

IN VITRO CULTIVATION OF SAFFRON (*CROCUS SATIVUS* L.) AND ASSESSMENT OF GENETIC HOMOGENEITY USING IPBS MARKERS

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Abstract

This research was an attempt to develop a protocol for the most optimal in vitro cultivation of saffron (*Crocus sativus* L.). The dried stigmas of the plant are known as spice imparting colour, flavour and aroma to foods and beverages. The appropriate disinfection procedure to introduce corm explants into in vitro culture was established. The optimum corm formation and corm growth (mean number of 3.6 per explant) was achieved on Murashige and Skoog (MS) medium containing 5 mg/l 6-benzylaminopurine (BAP) and 1 mg/l indole-3-acetic acid (IAA), while MS supplemented with 1 mg/l indole-3-butyric acid (IBA) was the best medium for root development. The fingerprinting profiles of the field saffron and the in vitro cultures assessed by iPBS markers were found identical which implies that no genetic alterations have occurred in response to in vitro cultivation.

Key words: corm, tissue culture, organogenesis, surface sterilization, molecular markers

Introduction. *Crocus sativus* L. (Iridaceae) is a fall-flowering species producing saffron and has been cultivated for centuries because of its aromatic, colouring and medicinal properties [1]. The dried stigmas of saffron flowers are the most expensive unique spice, popularly known as the “Golden Condiment”. *C. sativus* is

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a triploid perennial sterile plant, which does not produce seeds and is propagated vegetatively by annually replacing corms. A corm survives for only one season, reproducing via division into “cormlets” that eventually give rise to new plants. The productivity of saffron crocus under natural field conditions is limited due to the low propagation rate of cormlets and the high contamination levels of field-grown corms caused by fungi and bacteria [2]. The field cultivation of saffron and its manufacture requires large input of manual work including collecting stigmas from flowers in autumn [3]. The micropropagation as an alternative method of propagation allows large-scale production of quality, disease-free plants, regardless of climatic conditions [4]. There are many reports concerning micropropagation of *C. sativus* describing the process with different efficiency [2,3,5,6]. Due to the high economic value of saffron and its commercial significance, Bulgarian farmers are interested in growing *C. sativus* on the site of tobacco plantations.

The aim of the study was to establish the optimal culture conditions to introduce in vitro the valuable crop *C. sativus*, to examine the effect of plant growth regulators (PGRs) on shoots, corms and roots production as well as to assess genetic homogeneity of in vitro obtained plant material through iPBS markers.

Materials and methods. Plant material and disinfection of saffron corms. The saffron corms were collected from the experimental field of Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, by courtesy of Dr. Grigor Zehirov. The corms (harvested before the onset of flowering) were washed under running tap water with liquid soap for 30 min. Various sterilizing agents (ethanol, hydrogen peroxide, mercuric chloride, fungicide topsin and commercial bleach containing 4.85% sodium hypochlorite) applied in different concentration and combinations were tested. Each procedure was followed by 5-fold rinse in autoclaved distilled water. Six different disinfection procedures, each consisting of several steps, were applied: A: 75% C₂H₅OH (1 min) → 0.1% HgCl₂ (20 min); B: 75% C₂H₅OH (1 min) → 0.01% topsin (10 min) → 50% bleach (10 min) → 1.6% HgCl₂ (5 min); C: 70% C₂H₅OH (5 min) → 50% bleach (10 min) → 7% H₂O₂ (15 min); D: 0.01% topsin (10 min) → 50% bleach (10 min) → 0.1% HgCl₂ (10 min); E: 70% C₂H₅OH (6 min) → 100% bleach (5 min) → 7% H₂O₂ (15 min); F: 70% C₂H₅OH (5 min) → 100% bleach (5 min) → 0.1% HgCl₂ (20 min). The screening of disinfection was performed after 10 days and 2 months of explants treatment.

