Final published version can be accessed at https://doi.org/10.1007/s11240-020-01820-7

1 **Assessments of rooting, vegetative growth, bulb production, genetic**
2 **integrity and biochemical compounds in cryopreserved plants of**
3 **shallot**

4

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21 *Key message* Rooting, vegetative growth, bulb production, genetic stability
22 and biochemical compounds were maintained in cryopreserved plants of
23 shallot. Our results support use of cryopreservation for long-term
24 preservation of shallot germplasm.

25

26 Abstract
27 Shallot (*Allium cepa* var. *aggregatum*), a small bulb onion, is widely grown
28 in the world. We previously reported a droplet-vitrification for
29 cryopreservation of *in vitro*-grown shoot tips of shallot genotype ‘10603’.
30 The present study further evaluated rooting, vegetative growth, bulb
31 production and contents of biochemical compounds in bulbs, as well as
32 genetic stability in cryo-derived plants. The results showed no significant
33 differences in rooting, vegetative growth, bulb production and contents of
34 soluble sugars and flavonols between the cryo- and *in vitro*-derived plants.
35 Analyses of ISSR and AFLP markers did not detect any polymorphic bands
36 in the cryo-derived plants. These results indicate rooting and vegetative
37 growth ability, biochemical compounds and genetic stability were
38 maintained in cryo-derived plants. The present study provides
39 experimental evidences that support the use of cryopreservation method
40 for long-term preservation of genetic resources of shallots and other *Allium*
41 species.

42

43 Keywords Biochemical compounds • Cryopreservation • Genetic
44 stability • Rooting • Shallot • Vegetative growth

45

46 Abbreviations

47 AFLP Amplified fragment length polymorphism

48 6-BP 6-benzylaminopurine,

49 ISSR Inter-simple sequence repeat

50 LN Liquid nitrogen

51 LNV Liquid nitrogen vapor

52 MS Murashige and Skoog (1962)

53 NAA 1-naphthylacetic acid

54 PCO Principal Coordinate Analysis

55 SPMM Stock plant maintenance medium

56 UPGMA Unweighted pair group method with arithmetic mean

57

58

59 Introduction

60 Shallot (*Allim cepa* var. *aggregatum*) is a small-bulb onion and mainly
61 cultivated in Europe, South America and Asia (Fritsch and Friesen 2002;
62 Rabinowitch and Kamenetsky 2002). Shallot leaves and bulbs are

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63 consumed, due to their milder and sweeter taste than common onions, and
64 high levels of biochemical compounds such as flavonoids and polyphenols,
65 which have antioxidant and anti-fungal activities (Yang et al. 2004;
66 Leelarungrayub et al. 2006; Ferioli and D'Antuono 2016; Sittisart et al.
67 2017).

68 Cryopreservation is at present time considered a favorite method for the
69 long-term storage of plant genetic resources (Li et al. 2018; Wang et al.
70 2018). Theoretically, once cryopreserved, cellular divisions and metabolic
71 processes of the stored samples cease, and thus plant materials can be
72 preserved for long durations, while maximally maintaining their genetic
73 stability (Harding 2004; Benson 2008).

74 For vitrification-based cryopreservation of *in vitro*-grown shoot tips, an
75 entire cryopreservation procedure involves several major steps including
76 establishment of *in vitro* stock cultures, shoot tip preculture, exposure of
77 shoot tips to plant vitrification solution (PVS), storage in liquid nitrogen
78 and finally *in vitro* post-thaw culture for recovery (Sakai et al. 2008). *In*
79 *vitro* culture has risks of inducing genetic variations in *in vitro* regenerants
80 (Bednarek and Orłowska 2020). Preculture and exposure to PVS impose
81 osmotic and chemical stresses to the samples, and consequently may
82 induce genetic or morphological variations in cryo-derived regenerants

83 (Harding 2004; Benson 2008). Therefore, assessments of genetic stability
84 in cryo-derived plants are necessary (Harding 2004; Benson 2008).
85 Molecular markers like inter-simple sequence repeat (ISSR) and
86 amplified fragment length polymorphism (AFLP) have been widely used
87 to assess genetic stability in cryo-derived regenerants of many plant species
88 (Wang et al. 2014a, b, 2017, 2018; Li et al. 2015; Zhang et al. 2015; Bi et
89 al. 2016). However, such studies have been quite limited in cryo-derived
90 regenerants of *Allium* spp. (Liu et al. 2017). Evaluations of field
91 performance in cryo-derived plants provided valuable information for use
92 of cryopreservation for establishment of cryo-banks (Salama et al. 2018).
93 In addition, analysis of the biochemical compounds is also necessary in the
94 cryopreserved plants that have culinary and medical values (Ahuja et al.
95 2002, Bi et al. 2016).
96 In the genus *Allium*, cryopreservation has been well-established for
97 garlic (Keller 2002, 2005; Keller et al. 2011; Ellis et al. 2006; Kim et al.
98 2006, 2007; Liu et al. 2017), while quite a few addressed shallot (Kim et
99 al. 2007). We previously reported a droplet-vitrification for efficient
100 cryopreservation of shallot shoot tips (Wang et al. 2019, 2020). The present
101 study was, therefore, to further assess rooting, vegetative growth, bulb
102 production, genetic stability and contents of soluble sugars and flavonols
103 in cryo-derived plants. Results reported here support use of the droplet-

