

1 Secondary Somatic Embryogenesis in *Crocus vernus* (L.) Hill

2 Iyyakkannu Sivanesan¹, Sonali Jana², and Byoung Ryong Jeong^{1,2,3*}

3 ¹Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Korea 660-701

4 *Fax: + 82-55-757-7542, *E-mail: brjeong@gnu.ac.kr

5 ²Research Institute of Life Science, Gyeongsang National University, Jinju, Korea 660-701

6 ³Division of Applied Life Science (BK21 Program), Graduate School, Gyeongsang National

7 University, Jinju, Korea 660-701

Plant regeneration through

8 **Abstract** An efficient procedure for secondary somatic embryogenesis was developed in
9 *Crocus vernus*. The primary somatic embryo (PSE) was induced from the corm explant of *C.*
10 *vernus* on the Murashige and Skoog (MS) medium supplemented with 1.0 mg l⁻¹ 2,4-
11 dichlorophenoxyacetic acid (2,4-D) and 0.5 mg l⁻¹ thidiazuron (TDZ) with a mean number of
12 52 somatic embryos per explant. The effects of medium type (Schenk and Hildebrandt (SH),
13 Gamborg's (B-5), Chu (N6), Anderson's (AM), and MS) and light intensity (10 and 45
14 μmol·m⁻²·s⁻¹ photosynthetic photon flux density; PPFD) were studied for primary somatic
15 embryo induction. The greatest number of somatic embryo induction was obtained on the SH
16 medium amended with 1.0 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ TDZ under 10 μmol·m⁻²·s⁻¹ PPFD. The
17 primary somatic embryos were inoculated on the SH medium amended with 2-isopentyl
18 adenine or N⁶-benzyladenine (BA), in combination with α-naphthalene acetic acid (NAA) for
19 secondary somatic embryo induction. After 45 days, the SH medium fortified with 2.0 mg l⁻¹
20 BA and 0.5 mg l⁻¹ NAA was found to be the best for secondary embryo induction, secondary
21 embryos were induced on surface of globular (88.9%) and heart-shaped (95.2%) PSE's. At
22 1.0 mg l⁻¹ gibberellic acid (GA₃) 92.3% embryo maturation and conversion were observed.
23 Out of the various sucrose concentrations tried, 6% (w/v) sucrose was found to be the best and
24 with 100% embryo conversion. Finally it will be interesting to validate this protocol of
25 reproducible direct somatic embryogenesis from the corm explants for other *Crocus* species

According to Wikipedia Dutch crocus is *Crocus flavus*, not *C. vernus*. Authors, please give references to the name and origin of the species. *C. vernus* is known as giant crocus.

26 as well.

27 **Keywords:** corm, Dutch crocus, light intensity, somatic embryogenesis, thidiazuron

28 Introduction

29 *Crocus* belongs to the family Iridaceae which mainly embraces herbs with rhizomes,
30 corms or bulbs. The genus *Crocus* includes about 80 species distributed from the south-
31 western Europe, through central Europe to Turkey and south-western parts of Asia, as far as
32 east and western China (Mathew 1982). This genus is mainly known for saffron (*C. sativus*),
33 as one of the most important spices in the world, but other *Crocus* species are also
34 economically important. *Crocus vernus* Hill is an ornamental species, which acts as a
35 temperate forest spring ephemeral (Badri et al. 2007). It is commonly known as the Dutch
36 crocus that was originated in Russia and Eastern Europe, and is highly prized for its colorful
37 flowers and thus used extensively in gardening.

2 Dutch crocus is
38 It is propagated vegetatively by the daughter corms, which are annually formed at the top of a
39 mother corm. The rate of the natural propagation is very slow. The low rate of corm
40 production limits the availability of propagation materials (Sivanesan et al. 2011a).
41 Micropropagation is an alternative for large scale production of the disease-free,
42 economically important plants. In recent years, there has been a growing interest to exploit
43 tissue culture and genetic engineering techniques for improvement of the *Crocus* species.

44 Somatic embryogenesis can be the most promising technique for the *in vitro* propagation of
45 the plants. An exceptional uniqueness of somatic embryogenesis is the continuation of growth
46 and development of many embryos. Moreover, secondary somatic embryogenesis is a process
47 whereby new somatic embryos are initiated from the originally-formed primary somatic
48 embryos. It has certain advantages as compared to the primary somatic embryogenesis such
49 as very high multiplication rate (Te-chato and Hilae 2007), independence of an explant
50 source (Jariteh et al. 2011), and reproducibility (Karami et al. 2008). Additionally,

Did the authors use the same protocol for PGR induction?

