Review of JPOP647

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

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Abstract An efficient procedure for secondary somatic embryogenesis was developed ~~in~~ for *Crocus vernus*. The primary somatic embryo (PSE) was induced from the corm explant of *C. vernus* on ~~the~~ Murashige and Skoog (MS) medium supplemented with 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg l^{-1} thidiazuron (TDZ) with a mean ~~number~~ of 52 somatic embryos per explant. The effects of medium type (Schenk and Hildebrandt (SH), Gamborg's (B-5), Chu (N6), Anderson's (AM), and MS) and light intensity (10 and 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ ~~photosynthetic photon flux density; PPFD~~) were studied for primary somatic embryo induction. The greatest number of somatic embryo induction was obtained on ~~the~~ SH medium amended with 1.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} TDZ under 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ ~~PPFD~~. The primary somatic embryos were ~~inoculated~~ cultured on ~~the~~ SH medium amended with 2-isopentyl adenine or N^6-benzyladenine (BA), in combination with α-naphthalene acetic acid (NAA) for secondary somatic embryo induction. After 45 days, ~~the~~ SH medium fortified with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA was found to be the best for secondary embryo induction, and secondary embryos were induced on the surface of globular (88.9%) and heart-shaped (95.2%) PSE's. At 1.0 mg l^{-1} gibberellic acid (GA_3) 92.3% embryo maturation and conversion were observed. ~~Out of the various sucrose concentrations tried,~~ Six-percent ~~6%~~ (w/v) sucrose was found to be the best with 100% embryo conversion. ~~Finally it will be interesting to validate this protocol of reproducible direct somatic embryogenesis from the corm explants for other *Crocus* species~~

* Report all plant growth regulators in microMolar (μM) not milligrams per liter.
* Do not italicize the words (in vitro). *

as well.

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

Introduction

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

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

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

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 of the auxin/cytokinin ratio. Somatic
57 embryogenesis has been accomplished for a few *Crocus* species viz., *C. cancellatus*, *C.*
58 *caspius*, 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 on *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, GA_3 and sucrose on embryo maturation and
68 conversion.

69 **Materials and Methods**

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

distilled water.

The medium consisted of MS basal salts and vitamins (Murashige and Skoog 1962), supplemented with 3% (w/v) sucrose, and solidified with 0.8 % (w/v) agar. The pH of the medium was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCl before autoclaving at 15 psi and 121 °C for 15 min. Gibberellic acid ~~(GA_3)~~ and thidiazuron (TDZ) were filter sterilized and added to autoclaved medium. Other ~~plant growth regulators~~ were added to the basal medium prior to pH adjustment and ~~sterilization~~.

~~The~~ explants were cut into 0.5-1.0 cm long segments and cultured ~~on~~ 100mm X 40mm (diameter X depth) petridishes (SPL, Korea) containing MS medium supplemented with 0.25, 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} ~~thidiazuron~~ (TDZ). The explants were maintained for 14 days at 25 ± 1°C in dark~~ness~~ and then exposed to light of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ ~~photosynthetic photon flux density (PPFD)~~ provided by cool white fluorescent light (40 W tubes, Philips) with a daily light/dark cycle of 16/8 h.

Influence of ~~media~~ and light intensity on somatic embryogenesis

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

Secondary somatic embryo induction

To obtain the secondary embryos, the primary embryos at different developmental stages were transferred to SH medium containing 1.0 or 2.0 mg l^{-1} of either 2-isopentyl adenine (2-iP) or N^6-benzyladenine (BA) combined with 0.1 or 0.5 mg l^{-1} α-naphthalene acetic acid (NAA).

Somatic embryo maturation and conversion

Secondary somatic embryos were cultured on SH medium supplemented with activated

State brand or type

charcoal (0.3%, w/v), and 0, 0.5, 1.0, 2.0 or 4.0 mg l^{-1} GA_3 for maturation and conversion. The somatic embryos were subcultured ~~at an interval of~~ every 3 weeks. The percentage of embryo conversion was calculated as the percentage of the number of germinated somatic embryos divided by the total number of somatic embryos.