104 vitrification method described by Wang et al. (2019, 2020) for
105 establishment of cryo-banks of shallot germplasm.
106
107 Material and Methods
108 Preparation of cryo-derived regenerants
109 *In vitro* stock cultures of shallot genotype '10603' (*A. cepa* var. *aggregatum*)
110 were maintained on a stock culture medium (SCM) composed of
111 Murashige and Skoog (1962) medium (MS) supplemented with 30 g/l
112 sucrose, 0.5 mg/l 6-benzylaminopurine (6-BA), 0.1 mg/l 1-naphthylacetic
113 acid (NAA) and 8 g/l agar (pH, 5.8). The cultures were grown at a constant
114 temperature of 22 ± 2 °C under a 16-h photoperiod of a light intensity of
115 50 μmol $s^{-1}m^{-2}$ provided by cool-white fluorescent tubes. Subculturing was
116 conducted once every 4 weeks. Shoot tips (2-3 mm in length) with 4-5 leaf
117 primordia (LPs) were taken from 4-week old *in vitro* stock cultures and
118 used for droplet-vitrification, as described by Wang et al. (2020). Briefly,
119 shoot tips were precultured, loaded and exposed to PVS3 (Nishizawa *et al.*,
120 1993) at 24 °C for 3 h. At the end of exposure to PVS3, each of shoot tips
121 was moved into 5 μl PVS3 droplets made on aluminum foils (2 x 0.8 cm),
122 followed by direct immersion in LN for storage for 1 h. Following thawing,
123 cryopreserved shoot tips were post-thaw cultured on SCM in the light
124 conditions for recovery. Shoots with roots developed after 8 weeks of post-

125 thaw culture. Subculture was conducted once every 4 weeks. Regenerants
126 recovered from cryopreserved shoot tips were designed as cryo-derived
127 samples, while those from non-cryopreserved shoot tips as *in vitro*-derived
128 samples (control).
129
130 Assessments of rooting, vegetative growth and bulb production
131 Rooting and vegetative growth were assessed in cryo- and *in vitro*-derived
132 plantlets cultured *in vitro*. After 16 weeks (4 rounds of subculture) of post-
133 thaw culture following cryopreservation, these two types of plantlets were
134 transferred onto new SMM. Root number and length of the longest root,
135 leaf number and length of the longest leaf, and number of proliferating
136 shoots were measured after 4 weeks of culture.
137 Vegetative growth and bulb production were measured in cryo- and *in*
138 *vitro*-derived plantlets grown in greenhouse conditions. For preparation of
139 greenhouse-grown plants, both cryo-derived and *in vitro*-derived plantlets
140 were transferred into 9-cm pots containing soil substrates (PINDSTRUP,
141 Ryomgaard, Denmark) on black plastic trays. The cultures were covered
142 with white plastic bags to maintain high humidity and prevent the shoots
143 from wilting, and grown in a greenhouse set at a constant temperature of
144 22 ± 2 ºC under an 18-h photoperiod of light intensity of 200 μmol m^{-2} s^{-1}
145 provided by cool-white fluorescent tubes. The bags were gradually

146 uncovered to reduce the humidity and removed totally after 1 week of
147 culture. Regular managements, including watering, fertilizing and pest
148 control, were applied to the greenhouse-grown plants. Number of leaves,
149 length of the longest leaf and number of the dividing plants were measured
150 after 3 months of growth. Bulb production was recorded after 5 months of
151 growth. After fresh weight measurement, bulbs were quartered vertically
152 and dried at 102 °C for 48 h to measure bulb dry weight.

153

154 Assessments of genetic integrity

155 Cryo- and *in vitro*-derived plants were grown in the greenhouse for 3
156 months, and then leaves were taken and used for assessments of genetic
157 stability using inter-simple sequence repeat (ISSR) and amplified fragment
158 length polymorphism (AFLP) molecular markers, as described below.

