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IN VITRO SHOOT REGENERATION AND MICROCORM DEVELOPMENT IN *CROCUS VERNUS* (L.) HILL

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Abstract

An efficient method has been developed for *In vitro* regeneration of shoot and microcorm from corm explants of *Crocus vernus*. Corms were cut into 0.5-1.0 cm long segments and cultured on the SH medium supplemented with 0.5, 1.0, 2.0, or 4.0 mg L^{-1} 2-isopentyl adenine (2-iP), N^6-benzyladenine (BA), and N^6-furfuryladenine (kinetin, Kin) alone or combination with 0.5 or 1.0 mg L^{-1} α-naphthalene acetic acid (NAA) for shoot regeneration. Of the three cytokinins tested, BA was found to be the most effective cytokinin for shoot formation. The number of shoots induced per explant was more when BA was combined with 0.5 mg L^{-1} NAA than with 1.0 mg L^{-1} NAA. The greatest percentage of shoot induction (97.2) with the mean number of 11.8 shoots per explant was obtained when the SH medium was supplemented with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA. The frequency of microcorm induction was significantly affected by the concentrations of sucrose. The greatest number of 6.1 microcorms per explant was obtained when the SH medium was supplemented with 2.0 mg L^{-1} BA, 0.5 mg L^{-1} NAA and 6.0% sucrose. The microcorms formed *In vitro* developed daughter corms when they were cultured on this medium. Microcorms were separated from the culture and planted out in acclimatization boxes containing a commercial medium. About 85% of corms developed shoot and root after 30 days. This protocol could be utilized for genetic transformation and mass clonal propagation of *C. vernus*.

Introduction

The genus *Crocus* (*Iridaceae*) includes about 90 species and occurs in Asia, Europe, and North Africa (Mathew, 1982). Many *Crocus* species are cultivated as ornamental plants (Kandemir *et al*., 2012). *Crocus vernus*, commonly known as Dutch crocus, is an ornamental species which acts as temperate forest spring ephemeral. It is highly prized for its colorful flowers and so used extensively in gardening. It is conventionally propagated through corms. However, the low rate of corm production limits the availability of propagation materials (Sivanesan *et al*., 2012). Further, lack of disease-free or quality corms may affect the plant growth and yield (Plessner *et al*., 1990). Micropropagation is an alternative technique for large-scale production of disease-free plantlets (Abbas *et al*., 2010; Hussain *et al*., 2011; Silva *et al*., 2012). Several reports are available on *In vitro* propagation (Karamian, 2004; Ascough *et al*., 2009; Demeter *et al*., 2010) and microcorm production (Sharma *et al*., 2008; Mir *et al*., 2010; Devi *et al*., 2011) in *Crocus* species. However, only two reports are available on *In vitro* propagation of *C. vernus* (Chub *et al*., 1994; Sivanesan *et al*., 2012), and to date, there are no reports on microcorm production. Several factors such as explant source, basal medium composition, plant growth regulators, sucrose, and temperature influence the microcorm formation *In vitro* (Milyaeva *et al*., 1995; Bhagyalakshmi, 1999; Raja *et al*., 2007; Sharma *et al*., 2008; Mir *et al*., 2010; Devi *et al*., 2011; Ahouran *et al*., 2012). The goals of this study were to find the effect of plant growth regulators on shoot regeneration from corm explants of *C. vernus*, and to test the effect of sucrose on microcorm formation.

Materials and Methods

Plant materials: The corms were collected from three years old greenhouse-grown plants, washed under running tap water for 30 min and soaked in Teepol solution (0.1%, v/v) for 5 min, and then washed three times with distilled water. Thereafter, in aseptic conditions, the explants were disinfected in 70% (v/v) ethanol for 60 sec, 2.0% (v/v) sodium hypochlorite for 10 min followed by three times washes with sterile distilled water, and 0.01% (w/v) mercuric chloride for 15 min followed by four times washes with sterile distilled water.