51 embryogenicity can be maintained for a long period of time by repeated cycles of secondary
52 embryogenesis.

53 The induction of somatic embryos in *Crocus* species has been reported earlier by
54 manipulating ~~adjustments such as decrease~~ of plant growth regulator (PGR) concentrations
55 and subsequent use of PGR-free medium (Plessner and Ziv 1999, Karamian and
56 Ebrahimzadeh 2001, Karamian 2007), or changes ⁱⁿ of the auxin/cytokinin ratio. Somatic
57 embryogenesis has been accomplished for a few *Crocus* species viz., *C. cancellatus*, *C.*
58 *caspicus*, and *C. michelsonii* (Karamian 2004), *C. heuffelianus* (Demeter et al. 2010), and *C.*
59 *sativus* (Rajabpoor et al. 2007, Sheibani et al. 2007). However, till date, there is no report on
60 the secondary somatic embryo induction ⁱⁿ *Crocus* species. The callus and organ culture of
61 *C. vernus* has been previously reported by Chub et al. (1994) and somatic embryogenesis by
62 Sivanesan et al. (2011a). It is a well known fact that plant growth regulators influence
63 somatic embryo formations. Other than this, culture conditions, medium, tissue or organ type,
64 and its physiological status also have some pivotal role. Hence, the objectives of the present
65 study were to assess the influence of medium, plant growth regulators (PGR), and culture
66 conditions on somatic embryo induction, to assess the effects of plant growth regulators on
67 the secondary somatic embryo formation, GA₃ and sucrose on embryo maturation and
68 conversion to plants.

69 Materials and Methods

Plant materials

70 Surface sterilization and culture conditions

71 The corms were collected from greenhouse-grown plants, washed thoroughly under running
72 tap water for 30 min, and then washed with distilled water. The explants were
73 decontaminated with 70% (v/v) ethanol (Yakuri Pure Chemicals, Japan) for 60 sec, 2.0%
74 sodium hypochlorite (NaOCl) (Yakuri Pure Chemicals, Japan) for 10 min, and 0.01% (w/v)
75 mercuric chloride for 15 min. Each treatment was followed by 3-4 washes with sterile

76 distilled water.

77 The medium consisted of MS ~~basal~~ salts and vitamins (Murashige and Skoog 1962),
78 supplemented with 3% (w/v) sucrose, and solidified with 0.8 % (w/v) agar.^(Brand name?)
79 medium was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCl before autoclaving at 15 psi and
80 121 °C for 15 min. Gibberellic acid (GA₃) and thidiazuron (TDZ) were filter sterilized and
81 added to autoclaved medium. Other plant growth regulators were added to the ~~basal~~ medium
82 prior to pH adjustment and sterilization.

83 The ~~corms~~ explants were cut into 0.5-1.0 cm long segments and cultured ⁱⁿ on 100mm X 40mm ✓
84 ² Petri (diameter X depth) petridishes (SPL, Korea) containing MS medium supplemented with 0.25, ✓
85 0.5, 1.0 or 2.0 mg l⁻¹ 2,4 dichlorophenoxy acetic acid (2,4-D) combined with 0.5 mg l⁻¹
86 thidiazuron (TDZ). The explants were maintained for 14 days at 25 ± 1°C in darkness and
87 then exposed to light of ^{at} 45 µmol·m⁻²·s⁻¹ photosynthetic photon flux density (PPFD) provided ✓
88 by cool white fluorescent light (40 W tubes, Philips) with a daily light/dark cycle of 16/8 h.

89 *Influence of media and light intensity on somatic embryogenesis*

90 The ~~decontaminated~~ corm explants (0.5-1.0 cm) were cultured on Anderson's (AM, 1984),
91 Chu (N6, 1975), Gamborg's (B-5, 1968), ^z MS, or Schenk and Hildebrandt (SH, 1972) media
92 amended with 1.0 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ TDZ. The two light intensities 10 and 45
93 µmol·m⁻²·s⁻¹ PPFD ^{were tested} was also taken into account for SE induction.

This media
is described
above.

