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Influence of nutrient media on callus induction, somatic embryogenesis and plant regeneration in selected Turkish crocus species

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ABSTRACT

Callus induction, somatic embryogenesis and plant regeneration were initiated in selected five species of Turkish crocus using three diffrent explants (leaf, stem and corm) cultured on four different media (MS, GB5, LS and CHE). The highest frequencies of callus induction (100%) and shoot regeneration (70%, with 7.2 shoots/callus) were found in the crocus species *Crocus oliveri* ssp. *Oliveri*, using the MS medium containing 5% (w/v) sucrose supplemented with (4 mg/L NAA + 4 mg/L TDZ) and (2 mg/L IAA + 2 mg/L TDZ + 2 mg/L BAP). When the embryogenic calli were transferred into the four nutrient media containing (2 mg/L IAA + 2 mg/L TDZ) and 100 mg/L ABA, these further developed into cotyledonary embryos. Maximum number of somatic embryos (2.9 embryos per leaf explant, with a frequency 46.6%) was obtained in *C. oliveri* ssp. *Oliveri*. During subculture using the half strength media, cotyledonary embryos gradually developed into plantlets.

1. Introduction

The genus *Crocus* belongs to the large Iridaceae family that includes more than 80 species, of which approximately 30 are cultivated worldwide. The name saffron applies indistinctly to *Crocus sativus* L., an herbaceous plant with corms, which grows widespread throughout the tropical and subtropical regions of the northern hemisphere. However, saffron (the dried orange-red trifid stigma of *C. sativus* and the costliest culinary spice of the world) is an autumn-flowering, triploid ($2n = 3\times = 24$) male-sterile plant, propagated vegetatively by means of corms. Saffrons have been used for their smell, color and healing qualities and are cultivated for their spice value for more than 4000 years [1]. Spain and Iran are the largest producers of the spice, accounting together for more than 80% of the world production. In the Mediterranean region, in Italy, Greece, and Turkey, saffron is also cultivated, though on a smaller scale. However, Turkey is among the richest country in producing the *Crocus* species. There are almost 70 *Crocus* taxa and 31 of them are endemic to Turkey [2]. When the diversity of the taxa is taken into consideration, Turkey is regarded as the gene center of this particular species. Currently, the production of saffron in the Turkish region has decreased owing to the drift of agricultural practices.

Saffron is a spice of great economic value; one kilogram of good quality saffron produced from *C. sativus* L. can cost more than 2000 US dollars [3]. Approximately 150,000 flowers are needed to produce one kilogram of dried saffron and in order to grow this amount, one would need about 2000 m^2 of land under cultivation [4]. According to Sobolev et al. [5], biologically active compounds such as crocetin, picrocrocin, and safranal are found in saffron. These compounds make saffron a promising candidate for imparting flavor and nutritional ingredients into the prepared food. The highly water soluble crocins (or, crocetins) are widely used as a natural food colourant and also act as antioxidants, thus protecting cells and tissues against oxidation [6]. Saffron is also used extensively in the Indian medicinal system (*Ayurveda*) for healing a variety of diseases including arthritis, cold, asthma, acne, skin disorders, impotence and infertility [7]. Saffron finds its applications as a textile dye and in perfume preparations [8]. It has

Abbreviations: ABA, abscisic acid; BAP, 6-benzylaminopurine; CHE, Chee and Pool medium; GB5, Gamborg's B-5 medium; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; LS, Linsmaier and Skoog medium; MS, Murashige and Skoog medium; NAA, α-naphthalene acetic acid; TDZ, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea.

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traditionally been considered as an anodyne, antidepressant, respiratory decongestant, antispasmodic, aphrodisiac, diaphoretic, emmenagogue, expectorant and sedative. Saffron is also used in folk medicine as a remedy against scarlet fever, smallpox, cold, asthma, eye and heart disease [9]. Apart from these, often saffron is used to help clear up sores and to reduce the discomfort of teething infants [10]. Recently, there has been a growing interest in its anti-carcinogenic compounds which can be used in the prevention of tumors [11].

Of late, there is an increasing interest in the exploitation of tissue culture and genetic improvement of *C. sativus* L., but the other members of genus *Crocus* have received very little attention. Saffron is a triploid plant conventionally propagated by corm, which makes its continued cultivation a bit problematic. Yield and quality improvement of saffron through breeding have not been possible because of the sterility problem [12]. *In vitro* culture is proved to be greatly advantageous for mass propagation and development of diseases-resistant cultivars, mainly for vegetatively propagated crops such as saffron [13]. Due to its ecological and economic importance, there is a need for germplasm preservation of the *Crocus* species. This could in turn contribute to the maintenance of genetic material of *C. sativus* allies, which is one of the major topics of the current saffron research [14].