Effect of concentration of sucrose on somatic embryo maturation and conversion How many explants per treatment?

Secondary somatic embryos were cultured on SH medium supplemented with activated charcoal (0.3%, w/v), 1.0 mg l^{-1} GA_3, and 0, 1.5, 3.0, 6.0, or 12.0% (w/v) sucrose for maturation and conversion. The percentage embryo conversion was calculated as the percentage of the number of germinated somatic embryos divided by the total number of somatic embryos.

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

Results and Discussion

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

concentration?

the explants turned brown, devoid of somatic embryos. When the cultures were kept ~~under~~ darkness for 14 days followed by expose to light, somatic embryos formed directly on the explants after 45 days at all concentrations tried (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 beyond 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 *Tricyrtis* species (Nakano et al. 2004).

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

Influence of media and light intensity on somatic embryogenesis

On this optimal plant growth regulator concentration, the effect of culture medium and light intensity was studied for somatic embryo induction (Table 2). Among various media (AM, B5, MS, N6, and SH) studied, SH was found to be the most effective for SE induction. Thus, the frequency of SE varied considerably with the medium constitution. The requirement of SH salts for SE shows the low salt requirement for the growth of *C. vernus*. Out of two light intensities, SE induction was best achieved at 10 µmol m^{-2} s^{-1} PPFD as compared to 45 µmol m^{-2} s^{-1} PPFD (Fig. 1). When the cultures were maintained at 45 µmol m^{-2} s^{-1} PPFD, the mean number of SEs per explant developed on MS medium and SH medium was 52.0 and 73.4, respectively. The highest frequency of SE induction (100%) was achieved when the explants were cultured on SH medium with 1.0 mg l^{-1} 12,4-D and 0.5 mg l^{-1} TDZ under 10 µmol·m^{-2}·s^{-1} PPFD (Table 2). Hence, the SH medium was found to be the best for SE induction and the mean number of SEs (124.7) induced per explant increased many folds than previous reports on *Crocus* species (Karamian 2004, Rajabpoor et al. 2007, Demeter et al. 2010, Sivanesan et al. 2011a).

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

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

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

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

How do your results compare to other studies of SSE?

Effect of GA_3 on somatic embryo maturation and germination

For embryo maturation and germination, embryos were cultured on SH medium supplemented with 0.3% AC, GA_3, and 3% sucrose. In the present endeavor, embryo germination and maturation were achieved at all concentrations of GA_3, although it was highest (92.3%) at 1.0 mg l^{-1} GA_3 (Table 4, Fig. 3). Thus, both the activated charcoal and GA_3 appeared to be necessary for the maturation and germination of SE's.

The fact that GA_3 stimulates the formation and conversion of somatic embryos is well known. Embryo maturation and simultaneous conversion to obtain plantlets is one of the important steps in *in vitro* embryogenesis, which partially depends on the embryo quality also. In the present study conducted, addition of GA_3 at higher concentrations (>2.0 mg l^{-1}) resulted in

green dormant embryos with no further elongation. Contrary to this, high concentrations of GA_3 (0.75-5.79 µM) have been reported to enhance germination in wheat somatic embryos (Miroshnichenko et al. 2009). In *Crocus heffuelianus*, conversion of somatic embryos was reported by addition of 0.5-2.0 mg l^{-1} BA together with 2.5 mg l^{-1} GA_3, 100 mg l^{-1} ascorbic acid, and 1% sucrose (Demeter et al. 2010). In our study, activated charcoal was added to the medium so as to absorb the phenolics secreted by the cells. Aboshama (2011) suggested that there is an important role of activated charcoal in reducing the inhibitory effect of residual plant growth regulators and stimulating embryo conversion.