Nutrient medium composition and culture conditions. Explants of 0.5 to 1 cm in diameter with meristematic nodes, as well as small whole corms were cultured on MS nutrient medium [7] supplemented with 6 mg/l BAP (MSB6) or with 2 mg/l BAP and 0.5 mg/l α -naphthalene acetic acid (NAA) (MSB2N0.5). Also, antibiotics 200 mg/l Ampicillin and 10 mg/l Amphotericin B were added to the abovementioned nutrient media. The sucrose and agar concentration were 3% (w/v) and 0.7% (w/v), respectively; the pH of the media was adjusted to pH 5.7.

Well-developed shoots were cultured on four different nutrient media con-

sisting of MS containing 4% (w/v) sucrose, 1 mg/l silver nitrate (AgNO_3), and supplemented with different PGRs i. e. BAP, IAA, thidiazuron (TDZ) and IBA. The composition of the media was as follows: i) MSB5I1 – MS supplemented with BAP (5 mg/l) and IAA (1 mg/l); ii) MST5I1 – MS containing TDZ (5 mg/l) and IAA (1 mg/l); iii) MSI1 – MS supplemented with IAA (1 mg/l); iv) MSIB1 – MS containing IBA (1 mg/l).

All media were sterilized by autoclaving at 120 °C for 20 min under the pressure of 1.1 kg/cm² and cultures were incubated at 23 ± 2 °C under 16 h photoperiod, illuminated with cool white fluorescent tubes. Each treatment contained 20 explants with 2 replications per treatment.

Analysis of genetic homogeneity of saffron material. The retrotransposon-based inter-priming binding sites (iPBS) method was performed to assess the level of genetic homogeneity of saffron material. The iPBS method is a high-throughput multi-marker method for identification of genome-wide variability related to the insertion/loss of LTR retrotransposons and/or to DNA sequence variations (nucleotide substitutions or indels) in adjacent regions [8]. Total genomic DNA was isolated from young leaf tissue of field grown plants (bulk probe) and from in vitro samples (in vitro regenerated shoots) using DNeasy Plant Mini kit (Qiagen). Two iPBS primers previously described by KALENDAR et al. [8] were used for single primer Polymerase Chain Reaction (PCR) – primer 2224 (5'-ATCCTGGCAATGGAACCA-3') and primer 2378 (5'-GGTCCTCATCCA-3'). The PCR was performed in 20 µL of the reaction mixture containing 50 ng of DNA template, 1X PCR buffer *DreamTaq* buffer, 0.25 mM dNTPs, 1 µM of primer for 12 nt primer or 0.6 µM for 18 nt primer, 1 units *Taq* DNA polymerase (*DreamTaq*, Fermentas) and 0.04 units Pfu DNA Polymerase (Fermentas). Fifteen-microliter aliquots of PCR products were resolved by 1.7% (w/v) agarose gel electrophoresis at 70 V for 6 h in 1X STBE buffer (10X STBE: 0.25 M Tris- H_3BO_3 , 40 mM $\text{Na}_2\text{B}_4\text{O}_7$, 10 mM EDTA, pH 8.6) and detected by ethidium bromide staining. Each PCR reaction was performed in 3 replicates.

Data analysis. Values of mean number of corms and roots were subjected to analysis of variance (ANOVA). The statistical significance between the mean values was assessed by applying Duncan's Multiple Range Test. A probability level of $P \leq 0.05$ was considered significant. Values in Table 1 are presented as means of 20 explants per treatment with standard errors (value ± SE).

Results. Optimization of corms disinfection. The surface sterilization efficiency (percentage of non-infected corms) was in the range 10–40% after 10 days culture period, and from 0 to 30% after 2 months, respectively. The highest rate of clean culture (40%) and the highest percentage of non-infected cormlets were achieved after disinfection procedure F. Furthermore, it was observed that the endogenous contamination (mostly by bacteria and fungi) that was latent in the developing explants reappeared 3 to 6 weeks after the culture initiation and hampered the obtaining of aseptic explants. Thus, the contamination level

of biological material after 2 months of incubation (F disinfection) decreased to 30%. Corm explants which were sterilized through disinfection procedures C, D, and E failed to overcome endogenous contamination after 2 months. Only 10% of cultures derived from A and B disinfection were sterilized after the period of 2 months.