Media and culture condition: The medium consisted of Schenk & Hildebrandt (1972) basal salts and vitamins (SH) supplemented with 0.3% (w/v) activated charcoal, 3% (w/v) sucrose, and gelled with 0.8% (w/v) agar. The medium was adjusted to pH 5.80 and autoclaved at 121°C for 15 min. Gibberellic acid (GA_3) was filter sterilized and added to autoclaved medium. Auxin (α-naphthalene acetic acid, NAA) and cytokinins (2-isopentyl adenine (2-iP), N^6-benzyladenine (BA), and N^6-furfuryladenine (kinetin, Kin) were added to the medium prior to pH adjustment and sterilization. Cultures were incubated in the culture room at 25±1°C under a 16 h photoperiod with 45 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) supplied by cool white fluorescent light.

The effect of plant growth regulators (PGRs) on shoot induction: Corms were cut into 0.5-1.0 cm long segments and cultured in 100 mm x 40 mm Petri dishes (SPL, Korea) containing SH medium supplemented with 0.5, 1.0, 2.0, or 4.0 mg L^{-1} 2-iP, BA, and Kin alone or combination with 0.5 or 1.0 mg L^{-1} NAA. The frequency of shoot induction was calculated by counting explants

forming shoots from the total number of explants after 45 days of incubation. The number of shoots per explant was also recorded.

The effect of sucrose concentration on microcorm induction: Corms were cut into 0.5-1.0 cm long segments and cultured on SH medium supplemented with 2.0 mg L^{-1} BA, 0.5 mg L^{-1} NAA and 0, 1.5, 3.0, 6.0 or 12.0% (w/v) sucrose. The frequency of microcorm induction was calculated by counting explants forming corms from the total number of explants after 60 days of incubation. The number of corms per explant was also recorded.

Acclimatization: Microcorms were removed from culture vessel, washed thoroughly with sterile water to remove residual nutrients and agar, transplanted into acclimatization boxes containing a commercial medium (Tosilee medium, Shinan Grow, Jinju, Korea), irrigated every alternative days with quarter strength SH salts solution, and maintained in the greenhouse. Germination was recorded after 4 weeks.

Statistical analysis: Each experiment was conducted in a completely randomized design and repeated thrice with 15 explants per treatment. Data were subjected to analysis of variance (ANOVA) followed by Duncan multiple range test using SAS computer package (Release 9.1, SAS Institute Inc., NC, USA).

Results

The surface sterilization method yielded 100% aseptic cultures. Corm explants failed to develop adventitious shoots on a PGRs-free SH medium. When the culture medium was supplemented with cytokinins, the explants developed adventitious shoots within 30 days of culture, but the frequency of shoot formation was much dependent on cytokinin type and their concentrations. Adventitious shoots were induced from the surface of the corm explants cultured on the SH medium containing 2-iP. The frequency of shoot regeneration ranged from 44.3% to 72.2%. Among the various concentrations of 2-ip studied, the maximum number of shoots (2.0) per explant achieved when the SH medium was supplemented with 2.0 mg L^{-1} 2-iP. The increase in concentration of 2-iP beyond optimal level shoot regeneration was decreased. Direct shoot regeneration from the corm explants was also observed when the SH medium was supplemented with different concentrations of BA. The highest frequency of shoot regeneration (93%) with a mean number of 5.8 shoots per explant was obtained on the medium supplemented with 2.0 mg L^{-1} BA (Table 1). When the corm explants were cultured on the SH medium supplemented with 4.0 mg L^{-1} BA dropped the regeneration frequency (72.8%) and number of shoots (2.4) per explant. When the SH medium was supplemented with 0.5 or 4.0 mg L^{-1} Kin, the corm explants produced a mean of 1.1 and 2.8 shoots, respectively. The best frequency of shoot induction (61.3%) was obtained when the corm explants were cultured on the SH medium containing 4.0 mg L^{-1} Kin (Table 1).