94 *Secondary somatic embryo induction*

95 To obtain the secondary embryos, the primary embryos at different developmental stages
96 were transferred to SH medium containing 1.0 or 2.0 mg l⁻¹ of either 2-isopentyl adenine (2-
97 iP) or N⁶-benzyladenine (BA) combined with 0.1 or 0.5 mg l⁻¹ α-naphthalene acetic acid
98 (NAA).

99 *Somatic embryo maturation and conversion*

100 Secondary somatic embryos were cultured on SH medium supplemented with activated

and washed? Brand name?

101 charcoal (0.3%, w/v), and 0, 0.5, 1.0, 2.0 or 4.0 mg l⁻¹ GA₃ for maturation and conversion.

102 The somatic embryos were subcultured at an interval of 3 weeks. The percentage embryo

103 conversion was calculated as the percentage of the number of germinated somatic embryos

104 divided by total number of somatic embryos.

105 *Effect of concentration of sucrose on somatic embryo maturation and conversion*

106 Secondary somatic embryos were cultured on SH medium supplemented with activated
107 charcoal (0.3%, w/v), 1.0 mg l⁻¹ GA₃, and 0, 1.5, 3.0, 6.0 or 12.0% (w/v) sucrose for
108 maturation and conversion. The percentage embryo conversion was calculated as the
109 percentage of the number of germinated somatic embryos divided by total number of somatic
110 embryos.

111 For each experiment, 50 explants were used and the experiment was repeated thrice. Data
112 were statistically analyzed by analysis of variance (ANOVA) followed by Duncan multiple
113 range test at a 5% probability level by using SAS computer package (SAS Institute Inc., NC,
114 USA).

115 **Results and Discussion**

116 In our previous study high frequency of somatic embryogenesis was achieved when corm
117 explants were cultured on MS medium with 0.5 mg l⁻¹ TDZ and 0.1 mg l⁻¹ NAA (Sivanesan et
118 al. 2011a). However, the mean number of PSE per explant was less. Somatic embryogenesis
119 SE is generally believed to be triggered by an auxin and for many plants, 2,4-D has been widely
120 regarded to be effective (Brown et al. 1995, Balaraju et al. 2011). In *Crocus* species, a
121 combination of 2,4-D and BA were reportedly being used for high frequency SE induction
122 (Karamian 2004, Rajabpoor et al. 2007). Thus in this present endeavor, an optimal
123 concentration of TDZ was combined with various concentrations of 2,4-D for somatic
124 embryo induction. Direct PSE was observed on the surface of the explants when MS medium
125 was supplemented with 2,4-D and TDZ. In contrast absence of PGR in the culture medium

or twice?
So, there were 4 experiments in total (200 explants)

Separate Results from Discussion,

How low

There is no logic between the sentences.
Re-write

and did not produce concentrations of what?

the explants turned brown, devoid of somatic embryos. When the cultures were kept under darkness for 14 days followed by exposure to light, somatic embryos formed directly on the explants after 45 days at all concentrations tested (Table 1). Somatic embryos at globular, heart-shaped, and cotyledonary stages were observed throughout the incubation period on the same medium. Direct somatic embryogenesis requires plant growth regulators and favorable conditions to allow the pre-embryogenic determined cells to undergo cell divisions and expression of embryogenesis (Sharp et al. 1980). In the present endeavor, embryos were white or pale yellow in color, small and globular in shape, appearing individually or in clusters. Morphologically, the early stage embryos appeared as shiny globular structures.

None of the embryos were found to be abnormal. The direct induction of globular somatic embryos as well as the number of induced globular embryos was assessed.

On increasing concentrations of 2,4-D from 0.25 to 1.0 mg l⁻¹, SE induction frequency and number of SE's induced per explant increased, but above 1.0 mg l⁻¹ they decreased (Table 1).

In contrast to our results, high frequency of SE induction was reported in *Crocus* species when the culture medium supplemented with 2.0 or 4.0 mg l⁻¹ 2,4-D in combination with 1.0 mg l⁻¹ BA (Karamian 2004, Rajabpoor et al. 2007). The TDZ has a dual role in the induction of somatic embryogenesis; a cytokinin-like activity that promotes cell division and differentiation and an auxin-like activity that seems to be crucial for induction of embryogenic competence. Thus, the presence of TDZ in the culture medium may be the reason behind low concentrations of 2,4-D requirement for SE. Our findings are in agreement with Chen (2012) who reported that TDZ lower down the requirement of dicamba for callus induction. The SE induction was best (74.6%) at 1.0 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ TDZ on MS medium containing 3% sucrose with a mean number of 52 somatic embryos per explants. Similar results were reported in other plant species such as *Cajanus cajan* (Aboshama 2011), *Pheonix dactylifera* (Sidky and Zaid 2011) and *Tricorytis* species (Nakano et al. 2004).