Effect of media composition and PGRs on direct organogenesis.

The sprouted shoots were developed after 2–3 weeks of culture from corm segments with meristematic nodes (Fig. 1A). MSB6 gave the best response on corm explants; as a result, approximately 90% of explants yielded sprouting shoots. Lower level of shoot induction (70%) was observed on MSB2N0.5. The results indicated that BAP alone or BAP combined with NAA are effective PGRs for enhancing organogenesis in *C. sativus*. The shoot length reached 10–14 cm after 8 weeks of culture (Fig. 1A). After this incubation period, the onset of development of full leaves was observed (Fig. 1C). In addition, multiple white leaved shoots formed directly from corm explants were noticed (Fig. 1B).

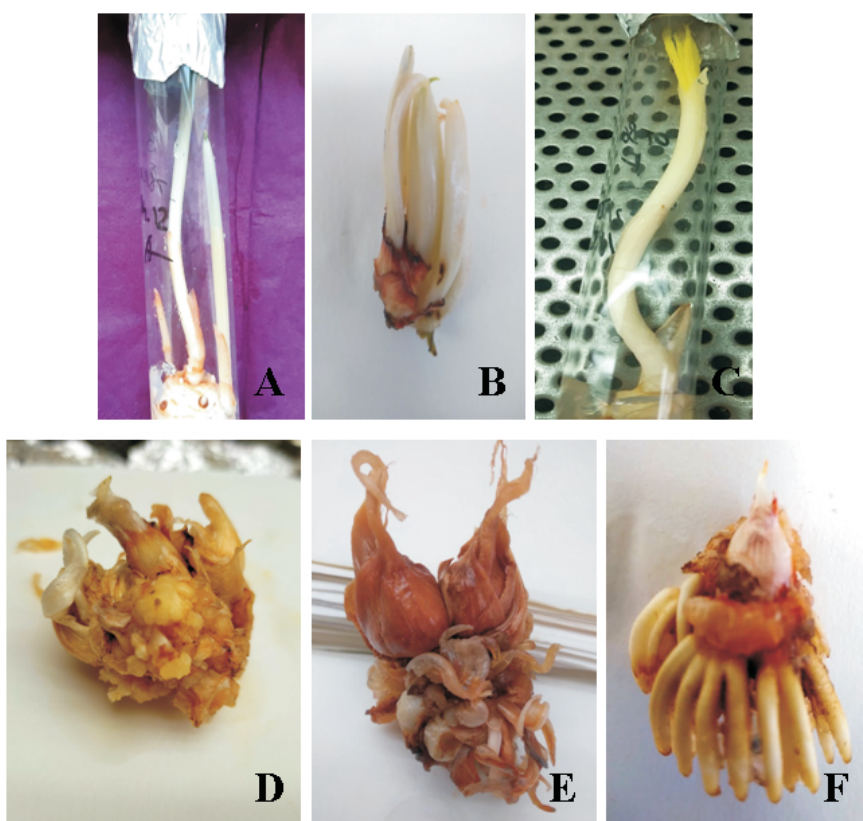


Fig. 1. Morphogenetic response of corm explants: A) sprouted shoots, B) multiple white leaved shoots, C) development of full leaves, D) microcorm induction, E) developed microcorms, F) cormlet with roots

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Effect of PGRs on microcorm and root formation

Nutrient medium	Microcorm formation %	Microcorm, number	Roots, number	Root length, cm
MSB5I1	60	3.6 ± 0.22^c	3.1 ± 0.27^a	0.8 ± 0.06^a
MST5I1	10	1.3 ± 0.21^{ab}	none	none
MSI1	20	0.9 ± 0.18^a	4.2 ± 0.39^a	1.24 ± 0.11^b
MSIB1	40	1.6 ± 0.16^b	5.4 ± 0.47^b	1.45 ± 0.09^b

