g.L⁻¹ on SRM1 and SRM3,
295 respectively, although we observed a decrease in the DNSO percentage on the other tested media (Fig. 3 and
296 Tables 3 and S3).

297 **Taken together, all of these results showed various behaviors that were genotype-dependent and sugar-**
298 **dependent with regard to their DNSO capacities in the regeneration process.**

299 4. Discussion

300 The aim of this study was to develop a protocol for rose DNSO with various steps corresponding to the initiation
301 of *in vitro* culture, then *in vitro* development and plant regeneration, as summarized in the Figure 5. For this study,
302 we observed the effects of the sugar concentration on *in vitro* growth, *in vitro* behaviour and the DNSO process in
303 combination with various PGR on six rose genotypes. The genotypes we used, differed according to their habit,
304 blooming mode, ploidy level and origin, or to their use (rootstock versus scion; old versus modern genotypes).
305 None of these differences were found to be correlated with the behaviour observed in the *in vitro* clonal cycle,
306 sugar use or DNSO process.

307 Between both tested media for plant elongation (SEM) and multiplication (SMM), basal multiplication was
308 observed on the SMM containing a greater amount of BAP and no rooting, contrary to the situation on SEM. The
309 carbon source and concentration were also tested. The carbon source is essential to sustain independent *in vitro*
310 growth and organogenesis as has been shown, particularly in strawberry, a species closely related to rose (Grout
311 and Price, 1987). This improved regenerative behaviour has not always been linked to the carbohydrate nutritional
312 status, but rather to the osmotic condition induced by carbohydrates (Gaj, 2004; Lou and Kako, 1995; Nakagawa
313 *et al.*, 2001). Sucrose is often assumed to be the sugar of choice in cell and tissue culture media because it is the
314 predominant sugar that is translocated in phloem (Brachi *et al.*, 2010; Faure *et al.*, 1998; Marino *et al.*, 1993; Pua
315 and Chong, 1985; Sharma *et al.*, 2008), and also due to its cheap and easy availability. In our study, we showed
316 that sucrose was not always the best carbohydrate and the explants grew well on media supplemented with other
317 carbohydrates, such as fructose and glucose. This is comparable to results obtained in other species, such as *Alnus*
318 spp. (Welander *et al.*, 1989) and *Quercus* (Romano *et al.* 1995), which grow better on medium supplemented with
319 glucose, whereas fructose is superior to sucrose for *in vitro* culture of *Morus alba* (Oka and Ohyama, 1986),
320 *Castanea sativa* (Chauvin and Salesses, 1988) and *Malus* rootstock (Welander *et al.* 1989).

321 Obviously, whilst sucrose is generally applied in tissue culture of plants, its effects on plant development,
322 photosynthetic performance and growth under these conditions seems to be variable and species dependent. Sugar
323 and starch dynamics in the medium-root-leaf system highlight possibilities for optimizing plant tissue culture.
324 Following invertase activity, sucrose is split into glucose and fructose (Straus, 1962). This hydrolysis can take
325 place both inside (within cells by cytosolic or vacuolar invertases) and outside the plant (in *in vitro* medium via
326 cell wall invertase). The amount of hydrolyzed sucrose and its reaction rate are species dependent (George, 1993).
327 Once hexoses are present in the medium (by adding it as the preferable carbon source or by invertase action on
328 sucrose), these sugars can also be used by *in vitro* plants (George, 1993; Wyse, 1979).

329 However, our results showed that sorbitol, a sugar alcohol, is detrimental to growth and organogenesis in any rose
330 tested genotypes, contrary to what has been previously shown in several studies on various members of the
331 *Rosaceae* family, in which sorbitol is an optimum source of carbon, energy and osmotic adjustment (Kadota and
332 Niimi, 2004; Marino *et al.*, 1993; Pua and Chong, 1984; Sotiropoulos *et al.*, 2006; Yaseen *et al.*, 2013). In apricot
333 microshoots (Marino *et al.* 2010), there are specific enzymes for sorbitol oxidation, including sorbitol
334 dehydrogenase, which convert sorbitol to fructose, thereby improving shoot production and development when
335 sorbitol is added to the medium. This is not true for *Rosa* spp that is known to be deprived of sorbitol-metabolizing
336 enzymes. In other species, sorbitol acts mainly as an osmotic regulator and, unlike sucrose, sorbitol neither
337 supports *in vitro* shoot growth nor is metabolized in most higher plants (Yaseen *et al.* 2013). On the other hand,
338 sorbitol was reported to be completely ineffective in stimulating shoot proliferation and root induction in *Quercus*
339 *suber* (Romano *et al.* 1995).

340 Our results showed that maltose yielded low results compared to fructose, glucose and sucrose. Maltose is derived
341 from starch degradation and is a carbon source while also being an osmotic agent. Such a low effect of maltose
342 could be assigned to its weak intake and/or metabolism by *in vitro* cultured tissue. Few studies have been carried
343 out on the effect of maltose on the micropropagation process, Bahmani *et al.* (2009) showed that the size and shoot
344 number of M9 apple rootstock were positively affected using maltose in *in vitro* culture media.

345 In this study, we also tested the impact of the concentration of various sugars on *in vitro* growth and development.
346 Source activities (e.g. photosynthesis, export) are upregulated under low sugar concentrations whilst sink processes
347 (e.g. growth, storage) are upregulated when carbon sources are abundantly present (Rolland *et al.*, 2006).
348 Lembrechts *et al.* (2017) concluded that, in horticultural production, higher carbohydrate contents (starch, hexoses
349 and sucrose) have substantial impacts since lower enriched media (e.g. 5 g.L⁻¹ instead of 25 g.L⁻¹) could potentially
350 be used to grow healthy *in vitro* plants able to perform photosynthesis immediately when transferred to the
351 greenhouse, while not compromising plant development and growth during the entire production cycle.
352 Concerning plant growth on medium supplemented with fructose, glucose and sucrose, we observed two trends,
353 either an increase or decrease depending on rose genotype in relation to the sugar concentration in the 10 g.L⁻¹ to
354 30 g.L⁻¹ range. This indicates that plant growth is a complex phenomenon that not only depends on the nature and
355 concentration of the sugars involved but also on the genotype of interest. Concerning organogenesis, it can be
356 concluded that optimal concentrations for the highest number of new shoots was 30 g.L⁻¹ for all sugars, except
357 sorbitol for two genotypes (GS and NB) for which the shoot number decreased with increasing sorbitol content.
358 In general, and regardless of the sugars involved, the highest tested concentration (40 g.L⁻¹) was detrimental to the

359 growth and development of all genotypes. Using 'Red Globe' grape plantlets, Mao *et al.* (2018) suggested that
360 lower glucose concentrations (10-20 g.L⁻¹) promoted photosynthesis and growth, and the decrease in the
361 photosynthesis rate at higher concentrations (40 g.L⁻¹) may be due to the downregulation of rubisco activity. In
362 line with this, Capellades *et al.* (1991) found that the difference between the photosynthesis rates of test tube *Rosa*
363 (*Rosa multiflora* L. cv.) seedlings grown at 1 and 3% sucrose concentrations were not significant, whereas they
364 decreased significantly at 5%. This rubisco downregulation in response to the accumulation of soluble sugars in
365 leaves could be mediated by a hexokinase signaling dependent pathway (Dai *et al.*, 1999). High carbon levels may
366 also affect cellular growth by affecting the water potential of the medium, which is a very important factor since
367 it determines the movement of water and mineral elements into plant tissues and also maintains better turgor for
368 the plant cells.