159

160 DNA extraction

161 Total DNA was extracted from the leaves (50-100 mg) using DNeasy Plant
162 Mini Kit (Qiagen GmbH, Hilden, Germany), according to manufacturer's
163 instructions.

164

165 ISSR analysis

166 ISSR was performed as described by Zhang et al. (2015). Eight primers
167 were selected from 20 candidate primers, based on the number of amplified
168 fragments in the range 500-2000 base pairs. PCR for DNA amplification
169 was performed in a 25-μL reaction solution containing 2.5 μL 10×PCR
170 buffer, 0.2 μL (1 U) Taq polymerase (Roche, Indiana, IN), 0.5 μL dNTP
171 (10 mM), 0.5 μL primer (100 μM), and 1 μL template DNA (100 ng/μL).
172 The PCR products were separated by electrophoresis in 2% (w/v) agarose
173 gel containing 0.1% (w/v) ethidium bromide and visualized under
174 ultraviolet light. The molecular 1-kb DNA ladder (New England BioLabs
175 Inc, Ipswich, UK) were used for estimating the size of the amplified
176 products. ISSR fingerprints were scored for the presence and the absence
177 of each band. Only clear monomorphic and polymorphic bands were
178 scored, while those with low visual intensity were not scored.
179
180 AFLP analysis
181 AFLP was conducted, according to Zhang et al (2015). Breifly, genomic
182 DNA (300 ng) was double-digested with EcoRI and the MseI isoschizomer
183 Tru1I. Following ligation of the restriction fragments to the adaptors, pre-
184 amplification PCR was carried out with non-selective primers in a total
185 volume of 25 μL, containing 5 μL of five-fold diluted ligation product. The
186 fluorescently labeled PCR products were analyzed, according to Zhang et

187 al. (2015). The data was collected using the software Data Collection v2.0
188 (Applied Biosystems), while GeneMapper v4.1 (Applied Biosystems) was
189 used to derive the fragment length of the labeled DNA fragments using the
190 known fragment lengths of the LIZ-labeled marker peaks. AFLP profiles
191 were manually scored for the presence and the absence of each band. Only
192 clear distinct monomorphic and polymorphic bands were scored, while
193 those showing low visual intensity were not scored.

194

195 Analysis of biochemical compounds

196 Bulbs were harvested from cryo- and *in vitro*-derived plants that had been
197 grown in the greenhouse for 5 months. Fresh bulbs were grounded into fine
198 powder and used for analysis of soluble sugars and flavonols.

199

200 Analysis of soluble sugars

201 Analysis of soluble sugars was conducted, according to Vågen and
202 Slimestad (2008). Briefly, 10 mg $CaCO_3$ were added to 15 mL centrifuge
203 tube containing 1 g sample powders. The samples were extracted twice
204 with 5 mL 80% ethanol at 75 °C for 20 min. Thereafter, the residue was
205 extracted twice with 2 mL of water at 75 °C for 10 min. The supernatant
206 was pooled from each extraction, with the volume adjusted to 14 mL, and
207 then filtrated through a 0.45 μm HPLC certified syringe filter for

208 measurement of the soluble sugars using an HPLC-instrument (Agilent
209 1100, Agilent Technologies) equipped with an evaporative light-scattering
210 detector (ELSD 800, Alltech). Separation was achieved by use of a Prevail
211 Carbohydrate ES-column (250 x 4.6 mm, 5 μm, Grace) and a gradient of
212 increasing amount of water in acetonitrile. Standards of fructose, glucose
213 and sucrose provided by Sigma (Sigma, Oslo, Norway) were used for
214 quantitative calculation of soluble sugar contents.
215
216 Analysis of flavonols
217 Analysis of flavonols was conducted, according to Vågen and Slimestad
218 (2008). In brief, 2 g sample powders were transferred into tubes and
219 extracted with 5 mL 0.5% methanol for 48 h at 4 °C. After centrifugation
220 at 1500 rpm for 10 min, supernatant was collected and passed through 0.45
221 μm filters and analyzed by UHPLC-DAD-MS (Agilent 1290 and 6120,
222 Agilent Technologies). Separation was achieved by gradient elution
223 (acetonitrile and 0.01% formic acid) and reversed-phase chromatography
224 (Zorbax Eclipse XDB-C8, 2.1 x 100 mm, 1.8 μm, Agilent Technologies).
225 (need instrument information). In-house standards of que, 3,4'-diglc and
226 que 4'-glc were used for quantitative calculation of flavonol contents.
227
228 Experimental design and statistical analysis of data

