Production and Characterization of Tissue Cultures of Four *Crocus* Species from the Carpathian Basin

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We aimed to produce tissue cultures and plant regeneration from endangered *Crocus* species: *C. scepusiensis*, *C. tommasinianus*, *C. vittatus* ("Verni" series of the genus) and *C. banaticus*. For initiation of cultures we used a plant growth regulator (PGR) combination used for *in vitro* culture of saffron and its relatives: 10 mg L^{-1} α-naphthaleneacetic acid (NAA) and 1 mg L^{-1} 6-benzyladenine (BA). Shoot tips of young seedlings (*C. scepusiensis*) and corms (for the rest of species) were used as explants. *C. scepusiensis* explants developed into organogenic calli. On media with decreased NAA and with or without increased BA concentration, calli produced stigma-like structures and/or shoots and whole plants. In the other species, callus initiation medium induced callus formation with abundant somatic embryos. In *C. tommasinianus*, embryos developed shoots when auxin content of medium was decreased. In *C. banaticus*, a decrease of auxin with or without an increase in cytokinin content led to shoot or whole plant regeneration, as in *C. scepusiensis*. In the case of *C. vittatus* and *C. banaticus*, initiation and/or maintenance of cultures on indole-3-butyric acid (IBA) and increased sucrose concentration stimulated whole plant regeneration and *in vitro* cormlet development. *C. scepusiensis* and the rest of cultures (organogenic vs. embryogenic) differed at the biochemical level: *C. scepusiensis* cultures had higher (yet still low) enzymatic antioxidant (catalase, peroxidase) activities. With respect to catalase isoenzyme patterns, *C. banaticus* was different from the rest of cultures, demonstrating its distinct taxonomical position. Besides germplasm preservation use of the present cultures, they have a potential biotechnological value.

Keywords: *Crocus*, Carpathian Basin, organogenesis, embryogenesis, plant regeneration, antioxidant enzymes

INTRODUCTION

Saffron (*Crocus sativus* L., Iridaceae) is a well-known crop species, cultivated mainly for its dried stigmas, the most expensive spice that contains apocarotenoids with antioxidant properties (Gantait and Vahedi, 2015). The genus *Crocus* includes a considerable number of wild-growing species, several of them being endangered, thus their conservation is important (Fernández et al., 2011). The species involved in our study were: (i) species taxonomically belonging to the "Verni" series: *C. scepusiensis* Borbás Ex Kulczyński endemic to Slovakia and Southern Poland, the Northern Carpathians, C. *tommasinianus* Herbert native to Hungary and the Balkans, *C. vittatus* Schlosser

& Vukotinović native to Croatia and Hungary (*C. heuffelianus* Herbert, another member of this taxonomical series, has already been successfully cultured *in vitro* in our laboratory, see Demeter et al., 2010); (ii) the taxonomically distinct *C. banaticus* Gay, native to the Banat (Romania, Serbia), as well as Romanian and Ukrainian regions of the Carpathian Basin. *C. tommasinianus* and *C. vittatus* are red list species in Hungary (Király, 2007), while *C. banaticus* is protected in Romania (Pătroescu and Rozyłowicz, 2000).

Tissue culture of saffron and its relatives is of double importance: (i) concerning *C. sativus*, it has biotechnological value mainly in the search for alternative to traditional breeding methods of producing biologically active compounds (Gantait

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and Vahedi, 2015); (ii) germplasm preservation involves not only different genotypes of saffron, but also of its wild relatives. The genetic diversity of saffron is decreasing, partly because, being a triploid hybrid, it does not reproduce sexually (Fernández et al., 2011). Thus, tissue culture may offer a good method to preserve valued genotypes. As for wild-growing species, their preservation via tissue culture is important not only for conservation purposes, but also in the search for novel biologically active compounds (Demeter et al., 2010).