369 To improve *de novo* shoot organogenesis, we produced explants from plants multiplied on medium containing
370 sugar that ensured a maximum of tested genotypes and a higher shoot number, i.e. glucose and fructose at 30 g.L⁻¹.
371

372 Several previous studies on DNSO (direct or indirect) showed that leaves (leaflets or trifoliate structure) and
373 petioles are the best tissues to induce *de novo* shoot organogenesis (Arene *et al.* 1993; Dubois *et al.* 2000; Li *et al.*
374 2002; Lloyd *et al.* 1988; Pourhosseini *et al.* 2013). Only one study dealt with the positive impact of sugar (glucose
375 versus sucrose) on rose DNSO (Hsia and Korban, 1996) on one genotype and no significant difference for another
376 one. Our results clearly showed high variability in the DNSO potential of the tested genotypes, as shown by Dubois
377 *et al.* (2000) and Nguyen *et al.* (2017).

378 In the present study, we highlighted optimal conditions to obtain 100% DNSO for three genotypes (RW and WP)
379 and the highest percentage for the four others (NB 87.5%; OB 74%, GS 56.7% and FP 37.5%). The average
380 regeneration rate per genotype ranged from 37.5% to 100%, whereas other studies indicated a range of 62% to
381 100% on 24 genotypes (Dubois *et al.* 2000) and 0.88% to 88.33% on 96 genotypes (Nguyen *et al.* 2017). The
382 origin of this variability is unknown and may be dependent on the genotype. By a GWAS approach, Nguyen *et al.*
383 (2017) found SNP markers linked to this variability and listed candidate genes for shoot regeneration. The
384 superiority of sucrose and its derivative hexoses (glucose, fructose) in promoting shoot regeneration from leaf
385 explants is consistent with the results obtained with rose cultivars and *R. chinensis minima*
386 (Hsia and Korban 1996), and with *Annona muricata* (Lemos and Baker, 1998). Conversely, for *Solanum*
387 *aculeatissimum*, the highest regeneration percentage was obtained with 30 g.L⁻¹ sucrose (Ghimire *et al.*, 2012),
388 and similar results have been reported for *Echinacea angustifolia* (Kim *et al.*, 2010) and *Harpagophytum* sp. (Jain

389 *et al.*, 2009). Fructose and maltose, on the other hand, were found to be better carbon sources than sucrose in
390 *Juglans regia* (Seo *et al.*, 2010). These findings suggest that the nature of the carbon source in the culture medium
391 differs among plants species and is a key factor to successfully manage the organogenic competence of plant tissue
392 cultures. These findings pave the way for new investigations into the exact role of sugars in these developmental
393 aspects of plant tissue culture.

394 In this study, a reliable regeneration system was developed with rose leaves. This method could be used for rapid
395 propagation and genetic transformation studies of this ornamental species.

396

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398

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Fig. 1 Morphological responses of Rw genotype *in vitro* plants after 6 weeks on SEM and SMM containing various sugars.

Fruc: fructose; Gluc: glucose; Malt: maltose; Sorb: sorbitol; Suc: sucrose at various concentrations (10-15-30 or 40 g.L⁻¹).

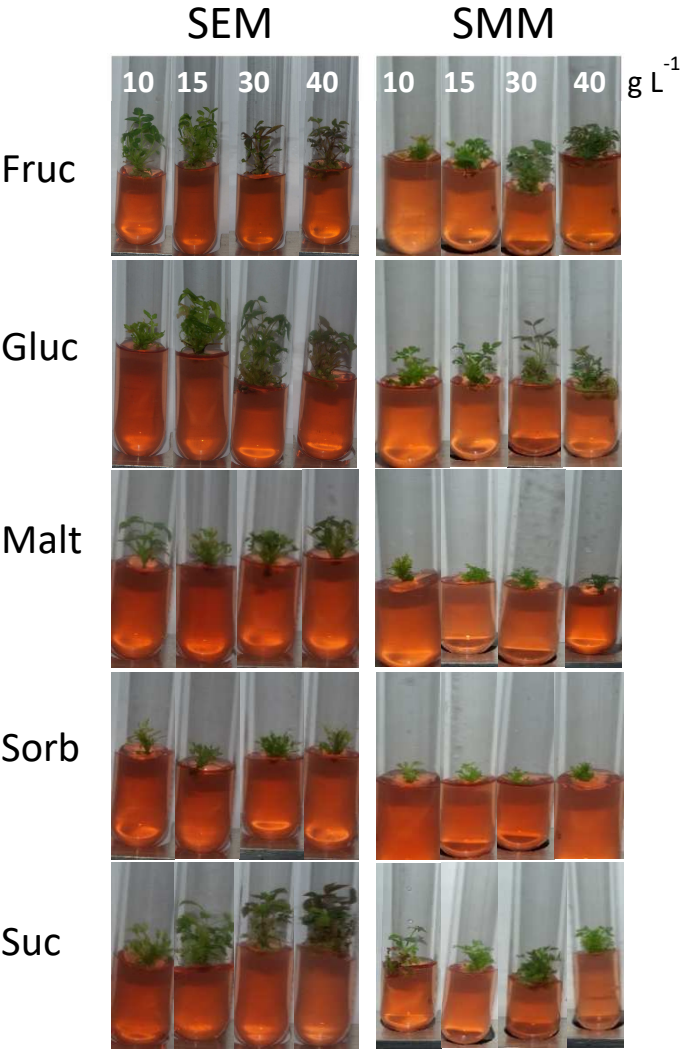
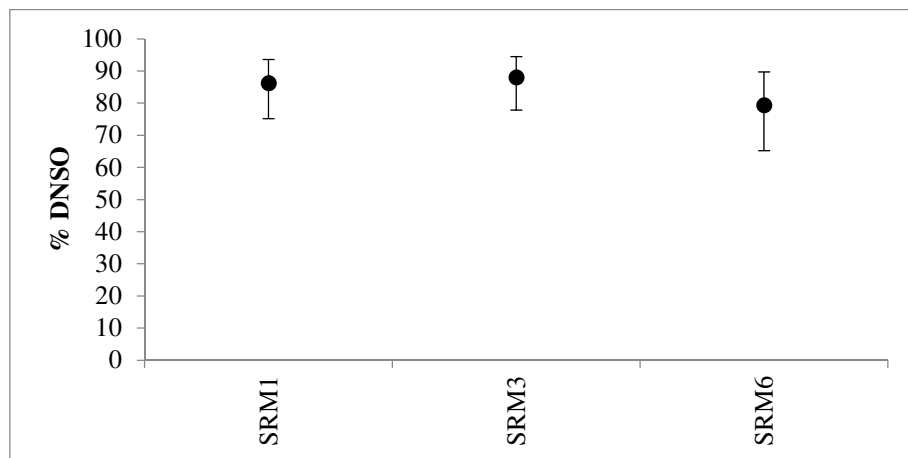


Fig. 2 Percentage of mean DNSO obtained from leaf explants of the *Rosa wichurana* hybrid excised from plants cultivated on SMM. The graph shows the percentage of mean DNSO on the different shoot regeneration media, i.e. SRM1, SRM3 or SRM6 (a), regardless of the sugar type and concentration and the percentage of mean DNSO according to the sugar type and concentration (b) and regardless of the medium.