229 All experiments of measurements of rooting, vegetative growth and bulb
230 production were conducted in a complete random design. Ten samples
231 were included in every treatment of three replicates in two independent
232 experiments. Twenty-four plants were randomly selected from 60 cryo-
233 derived plants and 24 plants from 100 *in vitro*-derived plants, and used in
234 experiments of ISSR and AFLP. The experiments were repeated twice to
235 confirm their repeatability. Bulbs were harvested from each of 10 cryo- and
236 *in vitro*-derived plants, and used for analysis biochemical compounds.
237 Each experiment contained 3 biological replicates and repeated twice.
238 Significant differences of data from different treatments were analyzed by
239 Student's *t*-test ($P<0.05$).
240

241 ## Results

242 ### Rooting and shoot growth in *in vitro* plantlets

243 Overall rooting and shoot growth of *in vitro*-cultured plantlets were similar
244 between the cryo- and *in vitro*-derived plantlets (Fig. 1a). All parameters
245 measured, including the number of roots, length of the longest root, the
246 number of leaves, length of the longest leaf and number of proliferating
247 shoots, were similar between the cryo- and *in vitro*-derived plantlets (Table
248 1).
249

250 Vegetative growth and bulb production in greenhouse-grown plants

251 Both cryo- and *in vitro*-derived plants were easily re-established in
252 greenhouse conditions, with more than 95% survival obtained for the two
253 types of plants. Overall growth of greenhouse-grown plants was similar
254 between the cryo- and *in vitro*-derived plants (Fig. 1b). The number of
255 leaves, length of the longest leaf and the number of dividing plants were
256 not significantly different between the cryo-derived plants and *in vitro*-
257 derived ones (Table 1). Similarly, no significantly differences were found
258 in bulb number and size, bulb yield and dry weight between the cryo-
259 derived plants and *in vitro*-derived ones (Fig. 1c, Table 2).

260

261 Assessments of genetic stability

262 ISSR analysis

263 Clear and reproducible bands were produced in all the 8 primers selected,
264 with their sizes ranging from 500 to 2000 bp (Fig. 2). Each primer produced
265 an mean of 6.1 bands, with 49 monomorphic bands obtained in the 8
266 primers (Table 3). A total of 1176 bands (number of bands/primer × number
267 of primers used × number of plants tested) were generated in the 24 cryo-
268 derived plants. No polymorphic bands were detected in all cryo-derived
269 plants tested (Table 3).

270

271 AFLP analysis

272 Clear and reproducible bands were produced in all the 5 primer
273 combinations selected (Fig. 3). Each primer combination produced a mean
274 of 65.8 monomorphic bands, with 329 clear monomorphic bands obtained
275 in the 5 primer combinations per plant (Fig. 3, Table 4). A total of 7896
276 bands (number of bands/primer combination× number of primer
277 combinations used × number of plants tested) were generated in cryo-
278 derived plants. No polymorphic bands were found in all samples analyzed
279 by the five primer combinations (Table 4).

280

281 Analysis of soluble sugars and flavonols

282 Three soluble sugars including fructose, glucose and sucrose were
283 analyzed in bulbs and compared between cryo-derived plants and *in vitro*-
284 derived ones. No significant differences were found in contents of fructose,
285 glucose and sucrose, as well as total soluble sugars in the bulbs harvested
286 between the two types of plants (Table 5). Three major flavonols including
287 Que, Que 3,4-Diglc and Que 4-Glc were analyzed in bulbs and compared
288 between cryo-derived and *in vitro*-derived plants. No significant
289 differences were found in contents of Que, Que 3,4-Diglc and Que 4-Glc,
290 as well as total flavonols in the bulbs harvested between the two types of
291 plants (Table 5).

292

293 ## Discussion

294 In the present study, rooting, vegetative growth, bulb production, genetic
295 integrity and biochemical compounds were compared between cryo- and
296 *in vitro*-derived plants of shallot genotype '10603'. No significant
297 differences were observed in rooting and vegetative growth between the
298 cryo- and *in vitro*-derived plantlets cultured *in vitro*. Similar results of
299 vegetative growth and bulb production were found in the two types of
300 plants grown in the greenhouse. Analyses of ISSR and AFLP did not detect
301 any polymorphic bands in the cryo-derived plants grown in greenhouse.
302 Contents of soluble sugars including fructose, glucose and sucrose, and
303 flavonols were similar in the bulbs produced in the cryo- and *in vitro*-
304 derived plants. All these data indicate that the droplet-vitrification
305 cryopreservation method can be considered safe for the long-term
306 preservation of shallot genetic resources. To the best of our knowledge, this
307 is the most empirical study on assessments of rooting, vegetative growth,
308 bulb production, genetic stability, and biochemical compounds in cry-
309 derived plants so far reported in cryopreserved *Allium* or bulbous? plants.

