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Somatic embryogenesis and plant regeneration in *Crocus vernus*

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***Abstract*—A simple and efficient protocol was developed for somatic embryogenesis from corm explants of *Crocus vernus*. Microcorms obtained from field grown plants were decontaminated and divided into several parts, cultured on the Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar, and different concentrations and combinations of plant growth regulators. Somatic embryos were induced both light and dark conditions but culturing the explants two weeks in the dark followed by three weeks under light resulted in high frequency of embryo formation. The greatest percentage of embryo induction was achieved when the explants cultured on the MS medium with 0.5 mg L^{-1} thidiazuron (TDZ) and 0.1 mg L^{-1} napthaleneacetic acid (NAA). Embryo maturation and germination were achieved on a MS medium with 2.0 mg L^{-1} BA and 1.0 mg L^{-1} GA_3. When the globular embryos were transferred to the MS medium containing 6% (w/v) sucrose, 2.0 mg L^{-1} BA, and 1.0 mg L^{-1} GA_3 resulted in the highest frequency of plant regeneration and microcorm formation. The microcorms developed new shoots when they were cultured on the half-strength MS medium with 1.0 mg L^{-1} GA_3.**

Keywords-Crocus; microcorm; somatic embryogenesis; Thidiazuron

I. INTRODUCTION

The genus *Crocus* with about 85 species comprises important medicinal and ornamental plants. *Crocus vernus* (L.) Hill is a perennial herbaceous species commonly known as Dutch crocus that has a wide distribution in Europe, North Africa, and temperate Asia. It is typically propagated by corms. Low rate of daughter corm formation under natural conditions is limited availability of propagating material for field cultivation. Tissue culture techniques can play an important role in the clonal propagation and qualitative improvement of this important plant species. Moreover, in vitro propagation of plants could help in raising disease free healthy clones on a large scale for the horticultural industry. Large scale production of elite clones through micropropagation or somatic embryogenesis solves this problem. Clonal propagation through somatic embryogenesis has become an essential method for the improvement of most economically important plants [1]. Direct embryogenesis reduces the time required for plant propagation, which may be beneficial to minimize culture-induced genetic changes. Somatic embryogenesis offers an excellent experimental system to study the physiological and biochemical aspects of embryo development [2]. Somatic embryos are of extensive practical importance in large scale propagation [3], genetic manipulations [4], cryopreservation [5], and artificial seed production [6]. Although tissue culture in the *C. vernus* has been previously reported [7], to date there is no report on somatic embryogenesis and plant regeneration. The aim of this study was to develop a reliable and efficient in vitro propagation protocol for *C. vernus*.

II. MATERIALS AND METHODS

The corms were collected from greenhouse-grown plants, washed thoroughly in running tap water for 30 min, and then washed with distilled water. The explants decontaminated with a 70% (v/v) ethanol for 60 sec, 2.0% sodium hypochlorite for 10 min, and 0.01% mercuric chloride for 15 min. Each treatment was followed by 3-4 washes with sterile distilled water, cut into 0.5-1.0 cm segments and cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of BA and NAA. Hormone-free MS medium was used as the control. Number of explants initiating shoot buds and average number of shoot buds per explant were recorded after 45 days. In each experiment, about 25 explants were used and the experiment was repeated thrice.

The medium consisted of MS basal salts and vitamins [8], 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 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 basal medium prior to pH adjustment and sterilization. All cultures were maintained at 25±2°C under a 16 h photoperiod with 30 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent light (PHILIPS 40 W tubes) unless otherwise stated.

Corm and shoot bud explants were cultured on the MS medium supplemented with TDZ and NAA. The explants were maintained for 0, 7 and 14 days at 25±2ºC in darkness and then exposed to light of 30 µmol m^{-2} s^{-1} with a light/dark cycle of 16/8 h. In each experiment, about 25 explants were used and the experiment was repeated thrice. The frequency of somatic embryo formation was determined by counting explants forming somatic embryos from the total number of the cultured explants after 45 days of culture. Somatic embryos (globular stage) were separated from the explants and cultured on the MS medium supplemented with sucrose

(3% or 6%, (w/v), 2.0 mg L^{-1} BA and 1.0 mg L^{-1} GA_3 for maturation and germination. Somatic embryos were sub-cultured at intervals of 3 weeks. Embryo germination percentage was calculated as (number of germinated somatic embryos/ total number of somatic embryos) × 100.

Data were statistically analyzed by analysis of variance (ANOVA) followed by Duncan multiple range test at 5% probability level. Data analysis was performed using SAS computer package (SAS Institute Inc., NC, USA).