The effect of BA and NAA combination was evaluated on shoot induction. The SH medium supplemented with various concentrations of BA and NAA most favoured adventitious shoot regeneration. The number of shoots induced per explant was more when BA was combined with 0.5 mg L^{-1} NAA than with 1.0 mg L^{-1} NAA (Table 2). The greatest percentage of shoot regeneration (97.2) with the mean number of 11.8 shoots per explant was obtained when the SH medium was supplemented with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA (Fig. 1A).

Table 1. Effect of cytokinins on shoot induction from corm explants of *C. vernus* after 45 days of culture.

Conc. (mg L^{-1})	Shoot induction (%)			No. of shoots per explant		
	2-iP	BA	Kin	2-iP	BA	Kin
0.0	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0c	0.0 ± 0.0d	0.0 ± 0.0c
0.5	44.3 ± 3.2d	65.2 ± 2.4d	34.2 ± 2.8d	1.1 ± 0.1b	2.1 ± 0.9c	1.1 ± 0.3b
1.0	55.5 ± 2.1c	84.0 ± 3.0b	41.8 ± 2.2c	1.7 ± 0.8ab	3.6 ± 1.7b	1.5 ± 0.7b
2.0	72.2 ± 2.5a	93.0 ± 2.0a	54.8 ± 2.3b	2.0 ± 0.7a	5.8 ± 1.0a	2.1 ± 0.6ab
4.0	62.7 ± 3.8b	72.8 ± 2.5c	61.3 ± 2.5a	1.4 ± 0.5b	2.4 ± 0.5c	2.8 ± 0.6a

Means followed by same letters within a column are not significantly different ($p \leq 0.05$)

Table 2. Effect of different concentrations and combination of BA and IAA on shoot induction from corm explants of *C. vernus* after 45 days of culture.

PGR (mg L^{-1})		Shoot induction (%)	No. of shoots per explant
BA	NAA		
1.0	0.5	93.1 ± 2.3b	7.4 ± 1.5b
2.0	0.5	97.2 ± 1.3a	11.8 ± 2.0a
1.0	1.0	90.2 ± 1.8c	5.1 ± 0.9c
2.0	1.0	95.2 ± 1.5ab	6.6 ± 1.4bc

Means followed by same letters within a column are not significantly different ($p \leq 0.05$)