310 Although there have been several studies assessing field performance of
311 cryo-derived plants, quite few focused in *Allium*. Bi et al. (2016) found that
312 the vegetative growth, morphologies and flower production were

313 maintained in the cryo-derived plants of *Chrysanthemum morifolium*
314 'Hangju'. Similar results were also reported in cryo-derived plants such as
315 *Dioscorea floribunda* (Ahuja et al. 2002), *Musa* (Agrawal et al. 2004) and
316 *Carica papaya* (Kaity et al. 2009). These results were consistent with ours.
317 However, there existed a few studies, in which field behavior of cryo-
318 derived plants differed from that of the control. Working on
319 *Argyranthemum*, Zhang et al. (2015) reported that root formation and plant
320 growth were reduced in the cryo-derived plants grown in the greenhouse,
321 but morphologies of the leaves and flowers remained unchanged. Harding
322 and Staines (2001) detected differences in plant height, leaf morphologies,
323 tuber size and weight between cryo-derived plants and the control in potato.
324 Recently, applying encapsulation-dehydration cryopreservation, Kulus at
325 el. (2019) reported there were significant differences in vegetative growth
326 including shoot length, internode length and leaf number between cryo-
327 derived plants and the control in chrysanthemum. But, the difference was
328 genotype-dependent. They further found no differences in flower
329 production including flower colour and diameter, fresh weight of
330 inforescences and length of ray forets between the two types of plants.
331 Therefore, differences in field behavior between cryo-derived plants and
332 the control may be attributed to plant species/genotypes, cryoprocedures
333 and *in vitro* culture system.

334 Studies of assessments of genetic stability in cryopreserved *Allium*
335 plants have been quite limited. Using SSR, Liu et al. (2017) did not detect
336 any polymorphic bands in the cryo-derived garlic plants. Since different
337 DNA makers are responsible for detecting polymorphisms in different
338 genomic regions, use of more than one molecular marker method certainly
339 produces more dependable results than those using only one marker (Wang
340 et al. 2014a, b, 2018). ISSR and AFLP did not detect any polymorphic
341 bands in *Solanum tuberosum* plants cryopreserved by droplet-vitrification,
342 encapsulation-vitrification and vitrification (Wang et al. 2014b; Li et al.
343 2017). Maintenance of genetic stability in cryopreserved plants has been
344 reported in various plants such as *Argyranthemum* (Zhang et al., 2015),
345 *Malus* (Li et al. 2015), *Chrysanthemum* (Bi et al. 2016), blue berries (Wang
346 et al. 2017), *Vitis* (Bi et al. 2018) and *Asparagus officinalis* (Carmona-
347 Martín et al. 2018). Similar results were obtained in the present study,
348 indicating that genetic stability is maintained in cryo-derived plants of
349 shallots. Indeed, polymorphic bands were detected by RAPD and ISSR in
350 chrysanthemum 'Lady Orange' (7.8%) and 'Lady Salmon' plants (3.2%),
351 but not in 'Richmond' plants recovered from encapsulation-dehydration
352 cryopreservation. Genomic variations at about 5% were detected in *Mentha*
353 × *piperita* plants following encapsulation-dehydration cryopreservation
354 (Martín et al. 2015) and *Hladnikia pastinacifolia* plants following

355 encapsulation-dehydration and encapsulation-vitrification (Ciringer et al.
356 2018). These data indicate that genetic variations in cryo-derived plants
357 may be affected by cryoprocedures and plant species/genotypes.

358 For plants that have culinary and medical values including shallots,
359 analysis for biochemical compounds is of great importance. No significant
360 differences in diosgenin contents were detected between cryopreserved
361 plants and the control in *Dioscorea floribunda* (Ahuja et al. 2002). Similar
362 levels of five major biochemical compounds were found in the flowers
363 harvested from the cryopreserved plants and the control in *Chrysanthemum*
364 *morifolium* 'Hangju' (Bi et al. 2016). Similarly, no differences in the level
365 of anthocyanins and carotenoids were detected in the flowers of cryo-
366 derived plants of chrysanthemum (Kulus et al. 2019). Soluble sugars and
367 flavanols are among the major biochemical compounds in shallots
368 (Slimestad et al. 2007; Vågen and Slimestad 2008). Analysis by HPLC-
369 ELSD for soluble sugars and by UHPLC-DAD-MS for flavanols found no
370 significant differences in greenhouse-grown plants that derived from
371 cryopreservation and *in vitro* culture. These results indicate that the
372 droplet-vitrification cryopreservation maintains contents of the
373 biochemical compounds in shallots.