Fruc=fructose, gluc=glucose, malt=maltose, sorb=sorbitol, suc=sucrose

15-30-40 indicate the sugar concentration corresponding to 15 g.L⁻¹, 30 g.L⁻¹ and 40 g.L⁻¹, respectively.

a



b

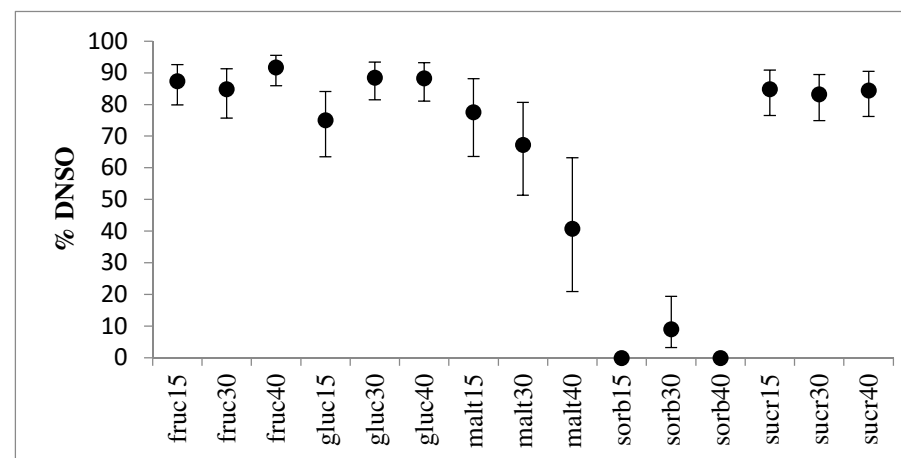


Fig. 3 Percentage (\pm confidence interval) of the mean DNSO obtained from explants of five genotypes excised from plants cultivated on SMM. The graph shows the percentage of mean DNSO on the various media, i.e. SRM1, SRM3 or SRM6 (a) to the sugar type and concentration and the percentage of mean DNSO according to the sugar type and concentration, regardless of the medium (b).

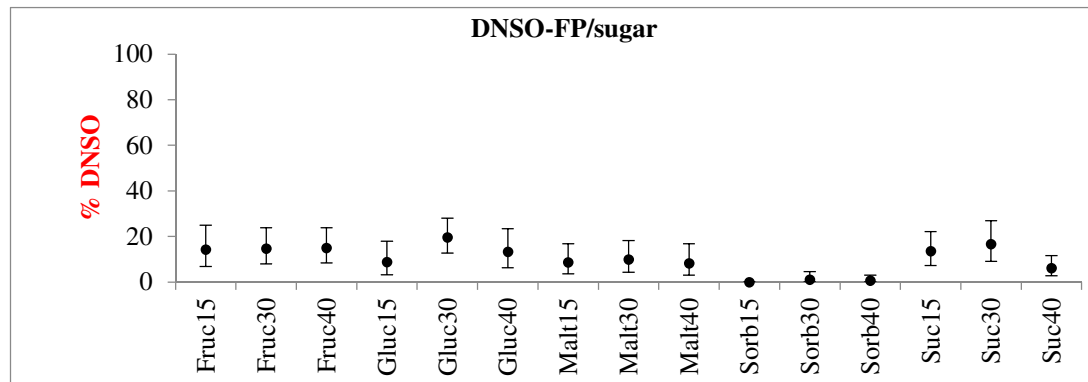
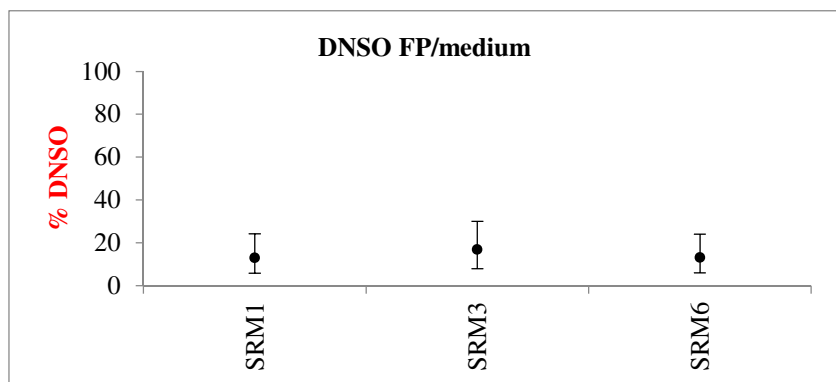
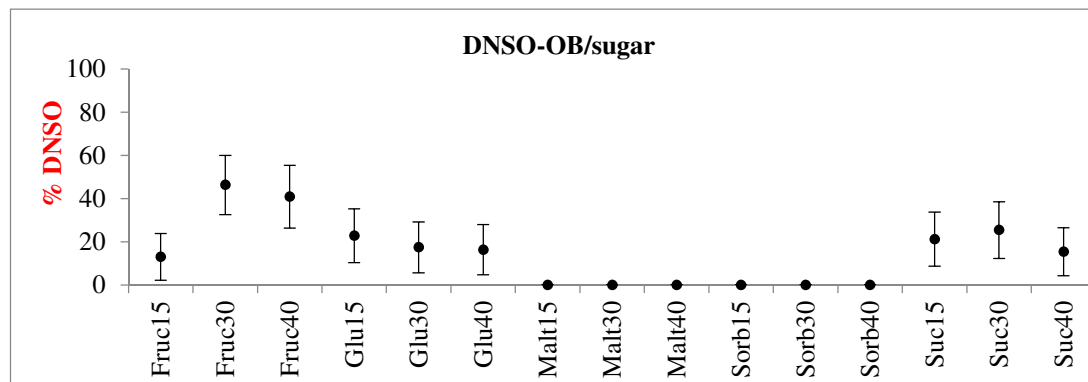
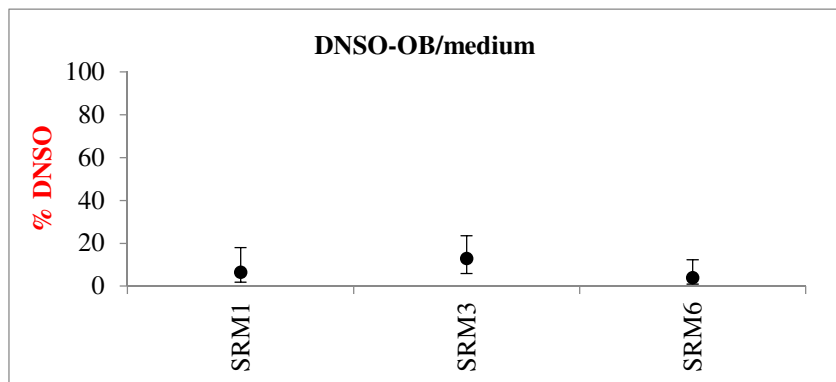
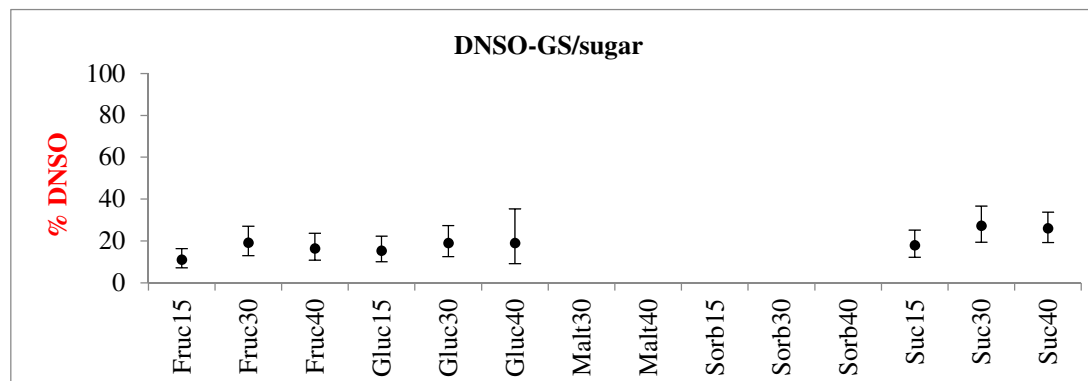
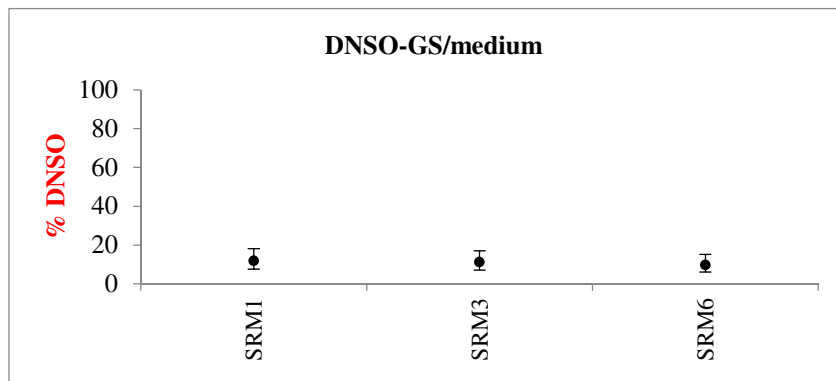
The genotypes are Rosa GUY SAVOY® ‘Delstrimen’ (GS), Rosa ‘Félicité et Perpétue’ (FP), Rosa ‘Natal Briar’ (NB), Rosa chinensis ‘Old Blush’ (OB) and Rosa ‘White Pet’.