III. RESULTS AND DISCUSSION

When corm explants of *C. vernus* were cultured on the MS basal medium, they did not induce shoot buds or callus. In contrast, when the same explants were grown on the MS medium containing PGRs they produced shoot buds. When MS medium was supplemented with different concentrations of BA, shoot buds emerged from the corm explants starting 4 weeks after culture. Plessner et al. (1990), reported that Shoot development on corm explants was promoted by cytokinins. The highest percentage of shoot formation with an average number of 2.0 shoots per explant was observed on MS medium supplemented with 2.0 mg L^{-1} BA (Table 1). In general, a combination of cytokinin and auxin enhanced shoot bud induction. The frequency of shoot induction and number of shoots increased when BA was combined with NAA in comparison to BA alone. The greatest percentage of shoot induction (94.2) with a mean number of 4.3 shoots per explants was achieved when the medium was supplemented with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA (Fig 1A). Similar results were obtained with the same hormones by Bhagyalakshmi [9]. and Karaoglu et al. [10].

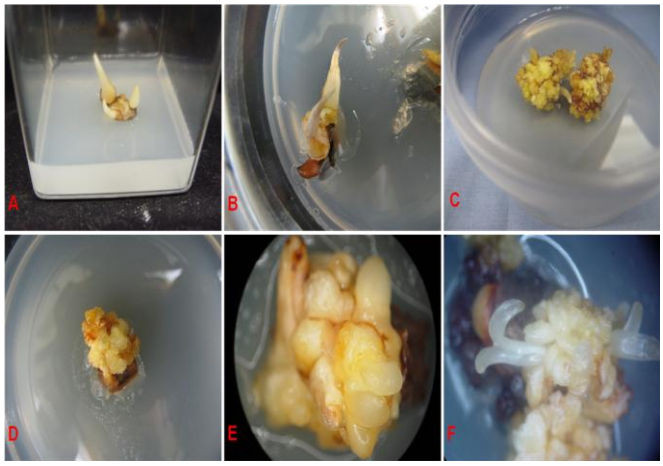

Fig. 1. Direct shoot regeneration and somatic embryogenesis of Crocus vernus. A, shoot regeneration from corm explants after 45 days of culture; B and C, somatic embryo formation from shoot tip explants of C. vernus after 45 days of culture; D and E, somatic embryo formation from corm explants of C. vernus; and F, cotyledonary stage.

TABLE 1. EFFECT OF PLANT GROWTH REGULATORS (PGR) ON SHOOT BUD INDUCTION FROM CORM EXPLANTS OF CROCUS VERNUS.

Conc. mg L^{-1}		Shoot induction (%)	No. of shoots/explant
BA	NAA		
0	0	0	0
0.5	0	72.8 e	1.3 d
1.0	0	80.3 d	1.8 cd
2.0	0	86.0 c	2.0 c
1.0	0.5	91.0 b	3.0 b
2.0	0.5	94.2 a	4.3 a

Means followed by same letter (s) within a column are not significantly different (P<0.05)

The formation of somatic embryos was observed when corm and shoot bud explants were cultured on the MS medium was supplemented with TDZ and NAA (Fig. 1B-E). Of the two explants, shoot bud explants found to be the most effective for somatic embryo induction. Corm and shoot bud explants produced maximum number of somatic embryos at 0.5 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA and the percentage of somatic embryo induction were 97 and 100, respectively (Table 2). Embryo maturation and germination were achieved on the MS medium with 2.0 mg L^{-1} BA and 1.0 mg L^{-1} GA_3 (Fig. 2B). When the globular embryos were transferred to the MS medium containing 6% (w/v) sucrose, 2.0 mg L^{-1} BA, and 1.0 mg L^{-1} GA_3 resulted in the highest frequency of plant regeneration and microcorm formation (Fig. 2A). The microcorms developed new shoots when they were cultured on the half-strength MS medium with 1.0 mg L^{-1} GA_3.

TABLE 2. EFFECT OF CONCENTRATIONS OF TDZ PLUS 0.1 MG L-1 NAA ON SOMATIC EMBRYO INDUCTION FROM CORM AND SHOOT TIP EXPLANTS OF C. VERNUS.

Conc. mg L^{-1}	Somatic embryo induction (%)	
TDZ	Corm	Shoot bud
0.1	78.2 d	92.0 c
0.5	97.0 a	100 a
1.0	90.3 b	96.4 b
2.0	88.7 c	89.0 d

Means followed by same letter (s) within a column are not significantly different (P<0.05)

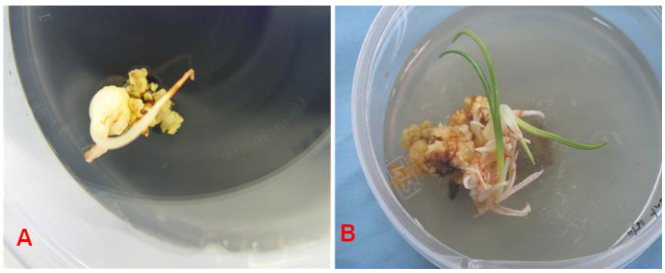

Fig. 2. Effect of BAP and GA3 on microcrom induction and plant regeneration of C. vernus. A, microcrom induction; and B, somatic embryos germinated on MS medium containing 6% (w/v) sucrose, 2.0 mg L-1 BA, & 1.0 mg L-1 GA3.

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