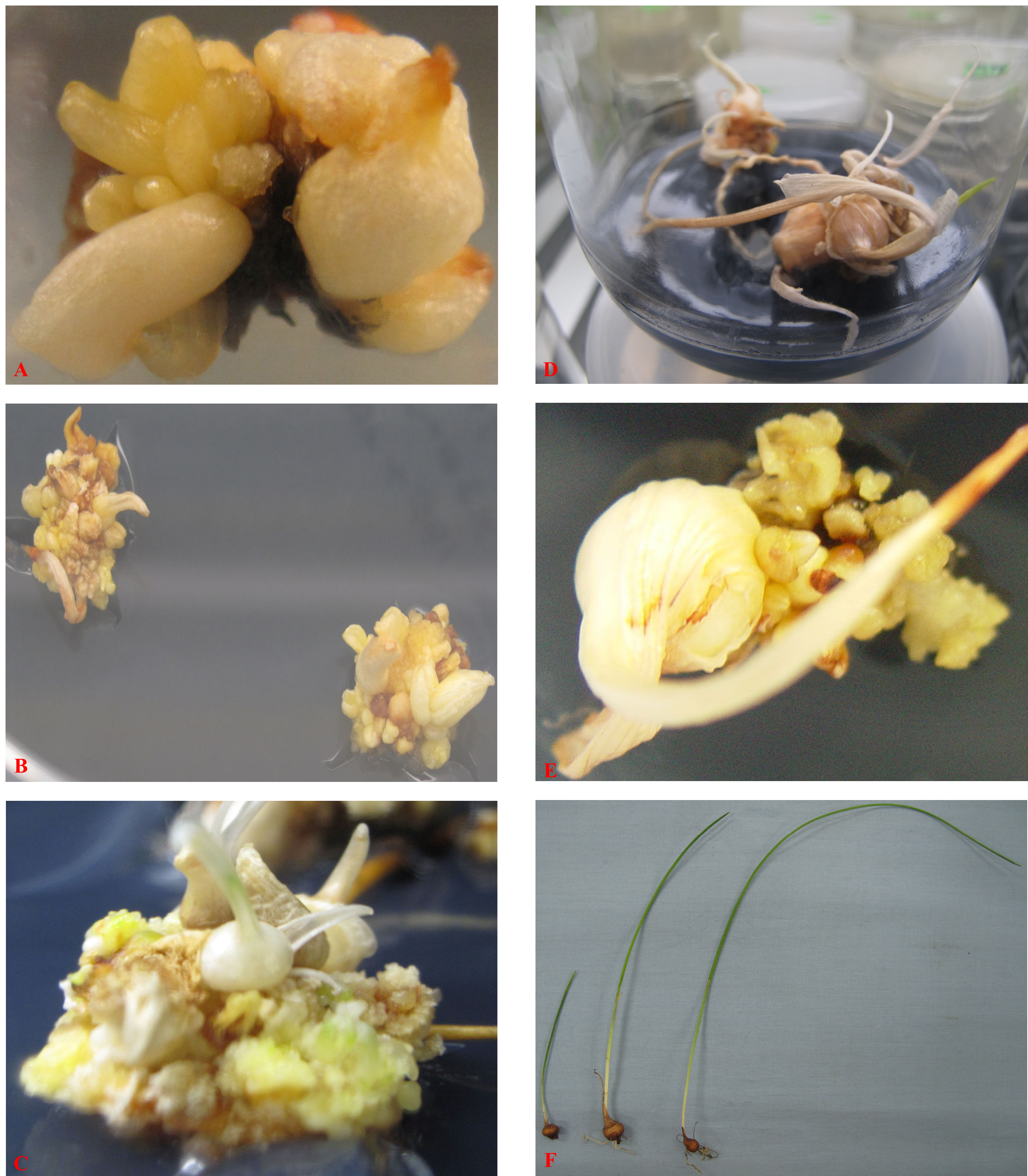

Fig. 1. Shoot regeneration and microcorm development in *Crocus vernus*. A) Adventitious shoot regeneration from corm explants cultured on the SH medium with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA, B-D) Induction and development of microcorms from corm explants cultured on the SH medium with 2.0 mg L^{-1} BA, 0.5 mg L^{-1} NAA and 6.0% sucrose, E) Daughter corms formation from *In vitro* derived microcorms cultured on the SH medium with 2.0 mg L^{-1} BA, 0.5 mg L^{-1} NAA and 6.0% sucrose, and F) Different stages of germinated microcorms.

We evaluated the effect of sucrose concentrations on microcorm induction from corm explants of *C. vernus* cultured in the SH medium containing 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA. The frequency of microcorm induction was significantly affected by the concentrations of sucrose. No microcorm was observed when the explants were cultured on the SH medium containing 0 or 1.5% sucrose. Microcorms were observed on the cut ends and surface of the explants when the induction medium was supplemented with above 1.5% of sucrose. The frequency of microcorm induction was 32.7, 70.9 or 45.5% when the SH medium was supplemented with 3.0, 6.0 or 12.0% sucrose, respectively (Table 3). The greatest number of 6.1 microcorms per explant was obtained when the SH

medium was supplemented with 2.0 mg L^{-1} BA, 0.5 mg L^{-1} NAA and 6.0% sucrose (Fig. 1B-D). The microcorms formed *In vitro* developed daughter corms when they were cultured on this medium (Fig. 1E). The increase in sucrose concentration above 6.0% reduced the number of 4.2 microcorms per explant. After 60 days, well developed microcorms were separated from the culture and planted out in acclimatization boxes containing a commercial medium. About 85% of corms developed shoot and root after 30 days (Fig. 1F).