Effect of sucrose concentration on somatic embryo maturation and conversion

Influence of various sucrose concentrations was evaluated on embryo maturation and conversion on the SH medium with 1.0 mg l^{-1} GA_3 and 0.3 % activated charcoal. The rate of conversion increased on increasing the concentration of the sucrose (Table 5). It was found that sucrose at 6% concentration resulted in 100% conversion. It has been reported by Corredoira et al. (2003) that carbon source and concentration had a marked influence on maturation and subsequent conversion ability of chestnut somatic embryos. The present findings are also in concomitance with Agarwal et al. (2004), who reported that best SSE was observed at 6% sucrose in the medium in *Morus alba*. It has been observed that increased concentrations of carbohydrates make them osmotically dynamic and they generate osmotic tension which helps in embryo formation and further conversion. However, sugars simply do not act as an osmoticum, but also provide energy and carbon source for somatic embryogenesis (Daigny et al. 1996). Contrary to these, Demeter et al. (2010) reported decrease in strength of culture medium (1/4th MS) and content of organic carbon source (1%) to be followed for *C. heuffelianus*.

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

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

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Abbreviations

SSE: Secondary somatic embryogenesis

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

TDZ: Thidiazuron

MS: Murashige and Skoog (1962)

SH: Schenk and Hildebrandt (1972)

B-5: Gamborg et al (1968)

N6: Chu et al (1975)

AM: Anderson's medium

GA_3: Gibberellic acid

2iP: 2-isopentyl adenine

BA: N^6-benzyladenine

PSE: primary somatic embryo

NAA: α-naphthalene acetic acid

~~PPFD: Photosynthetic photon flux density~~

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

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

[z]Means ± SD followed by ~~the~~ same letters within a column ~~are~~ were not significantly different (P≤ 0.05).

* Remove grid lines from all tables. *

Table 2. Influence of medium and light intensity on somatic embryogenesis of *Crocus vernus*[z].

Culture medium	Somatic embryo induction (%)		No. of somatic embryos induced per explant	
	10 µmol m^{-2} s^{-1} PPFD	45 µmol m^{-2} s^{-1} PPFD	10 µmol m^{-2} s^{-1} PPFD	45 µmol m^{-2} s^{-1} PPFD
AM[y]	87.4±1.6c[x]	60.2±1.7c	94.7±7.4b	66.2±2.6b
B5	96.4±1.4a	72.2±1.2ab	75.3±8.1d	41.4±1.0d
MS	98.2±1.7a	74.6±2.4a	82.4±2.7c	52.0±2.6c
N6	91.8±0.5 b	59.6±1.0c	86.0±5.0c	63.7±1.4b
SH	100a	68.0±3.1b	124.7±9.0a	73.4±1.0a

[z]Corm explants were cultured on the basal medium supplemented with 1.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} TDZ.

[y]AM, Anderson's; B5, Gamborg's; MS, Murashige and Skoog's; N6, Chu's; and SH, Schenk and Hilderbrandt medium.

[x]Means ± SD followed by same letters within a column are not significantly different ($P \leq 0.05$).

Table 3. Effect of plant growth regulators (PGRs) on secondary somatic embryogenesis (SSE) on primary somatic embryo of *C. vernus* after 45 days of culture on the SH medium.

PGRs (mg l^{-1})			SSE induction (%)			No. of SE's induced per embryo		
2-iP	BA	NAA	Globular	Heart	Torpedo	Globular	Heart	Torpedo
1.0	0	0.1	0.0g[z]	0.0f	0.0e	0.0f	0.0e	0.0e
2.0	0	0.1	0.0g	0.0f	0.0e	0.0f	0.0e	0.0e
1.0	0	0.5	32.7±0.6f	37.5±1.6e	28.6±0.8d	5.7±1.5e	4.3±2.0d	2.3±0.5c
2.0	0	0.5	40.4±1.6e	62.5±1.7d	42.9±1.2b	9.7±1.3d	7.0±0.6c	3.6±0.5b
0	1.0	0.1	52.3±1.8d	60.0±2.0d	38.1±0.5c	11.4±1.6c	6.2±0.4c	1.0±0.1d
0	2.0	0.1	70.4±2.6b	81.2±0.8b	43.8±1.5b	11.0±2.0c	9.0±1.5b	1.2±0.3d
0	1.0	0.5	67.5±3.0c	75.0±1.0c	40.7±1.1bc	18.4±2.4b	12.4±1.2ab	4.0±0.3b
0	2.0	0.5	88.9±1.0a	95.2±1.0a	55.6±1.7a	28.0±3.0a	14.6±0.6a	7.2±0.6a

[z]Means ± SD followed by same letters within a column are not significantly different ($P \leq 0.05$).