This paragraph is poorly written and confusing. Please rewrite!

176 The observed results indicate that lower light intensities were insignificantly associated with
177 induction and proliferation of increased number of somatic embryos. In contrast to this,
178 Rajabpoor et al. (2007) reported somatic embryo induction in *C. sativus* cultures when they
179 were maintained completely under dark ~~conditions~~ for 70 days.

180 *Effect of plant growth regulators (PGRs) on secondary somatic embryogenesis*

181 Induction of ~~secondary~~ somatic embryos (SSE) on primary SE's ~~on~~ the SH medium with
182 different PGR's was attempted. After 45 days of culture, ~~when~~ medium was fortified with 2-
183 iP and NAA (0.1), SSE was not induced, but 2-iP with higher concentration of NAA (0.5 mg
184 l⁻¹), SSE were induced (28.6 to 62.5 embryos per PSE). The best SSE induction was achieved
185 with 2.0 mg l⁻¹ BA and 0.5 mg l⁻¹ NAA (Table 3, Fig. 2). When primary somatic embryo was
186 transferred to ~~this~~ PGR combination, the highest SSE induction was achieved with heart-
187 shaped (95.2) followed by globular PE's (88.9). The percentage of torpedo-shaped PE's
188 conversion into SSE's was very low. Similarly, the number of embryos induced by these PE
189 stages (globular, heart, and torpedo) was also highest at 2.0 mg l⁻¹ BA and 0.5 mg l⁻¹ NAA
190 (Table 3).

The highest frequency of 2. Anulan
Rephrase,

191 *Effect of GA₃ on somatic embryo maturation and germination*

192 For ~~embryo~~ maturation and germination, embryos were cultured on SH medium
193 supplemented with 0.3% AC, GA₃ and 3% sucrose. In the present endeavour, embryo
194 germination and maturation were achieved at all concentrations of GA₃, although it was
195 the highest (92.3%) at 1.0 mg l⁻¹ GA₃ (Table 4, Fig. 3). Thus, both the activated charcoal and GA₃
196 appeared to be necessary for the maturation and germination of SE's.

197 The fact that GA₃ stimulates the formation and conversion of somatic embryos is well known.
198 Embryo maturation and simultaneous conversion to ~~obtain~~ plantlets is one of the important
199 steps in *in vitro* embryogenesis, which partially depends on the embryo quality also. In the
200 present study conducted, addition of GA₃ at higher concentrations (>2.0 mg l⁻¹) resulted in

151 In this study, all stages of somatic embryos were observed, while in *C. sativus* monopolar
152 embryos developed which later turned into bipolar structures during culture (Blazquea et al.
153 2009), and in *C. heuffelianus* bipolar embryos were directly differentiated from globular stage
154 embryos (Demeter et al. 2010).

155 *Influence of media and light intensity on somatic embryogenesis* ^{SE} Different media were tested that
156 ^{Repeat the "optimal PGR concentration here!"} contained the following PGRs: ^{which one}
On this optimal plant growth regulator concentration, the effect of culture medium and light
157 ~~intensity was studied for somatic embryo induction (Table 2). Among various media (AM, B5,~~
158 ~~MS, N6, and SH) studied, SH was found to be the most effective for SE induction. Thus, the~~ ~~delete~~
159 ~~frequency of SE varied considerably with the medium constitution. The requirement of SH has low~~
160 ~~concentration suggesting that *C. vernus* has~~ and SE.
161 ~~salts for SE shows the low salt requirement for the growth of *C. vernus*. Out of two light~~
162 ~~intensities, SE induction was best achieved at 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD as compared to 45 μmol~~
163 ~~$\text{m}^{-2} \text{s}^{-1}$ PPFD (Fig. 1). When the cultures were maintained at 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, the mean~~
164 ~~Somatic embryos~~
165 ~~number of SEs per explant developed on MS medium and SH medium was 52.0 and 73.4,~~
166 ~~respectively. The highest frequency of SE induction (100%) was achieved when the explants~~
167 ~~were cultured on SH medium with 1.0 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ TDZ under 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$~~
168 ~~PPFD (Table 2). Hence, the SH medium was found to be the best for SE induction and the~~
169 ~~mean number of Somatic embryos (124.7) induced per explant increased many folds than previous reports~~
170 ~~on *Crocus* species (Karamian 2004, Rajabpoor et al. 2007, Demeter et al. 2010, Sivanesan et~~
171 ~~al. 2011a).~~