Discussion

In this study, the influence of plant growth regulators (PGRs) and sucrose concentration on adventitious shoot regeneration and microcorm development of *C. vernus* was investigated. The inclusion of PGRs to the culture medium was required for shoot regeneration of many plant species. Cytokinin is one of the most important PGRs and influence the cell division and shoot induction. When the SH medium was supplemented with cytokinins, adventitious shoots induced without callus formation from the surface of the corm explants of *C. vernus*. The promoting effect of cytokinin on shoot regeneration has also been reported in *Crocus* species (Plessner *et al*., 1990; Ascough *et al*., 2009). Addition of 2-iP to the culture medium promoted adventitious shoot regeneration. Maximal shoot regeneration frequency (72.2%) was obtained on the culture medium containing 2.0 mg L^{-1} 2-iP. The stimulatory effect of 2-iP on shoot regeneration has also been reported earlier in saffron (Majourhat *et al*., 2007). In this study, the inclusion of BA or Kin also promoted shoot regeneration; however, the frequency of shoot induction was dependent on their concentrations. The frequency of shoot regeneration and mean number of shoots developed per explant increased with increasing concentrations of Kin in the SH medium. On the other hand, the increase in concentration of BA beyond optimal level both the frequency of shoot regeneration and mean number of shoots per explant was decreased. Similar result was also reported in *Crocus sativus* (Bhagyalakshmi, 1999). In contrast, maximum shoot formation from the corm explants of *C. sativus* was recorded when the medium was supplemented with high concentration of BA (Sharma *et al*., 2008; Devi *et al*., 2011). The optimal concentrations of BA vary according to species or varieties, because differences in their uptake, transport and metabolism. Among the three cytokinins investigated, BA was much effective for adventitious shoot regeneration. Majourhat *et al*., (2007) also reported that BA produced greater and more vigorous shoots than either 2-iP or thidiazuron in *C. sativus*.

In this study, the cytokinin BA alone was not able to induce much shoots in *C. vernus*. It has been reported that in many plant species, addition of low concentration of auxin along with optimal concentration of cytokinin enhanced shoot formation (Abbas & Qaiser, 2012; Sivanesan & Jeong, 2012). The frequency of shoot induction and mean number of induced shoots per explant significantly increased when the culture medium was supplemented with BA and NAA. Similar results were also obtained with the same hormones in *C. sativus* (Bhagyalakshmi, 1999; Karaoglu *et al*., 2007; Devi *et al*., 2011).

Sucrose concentrations affected *In vitro* microcorm induction in *C. sativus* (Raja *et al*., 2007; Sharma *et al*., 2008). In addition, both an auxin and a cytokinin required for maximum microcorm induction in *Crocus cancellatus* (Ahouran *et al*., 2012) and *C. sativus* (Mir *et al*., 2010). In this study, the greatest frequency of microcorm induction was recorded when the SH medium was supplemented with 2.0 mg L^{-1} BA, 0.5 mg L^{-1} NAA and 6.0% sucrose. The increase in concentration of sucrose beyond optimal level significantly decreased the frequency of microcorm induction. Similar result was also observed in *C. sativus* (Raja *et al*., 2007).

Table 3. Effect of different concentrations of sucrose on microcorm induction from corm explants of *C. vernus* after 60 days of culture.

Sucrose (%)	Microcorm induction (%)	No. of corms per explant
0.0	0.0 ± 0.0d	0.0 ± 0.0d
1.5	0.0 ± 0.0d	0.0 ± 0.0d
3.0	32.7 ± 5.1c	3.3 ± 1.1c
6.0	70.9 ± 2.6a	6.1 ± 1.7a
12.0	45.5 ± 3.7b	4.2 ± 1.5b

Means followed by same letters within a column are not significantly different ($p \leq 0.05$)

Conclusion

We developed a method for *In vitro* regeneration of shoot and microcorm from corm explants of *Crocus vernus*. Direct shoot regeneration from the corm explants without the formation of callus tissue may be important in maintaining genetic integrity. Further, its suitable for *Agrobacterium* mediated genetic transformation. Sucrose concentration is very important for induction of microcorms in *C. vernus*. The *In vitro* produced microcorms were successfully germinated in the greenhouse. Thus, this protocol could be utilized for mass clonal propagation of *C. vernus*.

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