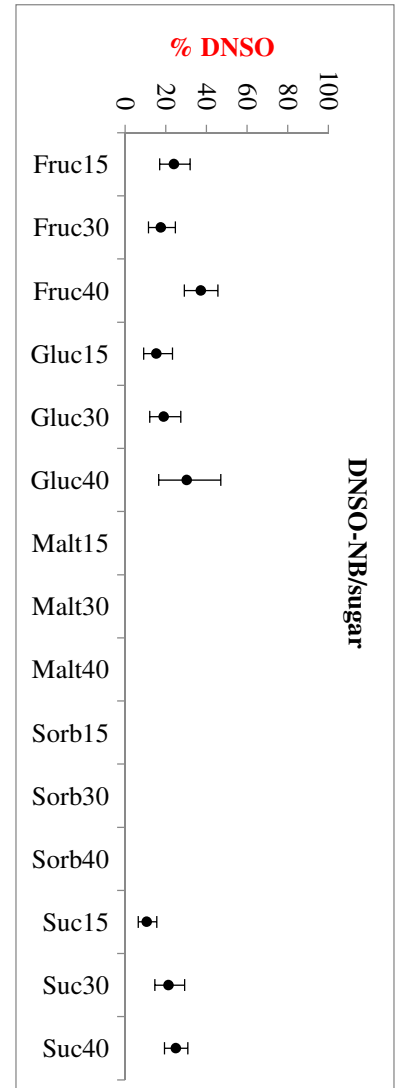
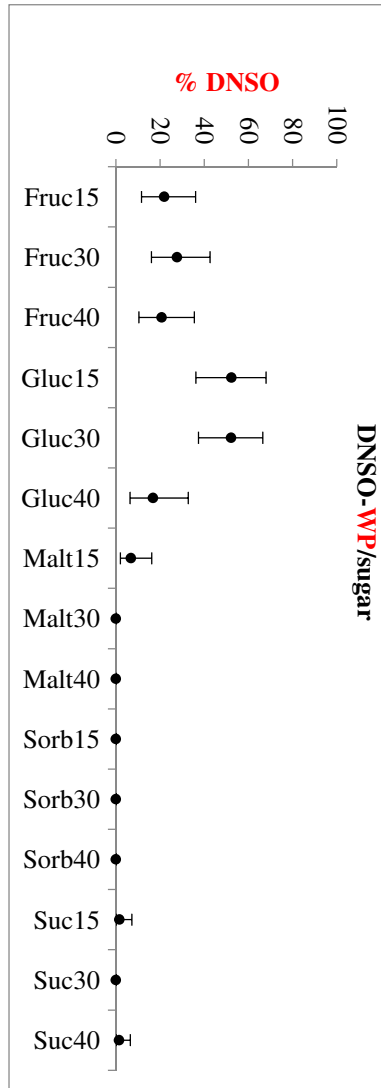
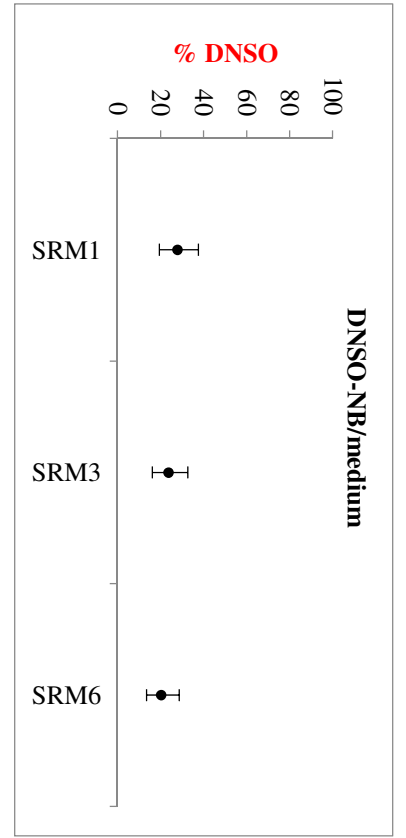
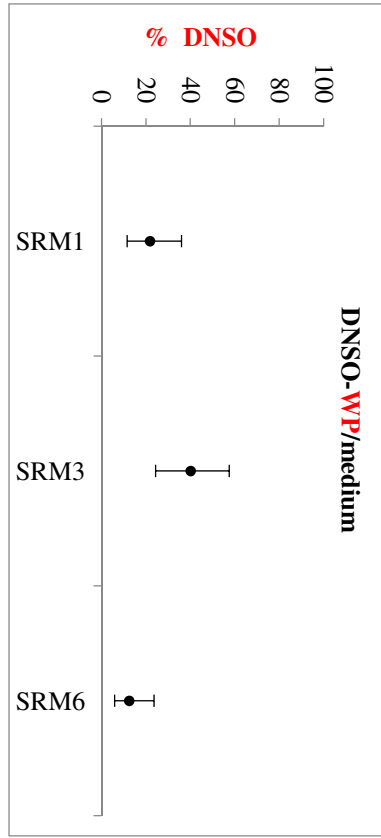
Fruc=fructose, Gluc=glucose, Malt=maltose, Sorb=sorbitol, Suc=sucrose