170 The quality and intensity of light has been reported to affect somatic embryogenesis in terms
171 of induction and growth of somatic embryos in *Cydonia oblonga* (Morini et al. 2000),
172 *Daucus carota* (Takanori and Cuello 2005), and *Coffea arabica* (Gatica et al. 2008). In most
173 of the reports, a dark period of few days has been necessary for induction of SE's (Koleva-
174 Gudeva et al. 2007, Sivanesan et al. 2011b). The results are in accordance to Konstas et al.
175 (2003) that a minimum initial incubation ^{in dark ???} was required for somatic embryogenesis to occur.

201 green dormant embryos with no further elongation. Contrary to this, high concentrations of
202 GA₃ (0.75-5.79 μM) have been reported to enhance germination in wheat somatic embryos
203 (Miroshnichenko et al. 2009). In *Crocus heuffelianus*, conversion of somatic embryos was
204 reported by addition of 0.5-2.0 mg l⁻¹ BA together with 2.5 mg l⁻¹ GA₃, 100 mg l⁻¹ ascorbic
205 acid, and 1% sucrose (Demeter et al. 2010). In our study, activated charcoal was added to the
206 medium ~~so as to absorb the phenolics secreted by the cells.~~ Aboshama (2011) suggested that ✓
207 there is an important role of activated charcoal in reducing the inhibitory effect of residual
208 plant growth regulators and stimulating embryo conversion.

209 *Effect of sucrose concentration on somatic embryo maturation and conversion*

210 Influence of various sucrose concentrations was evaluated on embryo maturation and
211 conversion on ~~the~~ SH medium with 1.0 mg l⁻¹ GA₃ and 0.3 % activated charcoal. The rate of ✓
212 conversion increased on increasing the concentration of the sucrose (Table 5). It was found
213 that sucrose at 6% ~~concentration~~ resulted in 100% conversion. It has been reported by
214 Corredoira et al. (2003) that carbon source and concentration had a marked influence on
215 maturation and subsequent conversion ability of chestnut somatic embryos. The present
216 findings are also in concormance with Agarwal et al. (2004), who reported that best SSE was
217 observed at 6% sucrose in the medium in *Morus alba*. It has been observed that increased
218 concentrations of carbohydrates make them osmotically dynamic and they generate osmotic
219 tension which helps in embryo formation and further conversion. However, sugars simply do
220 not act as an osmoticum, but also provide energy ^{as} and carbon source for somatic
221 embryogenesis (Daigny et al. 1996). Contrary to these, Demeter et al. (2010) reported
222 decrease in strength of culture medium (1/4th MS) and content of organic carbon source (1%) ✓
223 to be followed for *C. heuffelianus*. ^{? rephrase!}

224 To summarize a simple, reproducible, and efficient direct embryogenesis protocol which
225 involved almost single step has been standardized. In the present study, the frequency of

226 somatic embryogenic regeneration was found to be influenced by concentration of plant
227 growth regulators, type of medium and light intensity. However, the results clearly indicate
228 that low light intensity and the SH medium are best suited for this important species and
229 highest number of somatic embryos can be induced. Based on the above observations, the SH
230 basal medium was found to be the best for induction, maturation, and germination of SE. No
231 intervening callus phase was noted during embryo germination; hence, direct secondary
232 somatic embryo induction was achieved. This technique is important, since it could be used
233 as a possible micropropagation system and for the regeneration of transgenic plants.