SH: Schenk and Hildebrandt (1972).

2iP: - - -

BA: . . - -

NAA: . . - -

Tables must stand alone from the text.

Table 4. Effect of concentration of GA_3 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

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

[z]Means ± SD followed by same letters within a column are not significantly different (P≤ 0.05).

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

Secondary somatic embryos (globular) were cultured on SH medium supplemented with activated charcoal (0.3%, w/v) and 1.0 mg l^{-1} GA_3.

[z]Means ± SD followed by same letters within a column are not significantly different ($P \leq 0.05$).

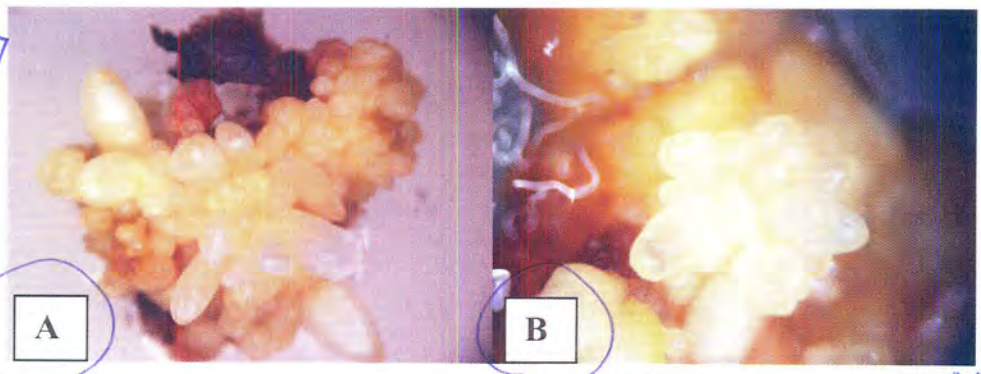

Fig. 1. Somatic embryos developed from the corm explants on the SH medium containing 1.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} TDZ after 45 days of culture under 10 µmol m^{-2} s^{-1} PPFD (A) and 45µmol m^{-2} s^{-1} PPFD (B).