15-30- 40 indicate the sugar concentration, corresponding to 15 g.L-1, 30 g.L-1 and 40 g.L-1, respectively

a

b





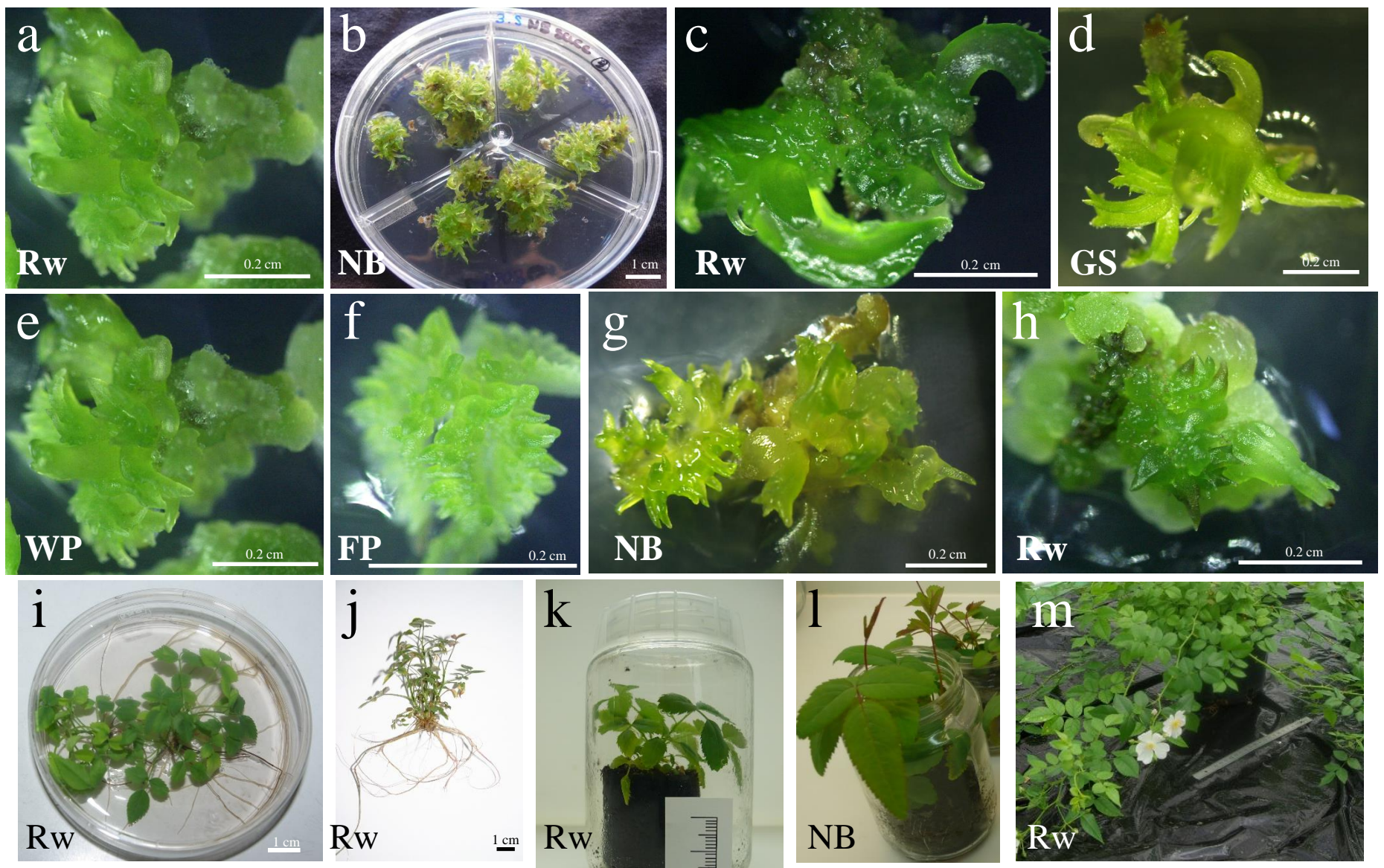


Fig. 4 Regeneration of various *Rosa* genotypes. **a)** callus formation on *R.wichurana* (Rw) leaf explants derived from donor plants cultivated on SEM during the clonal cycle **b)** compartmentalized petri dishes used to test three SRM. **c-h)** DMSO on various genotypes. **i-j)** Rooting of Rw plants. **k-l)** Acclimatisation of Rw and NB plants in Glass Jar. **m)** Regenerated Rw plant in the greenhouse.