234

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337

338

SE + somatic embryogen's

339

Abbreviations

340

PSE: Primary somatic embryogen's

341

SSE: Secondary somatic embryogenesis

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2, 4-D: 2, 4-dichlorophenoxyacetic acid

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TDZ: Thidiazuron

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MS: Murashige and Skoog (1962)

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SH: Schenk and Hildebrandt (1972)

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B-5: Gamborg et al (1968)

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N6: Chu et al (1975)

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AM: Anderson's medium

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GA₃: Gibberellic acid

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2iP: 2-isopentyl adenine

BA: N⁶-benzyladenine

351 NAA: α -naphthalene acetic acid

352 PPFD: Photosynthetic photon flux density

353 Table 1. Effect of concentration of 2,4-D plus 0.5 mg l⁻¹ TDZ on somatic embryogenesis from
354 corm explants of *Crocus vernus* culture ^{and} ~~on the~~ MS medium. ^{separate!} ✓

2,4-D (mg l ⁻¹)	Somatic embryo induction (%)	No. of somatic embryos induced per explant
0.25	49.2±3.2d ^z	18.7±2.6c
0.50	60.8±2.5c	30.4±3.0b
1.00	74.6±1.7a	52.0±4.0a
2.00	69.4±1.2b	10.2±1.6d

355 ^zMeans ± SD followed by same letters within a column are not significantly different (P≤
356 0.05).

delete and describe in the text.

368 Table 4. Effect of concentration of GA₃ on embryo maturation and conversion.

GA ₃ (mg l ⁻¹)	Conversion (%)
0.0	47.1±1.2d ^z
0.5	66.4±2.0c
1.0	92.3±1.6a
2.0	83.3±1.2b
4.0	21.0±1.0e

369 Secondary somatic embryos (globular) were cultured on SH medium supplemented with
370 activated charcoal (0.3%, w/v).

371 ^zMeans ± SD followed by same letters within a column are not significantly different (P≤
372 0.05).

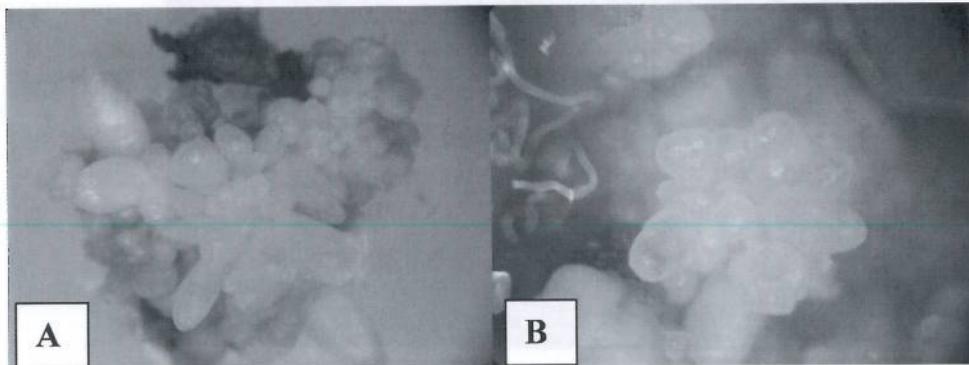
373 Table 5. Effect of various concentrations of sucrose on embryo maturation and conversion.

Sucrose (g l^{-1})	Conversion (%)
0	0.0e ^z
15	70.5±2.3c
30	92.3±1.6b
60	100a
120	27.3±0.6d

374 Secondary somatic embryos (globular) were cultured on SH medium supplemented with
375 activated charcoal (0.3%, w/v) and 1.0 mg l^{-1} GA₃.