374 In conclusion, results obtained in the present study indicate that no
375 significant differences were detected in rooting, vegetative growth, bulb

376 production and levels of biochemical compounds in bulbs between the
377 cryo-derived plants and *in vitro*-derived ones. Genetic stability analyzed
378 by ISSR and AFLP was maintained in the cryopreserved plants. The results
379 reported in the present study provides experimental evidences that support
380 use of cryopreservation method for long-term preservation of genetic
381 resources of shallots and other members of *Allium* species.

382

383 Acknowledgements
384 We acknowledge financial supports from the Research Council of Norway
385 (Project No. 255032/E50), NIBIO, Sagaplant, Gartnerhallen,
386 Gartnerforbundet, and Landbruksdirektoratet/the Norwegian Genetic
387 Resource Centre (project No. 18/4272).

388

389 Author contributions

390 M-R Wang: performance of experiments of cryopreservation, rooting and
391 vegetative growth, collection and analysis of data, and preparation of
392 manuscript; Z Zhang: performance of experiments of genetic stability and
393 biochemical compounds, collection and analysis of data and preparation of
394 manuscript; R Slimestad: analysis of biochemical compounds; A Elameen:
395 performance of assessments of genetic stability, valuable discussion and
396 revision of manuscript; Dag-Ragnar Blystad: chief scientist of the project,

397 valuable discussion and providing financial supports; S Haugslien:
398 maintenance of *in vitro* cultures, and assistance to experiments of
399 cryopreservation and evaluations of *in vitro* rooting and vegetatitive
400 growth; G Skjeseth: maintenance of greenhouse-grown plants and
401 evaluations of vegetative growth in greenhouse-grown plants; Q-C Wang:
402 experimental design, analysis of data, and revision and editing of
403 manuscript.

404

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Final published version can be accessed at https://doi.org/10.1007/s11240-020-01820-7

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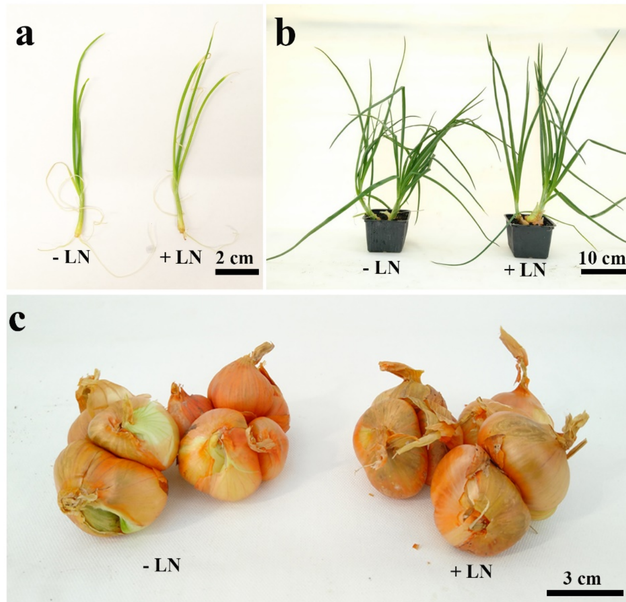

Fig. 1 Vegetative growth and bulb production in cryo-derived (+LN) and *in vitro*-derived plants (-LN) of shallot genotype '10603'. Vegetative growth of *in vitro* plantlets after 4 weeks of culture **(a)** and greenhouse-grown plants after 3 months of growth **(b)**. Bulbs harvested from greenhouse-grown plants after five months of growth **(c)**.

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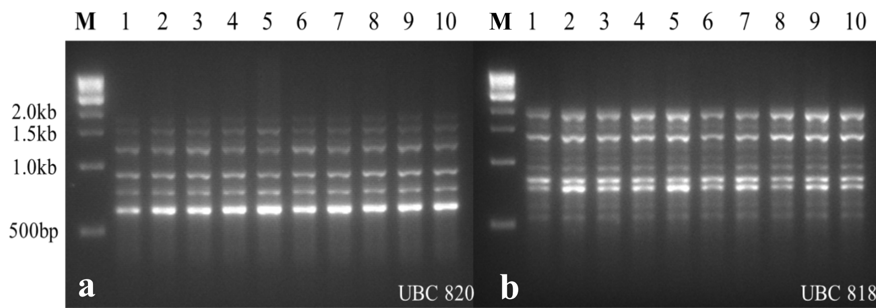

Fig. 2 ISSR banding patterns in cryo-derived and *in vitro*-derived plants of shallot genotype '10603' after 3 months of growth in greenhouse conditions. The ISSR finger prints amplified by the primers UBC820 (a) and UBC 818 (b). M=molecular marker; lanes 1-5=*in vitro*-derived plants; lanes 6-10 = cryo-derived plants.