GS: *Rosa* GUY SAVOY® ('Delstrimen'), WP: *Rosa chinensis* 'Old Blush', *Rosa* 'Félicité et Perpétue', *Rosa* 'Natal Briar' and *Rosa* 'White Pet'

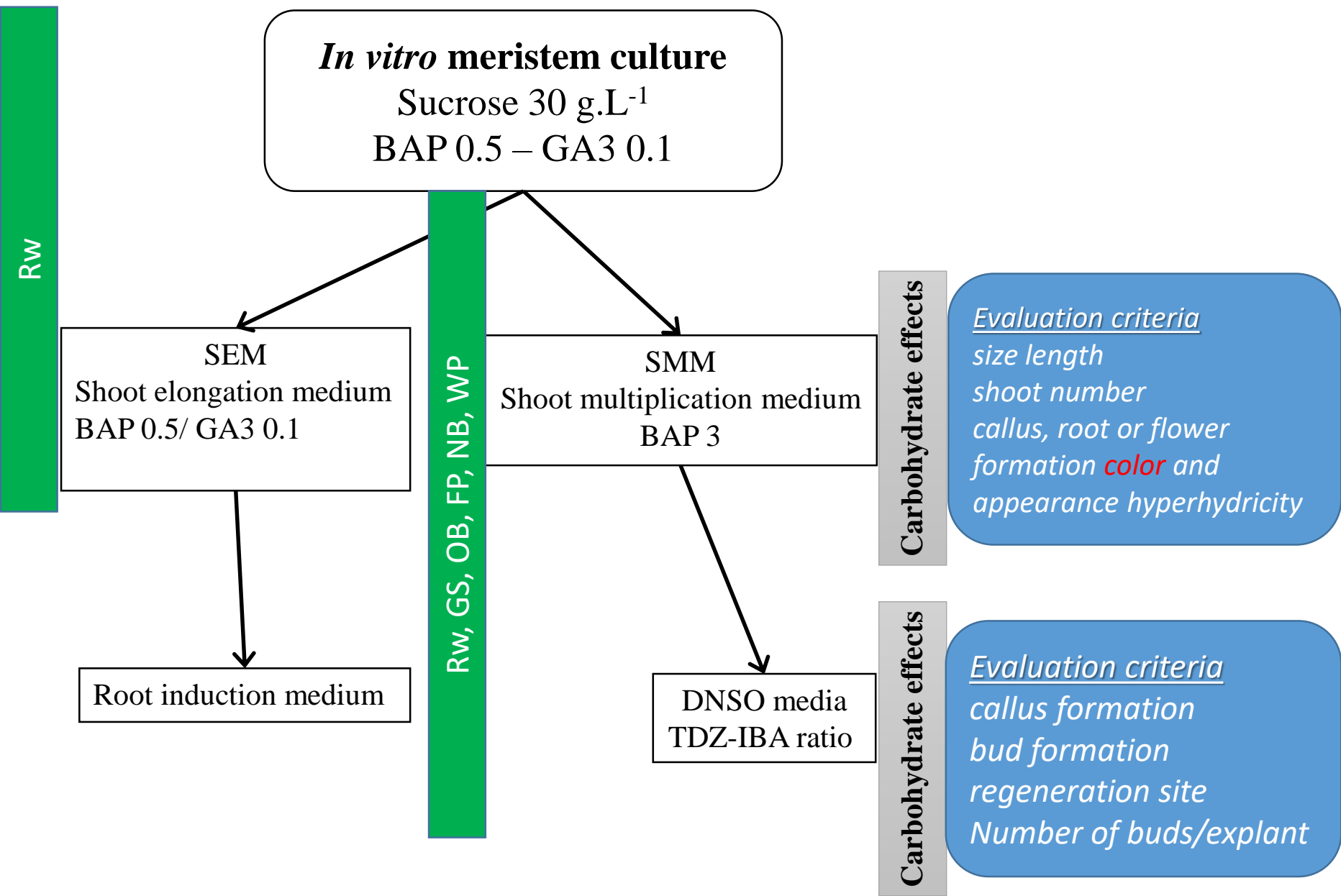


Fig. 5 Schematic diagram of different steps of rose tissue culture to obtain DNSO, different media components and criteria analysis. The medium (SEM and SMM) impact was evaluated on the genotype *Rosa wichurana* (Rw) and the best medium for DNSO was then used for all genotypes.

Table 1 Impact of SEM [BAP 0.5 mg.L⁻¹/GA3 0.1 mg.L⁻¹] (a) and SMM [BAP 3 mg.L⁻¹] (b) combined with various carbohydrate source types and concentrations on the callus formation, growth and development of *in vitro* *Rosa wichurana* (Rw) explants during the clonal cycle. The mean and standard deviation (SD) were calculated from 12 repetitions in two experiments for the height (cm) of shoots (Shoot size), the number of newly formed shoots derived from the base of the original axillary node (NoShoot), the scale of the size of a callus formed (Callus), the number of roots (Root), the number of formed nodes on the elongated stems (NoNode) and the number of axillary bud bursts on elongated stems (Ramification). Means of Shoot size and NoShoot that are not connected by the same letter are significantly different at 0.05 probability level.

a

SEM	Shoot size		NoShoot		Callus		Root		NoNode		Ramification	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
fructose10	1.90 ^{efghi}	0.55	3.63 ^{ab}	1.07	1.37	0.68	0.05	0.23	1.26	1.05	1.33	0.78
fructose15	2.54 ^{cdef}	0.77	3.40 ^{ab}	1.50	1.65	0.92	0.10	0.24	0.95	1.82	2.08	0.85
fructose30	3.43 ^{ab}	1.20	2.55 ^{abcde}	1.48	2.20	1.47	0.95	1.23	2.65	2.96	1.67	0.93
fructose40	2.75 ^{bcde}	0.79	3.35 ^{abc}	1.33	2.45	1.24	1.50	1.81	1.55	1.89	1.75	0.88
glucose10	1.48 ^{ghi}	0.32	3.33 ^{abc}	1.71	0.88	0.54	0.00	0.00	1.46	1.44	2.00	0.00
glucose15	2.20 ^{defg}	0.68	3.29 ^{abc}	1.82	1.14	0.73	0.05	0.22	0.86	1.42	1.80	0.63
glucose30	3.77 ^a	1.57	2.92 ^{abcd}	1.58	1.92	1.31	1.38	1.56	4.67	5.01	2.42	0.76
glucose40	2.60 ^{bcde}	1.13	3.16 ^{abc}	1.89	2.96	1.22	2.08	1.98	1.68	2.29	2.00	0.74
maltose10	1.25 ^{ghi}	0.53	2.00 ^{bcde}	1.03	0.11	0.32	0.00	0.00	1.89	1.13	0.82	0.40
maltose15	1.42 ^{ghi}	0.37	2.40 ^{abcde}	1.19	0.30	0.47	0.00	0.00	0.70	1.08	1.83	0.39
maltose30	1.59 ^{ghi}	0.38	3.42 ^{ab}	2.09	0.30	0.47	0.05	0.22	0.30	0.92	1.83	0.94
maltose40	1.65 ^{fghi}	0.25	3.85 ^a	1.79	0.35	0.49	0.00	0.00	0.85	1.18	1.77	0.93
sorbitol10	1.27 ^{hi}	0.78	1.53 ^{de}	0.70	0.00	0.00	0.00	0.00	2.42	1.30	0.73	0.47
sorbitol15	1.34 ^{ghi}	0.44	1.32 ^e	0.48	0.37	0.50	0.00	0.00	1.32	1.70	0.45	0.52
sorbitol30	1.20 ^{hi}	0.34	1.75 ^{cde}	0.79	0.30	0.47	0.00	0.00	0.85	1.50	0.83	0.72
sorbitol40	1.13 ⁱ	0.23	2.25 ^{abcde}	1.29	0.30	0.47	0.00	0.00	1.55	1.15	1.00	0.00
sucrose10	1.39 ^{ghi}	0.46	2.79 ^{abcde}	1.50	0.92	0.58	0.00	0.00	0.75	0.74	1.33	0.65
sucrose15	2.02 ^{efgh}	0.76	2.75 ^{abcde}	1.42	1.04	0.91	0.00	0.00	0.67	1.24	1.83	0.83
sucrose30	3.02 ^{abcd}	0.93	1.71 ^{de}	1.23	2.08	1.23	0.54	0.86	4.00	4.44	1.17	1.12
sucrose40	3.35 ^{abc}	1.01	2.67 ^{abcde}	1.49	3.00	1.44	1.29	1.33	3.21	3.66	1.33	0.65