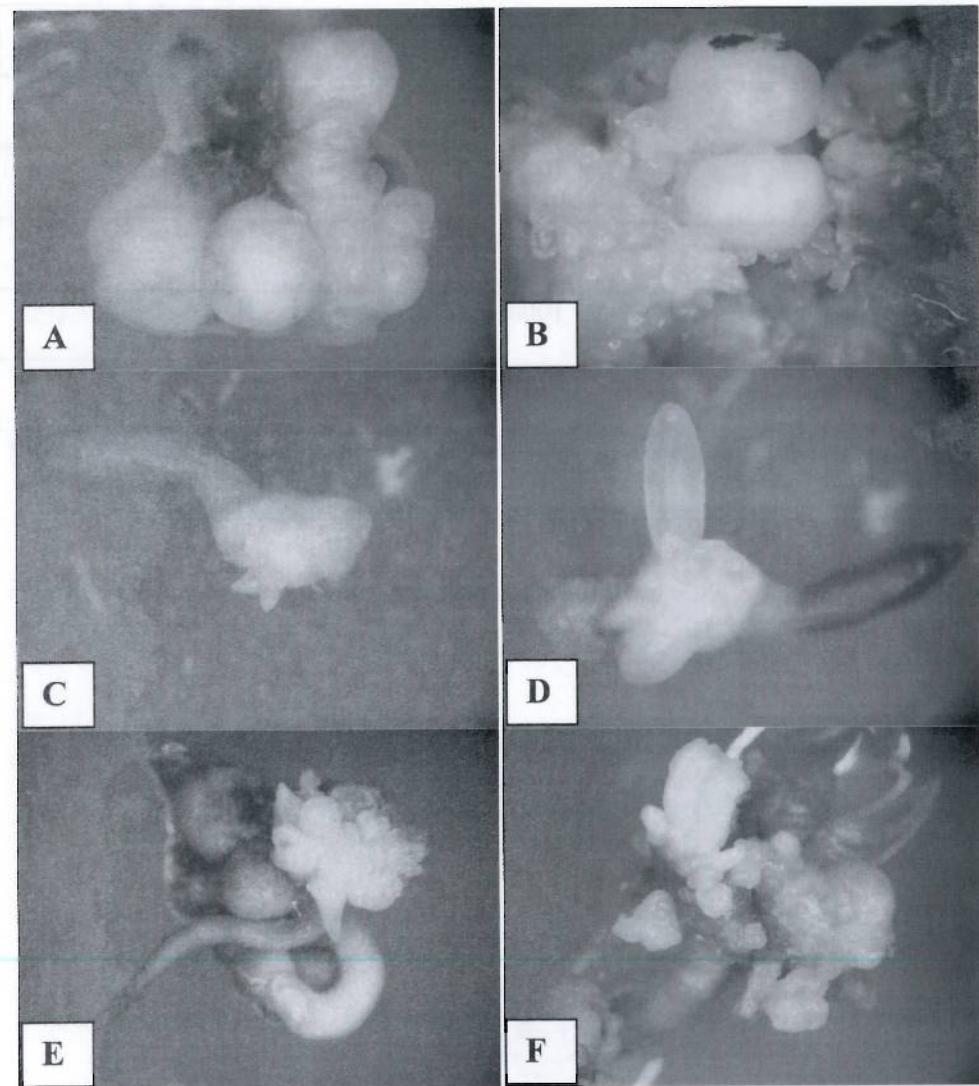
376 ^zMeans ± SD followed by same letters within a column are not significantly different ($P \leq$
377 0.05).

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380 Fig. 1. Somatic embryos developed from the corm explants on the SH medium containing 1.0
381 mg l^{-1} 2,4-D and 0.5 mg l^{-1} TDZ after 45 days of culture under $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (A) and
382 $45 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (B).

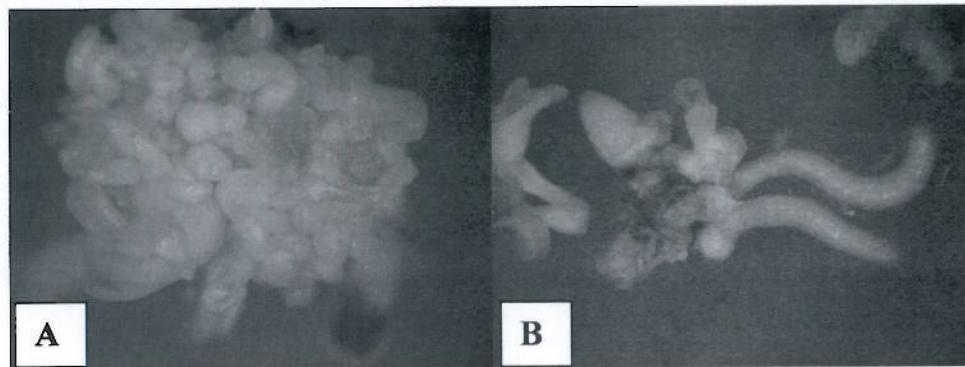


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Fig. 2. Formation of secondary somatic embryos on the primary embryos were cultured on
 the SH medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA. A, Primary somatic
 embryos; B, secondary embryogenesis from the primary somatic embryos; C & D, induction
 and development of secondary embryos from torpedo stage embryos; and E & F, induction
 and development of secondary embryos from cotyledonary stage embryos.

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↗ Is this a plantlet?



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393 Fig.3. Maturation and conversion of somatic embryos on the SH medium supplemented with
394 6% (w/v) sucrose and 1.0 mg l^{-1} GA₃. A, germination of somatic embryos after 4 weeks of
395 culture and B, embryo derived plantlet

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