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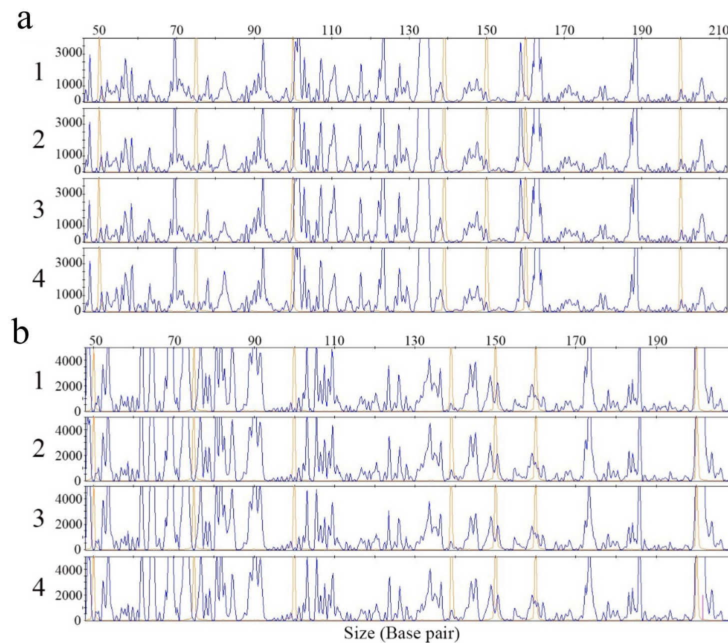

Fig. 3 AFLP patterns (presented by GeneMapper software) of cryo-derived and *in vitro*-derived plants of shallot genotype '10603' after 3 months of growth in greenhouse conditions. AFLP patterns of the primer E19 × M15 (a) and E19 × M16 (b). Numbers 1 and 2= cryo-derived plants. Numbers 3 and 4 = *in vitro*-derived plants.

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Table 1 Comparison in rooting and vegetative growth between cryo-derived and *in vitro*-derived plants of shallot genotype 10603 after 4 weeks of growth in *in vitro* culture conditions and 3 months of growth in greenhouse conditions

Growing conditions and types of plants	Number of roots/plant	Length of the longest root (cm)	Number of leaves/plant	Length of the longest leaf (cm)	Number of shoots/plant
In vitro culture					
Cryo-derived	2.0±0.2a	3.1±0.2a	3.9±0.3a	9.8±0.2a	1.6± 0.1a
In vitro-derived	1.9±0.2a	2.9±0.3a	3.8±0.3a	9.4±0.3a	1.4±0.1a
Greenhouse conditions					
Cryo-derived	-	-	27.5±2.4a	40.2±3.5a	2.8±0.4a
In vitro-derived	-	-	25.6±2.5a	39.6±3.3a	2.2±0.3a

Data are presented as ± standard errors (SE) and with the same letters in the same column indicate no significant differences at $P<0.05$ by student's t-test (n=30).

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Table 2 Comparison in bulb production between cryo-derived and *in vitro*-derived plants shallot genotype 10603 after 5 months of growth in greenhouse conditions

Types of plant	Bulb number/plant	Bulb size (cm)	Bulb yield /plant (g)	Dry weight content (g/100g FW)
Cryo-derived	9.5±0.5a	1.9±0.2a	88.1±5.0a	15.8 ± 0.3a
In vitro-derived	10.1±0.5a	2.1±0.3a	86.6±3.6a	15.7 ± 0.2a

Data are presented as means ± standard errors (SE) and with the same letters in the same column indicate no significant differences at $P<0.05$ analyzed by Student's t-test (n=30).

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Table 3 Names and sequences of the primers used for ISSR analysis, and number of monomorphic bands amplified in cryo-derived plants of shallot genotype 10603 after 3 months of growth in greenhouse conditions

Primer name	Primer sequence (5'-3')	Annealing temperature (ºC)	Number of monomorphic bands/primer	Number of polymorphic bands/primer
UBC 809	$(AG)_8G$	37	7	0
UBC 818	$(CA)_8G$	37	10	0
UBC 820	$(GT)_8C$	37	6	0
UBC 840	$(GA)_8YT$	37	10	0
UBC 857	$(AC)_8YG$	44	4	0
D2	$(GA)_9C$	42	4	0
D3	$(GT)_9C$	42	5	0
3A-37	$(CA)_7TGA$	39	3	0
Average			6.1	0
Total			49	0

Table 4 Names and sequences of the primer combinations used for AFLP analysis, and number of monomorphic bands amplified in cryo-derived plants of shallot genotype 10603 after 3 months of growth in greenhouse conditions.