b

SMM	Shoot size		NoShoot		Callus		Root		NoNode		Ramification	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
fructose10	1.10 ^{cde}	0.31	3.38 ^{bcdef}	1.47	0.83	0.82	0.00	0.00	0.00	0.00	0.00	0.00
fructose15	1.37 ^{bcd}	0.34	4.46 ^{ab}	1.38	1.21	1.14	0.00	0.00	0.00	0.00	0.00	0.00
fructose30	2.43 ^a	0.63	4.95 ^a	2.13	2.96	1.46	0.00	0.00	0.00	0.00	0.00	0.00
fructose40	1.65 ^b	0.59	3.00 ^{bcdefgh}	1.35	2.96	1.57	0.04	0.20	0.00	0.00	0.00	0.00
glucose10	1.07 ^{cde}	0.24	3.29 ^{bcdefg}	1.37	0.71	0.91	0.00	0.00	0.00	0.00	0.00	0.00
glucose15	1.33 ^{bcd}	0.56	3.75 ^{abcd}	1.15	0.96	0.95	0.00	0.00	0.00	0.00	0.00	0.00
glucose30	2.20 ^a	0.72	3.77 ^{abcd}	1.69	2.63	1.56	0.00	0.00	0.00	0.00	0.00	0.00
glucose40	1.42 ^{bc}	0.59	1.75 ^h	1.22	3.50	1.56	0.00	0.00	0.00	0.00	0.00	0.00
maltose10	1.02 ^{cde}	0.22	2.21 ^{efgh}	0.88	0.04	0.20	0.00	0.00	0.00	0.00	0.00	0.00
maltose15	1.16 ^{cde}	0.32	2.50 ^{cdefgh}	1.29	0.17	0.38	0.00	0.00	0.00	0.00	0.00	0.00
maltose30	1.31 ^{bcde}	0.44	4.10 ^{abc}	1.87	0.08	0.28	0.00	0.00	0.00	0.00	0.00	0.00
maltose40	1.39 ^{bcd}	0.52	4.13 ^{abc}	2.47	0.38	0.50	0.00	0.00	0.00	0.00	0.00	0.00
sorbitol10	0.81 ^e	0.21	1.86 ^{fgh}	1.04	0.14	0.35	0.00	0.00	0.00	0.00	0.00	0.00
sorbitol15	0.88 ^{de}	0.30	1.81 ^{gh}	0.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
sorbitol30	0.84 ^{de}	0.18	2.32 ^{defgh}	1.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
sorbitol40	0.79 ^e	0.21	2.00 ^{efgh}	0.93	0.06	0.25	0.00	0.00	0.00	0.00	0.00	0.00
sucrose10	1.07 ^{cde}	0.46	2.33 ^{defgh}	1.24	0.50	0.66	0.00	0.00	0.00	0.00	0.00	0.00
sucrose15	1.28 ^{bcde}	0.43	3.08 ^{bcdefgh}	1.10	0.92	1.06	0.00	0.00	0.00	0.00	0.00	0.00
sucrose30	2.25 ^a	0.75	3.63 ^{abcde}	1.56	2.42	1.38	0.00	0.00	0.00	0.00	0.00	0.00
sucrose40	1.37 ^{bcd}	0.62	2.63 ^{cdefgh}	1.66	2.50	1.72	0.00	0.00	0.00	0.00	0.00	0.00

Table 2 Results of HSD tests on the shoot size and number (NoShoot) relative to the sugar type and concentration for six genotypes (*Rosa* GUY SAVOY® 'Delstrimen', *Rosa* PIMPRENELLE® 'Deldog', *Rosa chinensis* 'Old Blush', *Rosa* 'Félicité et Perpétue', *Rosa* 'Natal Briar' and *Rosa* 'White Pet' during the clonal cycle on SMM. Means of Shoot size and NoShoot that are not connected by the same letter (column Groups) are significantly different at 0.05 probability level.

***Rosa* GUY SAVOY® ('Delstrimen')**

Shoot size			NoShoot		
Groups	Treatments	means	Groups	Treatments	means
a	fructose10	2.75	a	fructose15	6.423
ab	glucose10	2.361	ab	fructose10	5.545
ab	fructose15	2.308	abc	glucose30	5.208
abc	sorbitol10	2.264	abcd	fructose30	5.125
abc	glucose40	2.258	abcde	maltose10	5
abc	glucose30	2.233	abcde	sorbitol10	4.818
abc	sucrose10	2.227	abcde	glucose15	4.8
abc	glucose15	2.204	abcde	sorbitol15	4.538
abc	sucrose15	2.112	bcde	maltose30	4.417
abcd	maltose15	2.104	bcde	glucose10	4.217
abcd	maltose10	2.077	bcde	maltose15	3.917
bcd	sorbitol15	1.9	bcde	sucrose30	3.875
bcd	fructose30	1.838	bcde	sucrose15	3.692
bcd	maltose30	1.821	bcdef	sucrose10	3.591
bcd	sucrose40	1.762	bcdef	sorbitol30	3.5
bcde	sucrose30	1.638	cdef	glucose40	3.375
bcde	fructose40	1.621	def	maltose40	3.174
cde	maltose40	1.557	ef	fructose40	3.125
de	sorbitol30	1.364	ef	sucrose40	3.042
e	sorbitol40	0.9375	f	sorbitol40	1.708