*MS based medium supplemented with 1 mg/l AgNO₃ and 4% sucrose. The values present the means $\pm$ SE of two independent experiments; Mean values followed by the same letter are not significantly different at $P < 0.05$ by Duncan's multiple range test

It was evident that culture media composition had a significant effect on microcorm formation and root development (Table 1). According to the results of these experiments, MSB5I1 yielded the best responses (60%) with respect to microcorm formation (Fig. 1D). The mean number of microcorms per explant was 3.6 after 10 weeks of culture. In addition, 1.6 microcorms were formed on MSIB1 medium; on the same substrate, the highest number of roots (5.4) was induced (Table 1). Higher sucrose concentration (4%) was determined to be an important addendum to achieve a good response in corm initiation and growth. The whole process from the establishment of in vitro culture to the obtaining of mature new corms takes 7 months: 2 months for bud sprouting and shoots development; 3 months for induction and growth of microcorms (Fig. 1D, E); 2 months for development and rhizogenesis of cormlets (Fig. 1F).

Assessment of genetic homogeneity. It was found that the iPBS method generated easily scorable and reproducible bands with an amplification range 250–5000 bp. For both primers, marker fingerprints were found to be identical between the field saffron and the in vitro cultures which mean that no genetic alterations have occurred in response to in vitro cultivation (Fig. 2). In addition, a lack of polymorphism between different in vitro plants was also observed, which implies their genetic homogeneity and stability of in vitro obtained plant material.

Discussion. The possibility of high contamination levels, especially in cases where underground organs are used as a source of explants, is one of the key problems in introducing plants to tissue culture [9]. It was previously reported that the degree of contamination in corms and other underground organs can reach as high as 95 to 100% [4]. Thus, saffron as a geophyte bulbous species is considered a difficult material for in vitro introduction. The effectiveness of disinfection may be dependent on the type, concentration and kind of treatment with the sterilizing agent [2,6]. In our study, disinfection procedure F was the most successful; it involved several steps – sequentially immersing in 70% ethanol, 100% bleach and 0.1% HgCl₂. The sterilization procedure B was similar to those

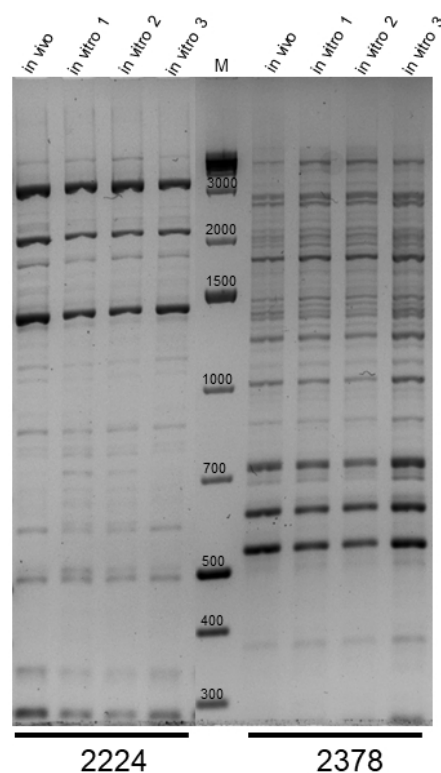


Fig. 2. Assessment of genetic homogeneity of the in vivo saffron and three in vitro samples using the iPBS method with primers 2224 and 2378, M – O'Gene Ruler 1 kb Plus DNA Ladder

reported by YASMIN et al. [4]; however, they observed higher percentage of clean viable saffron cultures. The observed difference demonstrates the important role of the source of initial corm explants. Our material was obtained from filed grown plants, while that used by the other authors was derived from healthy aseptic saffron corms from the nursery.