Primer combination	*Eco*RI primer seuences (5'-3')	*MseI* primer sequences (5'-3')	Number of monomorphic bands /primer combination	Number of polymorphic bands/primer combination
E19 x M15	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACA	70	0
E19 x M16	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACC	76	0
E12 x M17	GAC-TGC-GTA-CCA-ATT-CAC	GAT-GAG-TCC-TGA-GTA-ACG	68	0
E12 x M16	GAC-TGC-GTA-CCA-ATT-CAC	GAT-GAG-TCC-TGA-GTA-ACC	54	0
E19 x M17	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACG	61	0
Average			65.8	0
Total			329	0

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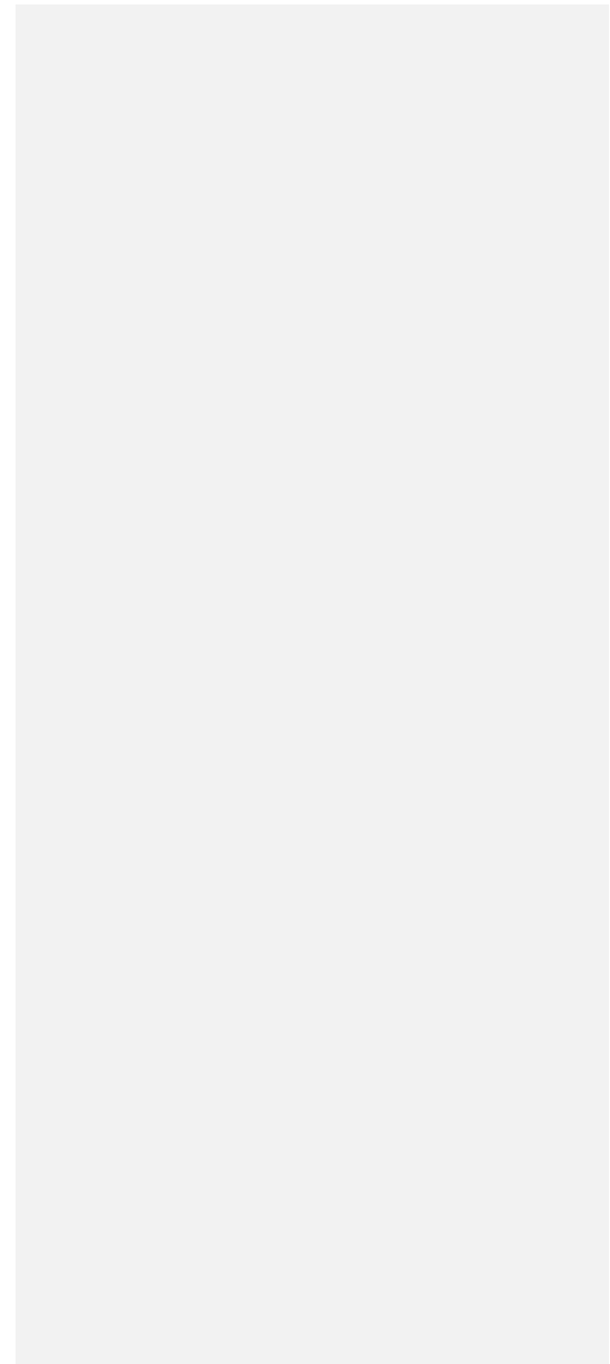

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Table 5 Comparison in contents of soluble sugars and flavonols of bulbs between cryo-derived and *in vitro*-derived plants of shallot genotype 10603 after 5 months of growth in greenhouse conditions

Types of plant	Fructose (g/100 g FW)	Glucose (g/100 g FW)	Sucrose (g/100 g FW)	Total soluble sugar (g/100 g FW)	Que (mg/100g FW)	Que 3,4-Diglc (mg/100g FW)	Que 4-Glc (mg/100g FW)	Flavonols (mg/100g WF)
Cryo-derived	1.69±0.09a	0.14±0.01a	1.43±0.05a	3.26±0.14a	32.2±5.4a	38.4±2.5a	37.2±2.6a	109.3±8.0a
In vitro-derived	1.58±0.09a	0.15±0.00a	1.36±0.05a	3.08±0.13a	37.2±4.4a	34.8±2.5a	43.6±3.9a	110.6±9.7a

Data are presented as means ± standard errors (SE) and with the same letters in the same column indicate no significant differences at $P<0.05$ analyzed by Student's *t*-test (n=~~6~~15).

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