***Rosa* PIMPRENELLE® ('Deldog')**

Shoot size			NoShoot		
Groups	Treatments	means	Groups	Treatments	means
a	glucose15	1.912	a	sucrose30	2.714
a	fructose10	1.86	ab	glucose30	2.571
ab	glucose30	1.786	abc	fructose30	2.286
ab	fructose15	1.75	abc	fructose40	2.286
ab	sucrose10	1.73	abc	fructose15	2.25
ab	fructose30	1.714	abc	maltose30	2.143
ab	maltose30	1.643	abc	sucrose40	1.857
ab	glucose10	1.63	abc	glucose40	1.714
ab	glucose40	1.629	abc	maltose40	1.571
ab	sucrose40	1.571	abc	glucose10	1.5
ab	sucrose15	1.475	bc	fructose10	1.4
ab	maltose15	1.457	bc	glucose15	1.375
ab	sorbitol10	1.412	bc	sucrose15	1.375
ab	sucrose30	1.357	bc	maltose15	1.286
ab	maltose10	1.35	c	sorbitol10	1.25
ab	fructose40	1.2	c	sorbitol30	1.143
ab	sorbitol15	1.071	c	sorbitol40	1.143
ab	maltose40	1.014	c	maltose10	1.125
b	sorbitol30	0.9	c	sucrose10	1.1
b	sorbitol40	0.9	c	sorbitol15	1

***Rosa chinensis* 'Old Blush'**

Shoot size			NoShoot		
Groups	Treatments	means	Groups	Treatments	means
a	sucrose30	1.633	a	fructose30	4.167
ab	fructose30	1.442	ab	sucrose30	3.5
abc	fructose40	1.333	abc	glucose30	3.167
abc	sucrose10	1.292	abcd	maltose30	3.083
abc	glucose15	1.25	abcde	glucose40	2.917
abc	glucose30	1.208	abcde	sucrose40	2.917
abc	fructose10	1.192	bcdef	fructose40	2.5
abc	maltose30	1.175	bcdef	maltose40	2.5
abc	fructose15	1.142	cdef	sorbitol40	1.75
abc	sucrose40	1.125	def	glucose10	1.583
abc	maltose15	1.05	def	glucose15	1.583
bc	glucose10	0.9917	def	maltose15	1.583
bc	maltose10	0.9917	ef	fructose15	1.417
bc	glucose40	0.9833	ef	sorbitol30	1.417
bc	sorbitol30	0.9417	f	sorbitol15	1.25
bc	sucrose15	0.875	f	fructose10	1.167
bc	maltose40	0.8667	f	maltose10	1.167
bc	sorbitol10	0.8333	f	sucrose10	1.083
bc	sorbitol40	0.8167	f	sorbitol10	1.001
c	sorbitol15	0.8	f	sucrose15	1.001

***Rosa* 'Félicité et Perpétue'**

Shoot size			NoShoot		
Groups	Treatments	means	Groups	Treatments	means
a	glucose40	1.667	a	glucose30	3.083
a	fructose30	1.533	a	fructose15	3
ab	glucose30	1.367	a	fructose30	2.917
ab	fructose40	1.356	a	maltose30	2.364
ab	maltose40	1.273	a	glucose15	2.167
ab	glucose15	1.1	a	glucose40	2.167
ab	fructose15	1.083	a	fructose40	2.111
ab	maltose30	1.027	a	sorbitol15	2
ab	sucrose30	1	a	sucrose10	2
ab	sucrose10	0.9889	a	sucrose40	2
ab	sorbitol30	0.9556	a	maltose40	1.818
ab	maltose15	0.8545	a	sucrose30	1.571
ab	sorbitol15	0.8125	a	sorbitol30	1.556
b	sorbitol40	0.6545	a	maltose15	1.545
b	sucrose40	0.6333	a	sorbitol40	1.455
b	sucrose15	0.4714	a	sucrose15	1.143

***Rosa* 'Natal Briar'**

Shoot size			NoShoot		
Groups	Treatments	means	Groups	Treatments	means
a	glucose30	1.456	a	fructose30	7.778
ab	fructose10	1.259	ab	glucose15	6.684
ab	glucose15	1.163	abc	fructose10	5.529
abc	fructose15	1.116	abcd	glucose30	5.167
abc	glucose10	1.082	abcd	maltose40	5.167
abc	fructose30	1.039	bcd	glucose10	5
abc	fructose40	1.039	bcde	fructose15	4.842
abc	maltose10	1.035	bcde	fructose40	4.778
abc	maltose15	1.028	bcde	maltose30	4.389
abc	maltose30	1.022	bcdef	maltose10	4.176
abc	sucrose30	0.9611	bcdef	maltose15	4.167
abc	sorbitol10	0.9412	bcdef	sucrose40	4.167
abc	glucose40	0.9235	cdef	sucrose30	3.833
abc	sucrose40	0.9	cdef	sucrose15	3.737
abc	maltose40	0.8778	cdef	glucose40	3.471
abc	UK40	0.8667	cdef	sorbitol10	3.412
abc	sorbitol15	0.8158	cdef	sucrose10	3.353
bc	sucrose15	0.7895	cdef	UK40	3.333
bc	sorbitol30	0.7278	def	sorbitol15	2.684
bc	sucrose10	0.7	def	UK15	2.667
bc	UK30	0.6	def	UK10	2.333
bc	UK15	0.5	def	UK30	2.333
c	sorbitol40	0.4944	ef	sorbitol30	2.222
c	UK10	0.3333	f	sorbitol40	1.444

***Rosa* 'White pet'**

Shoot size			NoShoot		
Groups	Treatments	means	Groups	Treatments	means
a	glucose40	1.892	a	maltose30	4.222
ab	fructose40	1.627	ab	glucose40	4
ab	glucose30	1.673	abc	fructose40	3.091
abc	fructose30	1.28	bcd	fructose30	2.5
abc	maltose30	1.344	cd	glucose30	2
abc	maltose40	1.42	cd	maltose40	1.9
bc	sucrose40	0.9182	cd	sorbitol30	2.111
c	sorbitol30	0.7556	cd	sucrose30	2.125
c	sucrose30	0.675	d	sucrose40	1.455

Table 3 Optimal conditions to obtain the best percentage of DNSO for each tested genotype.

Suc1 corresponds to the sugar type and concentration during the clonal cycle (*Suc*: sucrose, *fruc*: fructose; 30: 30 g.L⁻¹)

Medium corresponds to the medium for DNSO

Suc2 corresponds to the sugar type and concentration in DNSO medium

(*Fruc*: fructose, *Gluc*: glucose, *Suc*: sucrose; 15: 15 g.L⁻¹, 30: 30 g.L⁻¹, 40: 40 g.L⁻¹)

Rw: *Rosa wichurana*, *GS*: *Rosa GUY SAVOY*® ('*Delstrimen*'), *OB*: *Rosa chinensis* 'Old Blush', *FP*: *Rosa* 'Félicité et Perpétue', *NB*: *Rosa* 'Natal Briar', *WP*: *Rosa* 'White Pet'.

	Suc1	Medium	Suc2	% DNSO
		SRM1	Fruc15, Gluc30, Suc15	100
Rw	Suc30	SRM3	Suc15	100
		SRM6	Fruc40	100
GS	Suc30	SRM3	Fruc30	56.67
OB	Fruc30	SRM3	Fruc40	74
FP	Suc30	SRM1	Fruc30	37.5
NB	Fruc15	SRM1	Gluc30	87.5
WP	Suc30	SRM1	Gluc15	100
		SRM3	Gluc30	100