Combinations of cytokinins (BAP, kinetin, thidiazuron) with auxins (2,4-dichlorophenoxy acetic acid, IBA, NAA) are among the most commonly used PGRs for adventitious shoot induction of saffron [2,5]. In our study, the addition of BAP alone to culture medium at a high concentration (6 mg/l) was more effective for shoot sprouting and development compared to the combination of BAP and NAA. The data are in accordance with the results reported by SHARMA et al. [10] and ÇAVUŞOĞLU et al. [11], who also observed a high percentage of shoot induction from corm buds in a nutrient medium MS containing 6 mg/l BAP.

Previous studies have reported the formation of microcorms at the base of

developed shoots on nutrient media with different compositions [9]. Several authors emphasized the importance of BAP (from 2 to 5 mg/l) and NAA (0.5 mg/l) combination of daughter corm formation in saffron in association or with other additives like paclobutrazol (1.5 mg/l), high sucrose content (80 g/l) [3,9,10]. Our data showed that MS medium supplemented with 5 mg/l BAP, 1 mg/l IAA and 1 mg/l AgNO₃ is the best one for induction and growth of cormlets among the tested nutrient media. The presence of silver nitrate in the medium for microcorm and root induction that reduced the endogenous contaminants and increased the morphogenic response was also described in other plant species [12,13]. Furthermore, besides AgNO₃, 4% sucrose was also included in the growth media. It was observed that sucrose concentration is a critical factor in initiating root formation with a direct influence both on the rooting response and on the quality of roots formed [14]. In our study, the nutrient medium containing TDZ was less effective for cormlet induction and development. In contrast, high efficiency of microcorms per corm slice was described on half strength MS medium complemented with TDZ (20 µM) and IAA (10 µM) [2]. For rhizogenesis induction, auxins alone or in combination with cytokinins were applied [11]. The highest number of roots per corm was accomplished on nutrient medium containing 1 mg/l IBA. The results support the findings of ZEYBEK et al. [5], who reported the best rooting response on medium with IBA, although they used a high concentration (2 mg/l). The elaborated protocol in this study allows the obtaining of mature cormlets within 7 months. Sharma et al. [10] reported that microcorm formation from apical buds takes 9 months under in vitro conditions and 22 months under field conditions.

In vitro cultivation has been considered a stress factor that can lead to genetic variations in plants [15]. Therefore, the assessment of genetic uniformity of regenerated plants is a prerequisite step before proving the success of the micropropagation protocol. Molecular markers have been effectively applied to assess the genetic fidelity and stability of various plants after in vitro culture [16]. Previous studies have shown that different environmental stress stimuli including in vitro cultivation can trigger the dynamics of LTR retrotransposons – the most abundant fraction of plant genomes [17]. In this study, genetic homogeneity between in vitro regenerated saffron shoots and field grown plants were confirmed by transposon-based iPBS markers. Other authors also applied iPBS markers to confirm the genetic fidelity of date palm cultivars maintained in the field and those maintained under in vitro conditions [18]. Although LTR retrotransposon markers display high genome coverage, there still may exist mutations not detected by iPBS markers. To our knowledge, this is the first report on the assessment of genetic homogeneity in micropropagated plants of *C. sativus*.

Conclusions. The protocol reported in this study offers a potential system for in vitro micropropagation of saffron (*Crocus sativus* L.) through direct organogenesis. The application of silver nitrate and higher sucrose concentration in the culture media increased the morphogenetic response of corm explants. However,

further studies will be useful to improve the adaptation of in vitro plantlets to field conditions. By using iPBS markers no polymorphic bands were detected between field grown *C. sativus* plants and in vitro shoots, confirming the genetic homogeneity and stability of in vitro obtained plant material. The in vitro propagation of *C. sativus* may help in the growth and production of saffron thus offering the opportunity for its introduction into agriculture.

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