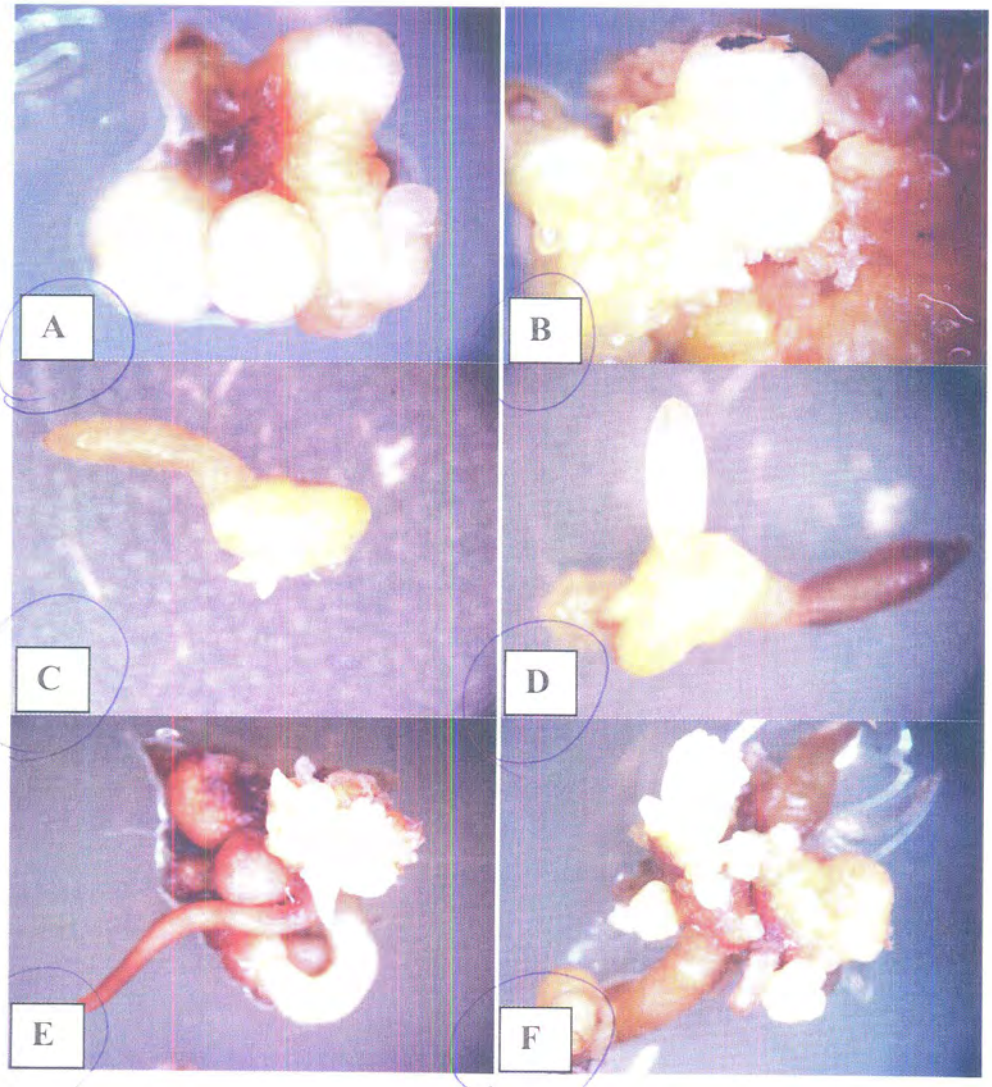

Fig. 2. Formation of secondary somatic embryos on the primary embryos were cultured on the SH medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA. (A) Primary somatic embryos; (B) secondary embryogenesis from the primary somatic embryos; (C & D) induction and development of secondary embryos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

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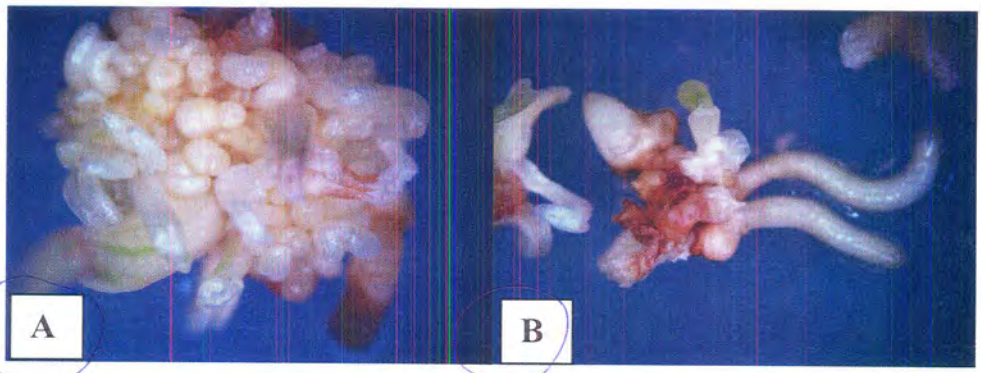

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399 Fig.3. Maturation and conversion of somatic embryos on the SH medium supplemented with

400 6% (w/v) sucrose and 1.0 mg l^{-1} GA_3. (A) germination of somatic embryos after 4 weeks of

401 culture and (B) embryo derived plantlet

402
403

Need to show a germinated somatic embryo as a plantlet. Were any plantlets acclimatized to the greenhouse?