Several reports have appeared on *in vitro* propagation of *Crocus* species [13,15–24]. An efficient *in vitro* tissue culture system is the need of the hour since the *Crocus* species are threatened in many countries where they are cultivated [25]. Therefore, improvement of an *in vitro* tissue culture protocol for the species is of great importance for germplasm conservation as well as for commercial propagation. However, there are no reports concerning *in vitro* regeneration of the selected five Turkish *Crocus* species. In this paper, for the first time, we describe a simple and very effective protocol for indirect somatic embryogenesis, organogenesis and plant regeneration of the five *Crocus* species [*Crocus speciosus* ssp. *Speciosus*, *Crocus oliveri* ssp. *Oliveri*, *Crocus pestalozzae* (endemic), *Crocus abantensis* (endemic) and *Crocus paschei* (*C. abantensis* × *C. oliveri* ssp. *Oliveri*) (endemic)]. The current protocol can easily be extended to other *Crocus* species for their propagation and conservation.

With respect to the commercial exploitation of the five crocus species under consideration in this article, all of these are sold in the local Turkish markets for their culinary use and also for use in folk medicines. Since the middle ages, the Persian, Mesopotamian and Arabian world have been well-known for their exotic food preparations in royal kitchens, where saffron was used as an integral ingredient. While data on individual species are not available, there are growing demands of the five crocus species among spice-loving consumers in the Middle East and in South East Asia.

2. Materials and methods

2.1. Plant materials

Crocus species were collected from natural populations of Bolu province [*C. speciosus* ssp. *Speciosus*: Abant Izeet baysal Campus (altitude 850 m, Coordinates: N 40° 42.864′, E 31° 31.166′); *C. oliveri* ssp. *Oliveri, C. abantensis,* and *C. paschei*: Abant lake, on the road of Mudurnu (altitude 1480 m, Coordinates: N 40° 35.549′, E 32° 16.956′)] and Istanbul [*C. pestalozzae* (altitude 130–155 m, N 40° 57′, E 29° 47.51′)] in Turkey. Identification of the collected species was done according to Davis et al. [2], and specimens were deposited at the Herbarium, Abant Izzet Baysal University, Turkey.

2.2. Sterilization

The corm, leaf, and stem were thoroughly washed under running tap water for 1 h and then cleaned with detergent Tween-20 with the help of a sable hair brush. These corm, leaf, and stem were then surface-disinfected with a savlon antiseptic (0.6 ml/100 ml; v/v) solution for 30 min. After rinsing with distilled water, the surface was sterilized with 70% (v/v) ethanol for 30–45 s and 0.1% (w/v) freshly prepared aqueous mercuric chloride ($HgCl_2$) for 10–20 min in a laminar flow cabinet, followed by washing with sterile distilled water. Small segments of the corm, leaf and stem were cultured in media for further experimentation.

2.3. Media preparation and culture conditions

The culture media used were MS (Murashige and Skoog [26]), GB5 (Gamborg's B-5, 1968) [27], LS (Linsmaier and Skoog [28]) and CHE' (Chee and Pool [29]) with 2% or 5% (w/v) sucrose and 0.8% agar. The pH of the medium was adjusted to 5.8 with 0.1 N HCl or 0.1 N KOH prior to autoclaving (121 °C and 1.06 kg cm^{-2} pressure for 15 min). The cultures were incubated at 23 ± 1 °C for the first two days in dark and then transferred to 16 h light: 8 h dark photoperiod (provided by cool-white fluorescent light, irradiance 50 μ mol^{-2} S^{-1}) and a relative humidity level of 50–60% were maintained.

2.4. Induction of callus and somatic embryogenesis

Three different types of explants (leaf, stem and corm) were used as explants for callus induction in MS, GB5, LS and CHE' media, each supplemented with 4 mg/L TDZ + 4 mg/L NAA as shown in Table 1. Callus and embryogenic callus induction frequencies were calculated as the percentage of cultured explants (leaf, stem and corm) producing callus and embryogenic callus respectively. After 2 months of culturing, calli were produced from all the explants. Nonembryogenic calli were compact and granular in shape. Embryogenic nature of the cultures was maintained by visual identification and selection of embryogenic sectors and removal of soft and translucent nonembryogenic portions were done at the time of subculturing. For the maintenance of embryogenic potential, embryogenic calli together with globular embryos were transferred to the media (MS, GB5, LS and CHE') containing 2 mg/L IAA + 2 mg/L TDZ + 100 mg/L ABA + 5% sucrose (w/v). The cultures were incubated at 23 ± 1 °C in 16 h light: 8 h dark photoperiod, for maturation of somatic embryos (cotyledonary stages). The embryos were then subcultured after 4 weeks in half strength media for further conversion into plantlets. All media formulations and plant growth regulators (PGR) were purchased from Duchefa Biochemie (Netherlands). Each experiment was repeated three times, each using 20 replicates (*i.e.*, a total of 60 explants per treatment). Both the frequency (%) of callus developing somatic embryos and a mean number of somatic embryos per explant derived callus were recorded after 8 or 12 weeks of culture, respectively.

2.5. Shoot regeneration

Selected nonembryogenic calli (Table 1) were further subcultured on media supplemented with 2 mg/L IAA + 2 mg/L TDZ + 2 mg/L BAP for shoot regeneration. The shoots obtained from calli were maintained through regular subculturing at 4 weeks intervals on fresh medium with the same composition in order to obtain more shoots. After sufficient growth, shoots (6–7 cm length) were excised from the parent culture and transferred to the MS rooting media containing IAA or IBA (0–2 mg/L). Root did not differentiate from the regenerated shoots.