There is a vast literature concerning tissue culture methods of saffron. These include plant regeneration from both floral explants and vegetative tissues via callus cultures, as well as direct micropropagation from corm explants by excluding the callus stage or protoplast culture (see Gantait and Vahedi, 2015 for a comprehensive review). As for wild relatives, we can find elegant plant regeneration methods for *C. cancellatus*, *C. pallasii,* etc. (Karamian and Ebrahimzadeh, 2001; Karamian, 2007). For species belonging to the *Verni* series of Crocus, there is only a limited number of tissue culture studies. Demeter et al. (2010) established an efficient plant regeneration method from embryogenic calli of *C. heuffelianus*. Sivanesan et al. (2012, 2014) reported efficient somatic embryo production and shoot regeneration from corm explants of *C. vernus*. Callus cultures of *C. scepusiensis*, *C. tommasinianus* and *C. banaticus* have been deposited in the tissue culture collection of the University of Debrecen (UD), Department of Botany, Hungary (Máthé et al., 2012), but to date, there is no international literature on plant regeneration from tissue cultures of the species used in the present study. Thus, the aim of this work was to: (i) establish and maintain tissue cultures of four wild-growing *Crocus* species for germplasm preservation and future experiments (not included in this paper) on their bioactive compound content; (ii) search for biochemical markers related to the type of tissue culture morphogenesis. Since antioxidant enzyme activities proved to be suitable markers (see, e.g., Blazquez et al., 2009), we chose the study of catalase (CAT) and peroxidase (POD) isoenzyme patterns on activity gels.

MATERIALS AND METHODS

PLANT MATERIAL

The location of plants serving as sources for tissue culture was as follows: *C. scepusiensis* – Vernár, central Slovakia; *C. tommasinianus* – Gyulaj, southwestern Hungary; *C. vittatus* – Pápasalamon, western Hungary; *C. banaticus* – Sovata, eastern Transsylvania, Romania. Corms of each species were collected in 2007 as part of a *Crocus* genebank collection program directed by the European Commission and called AGRI GEN RES 18 "CROCUSBANK" action (Fernández et al., 2011) that allowed the creation of a large collection of saffron crocus and its relatives. Two or three corms of the species involved in this study were further cultivated in the Botanical Garden of UD. In 2012, when shoots emerged, leaf and corm explants were sampled for further tissue culture. For *C. scepusiensis*, shoot tips from young seedlings (seeds produced in the Botanical Garden of UD and then surface sterilized) were also used for this purpose.

TISSUE CULTURE PROCEDURES AND CONDITIONS

Explants were surface sterilized by rinsing with 10% (v/v) Domestos bleach twice, followed by three consecutive washes with sterile water for 5 min. Sterile tissues were placed on plastic petri dishes (Sarstedt, Nümbrecht, Germany) with full-strength MS medium (Murashige and Skoog, 1962) modified in that it was supplemented with Gamborg's vitamins (Gamborg et al., 1968). Basal medium was then supplemented with 2 or 5% (w/v) sucrose (Molar, Halásztelek, Hungary) and solidified with 0.8% (w/v) Difco-agar (Difco, Lawrence, KS, USA). The following plant growth regulators (PGR) were used: α-naphthaleneacetic acid (NAA, Duchefa Biochemie, Haarlem, The Netherlands), indole-3-butyric acid (IBA, Sigma-Aldrich, St. Louis, Mo., USA) as auxins and 6-benzylaminopurine (BA, Duchefa Biochemie). Their concentrations depended on the culture condition tested (see Results section), but in principle they were: 0.5–10 mg L^{-1} (2.68-53.7 μM) NAA, 1 mg L^{-1} (4.9 μM) IBA and 1-5 mg L^{-1} (4.44-22.2 μM) BA. The physical conditions of the culture were as follows: continuous dim light of 5 μmol m^{-2} sec^{-1}, 22 ± 3°C. The age of cultures is specified in the Results section, Table 1.

Several tissue cultures were subject to histological analysis. In this case, calli were fixed with 4% (v/v) formaldehyde (Reanal, Budapest, Hungary) and then cryosectioned with a Leica Jung Histoslide 2000 microtome (Leica, Nussloch, Germany). 20–25 μm thick sections were examined by using the bright-field and fluorescence facilities of an Olympus Provis AX-70 (Olympus, Tokyo, Japan) fluorescence microscope. Autofluorescence of cell walls was detected by excitation at 320–360 nm.

IN-GEL ASSAY OF CAT AND POD ACTIVITIES

Typically, 45d old callus cultures grown on 5 mg L^{-1} NAA and 1 mg L^{-1} BA were used for enzyme activity assays, except when specified (see Results section). Calli were extracted at a 1:2 ratio (callus fresh weight : buffer volume) at 4°C with

TABLE 1. Main characteristics of *Crocus* tissue cultures involved in this study. The best culture conditions for plant regeneration are indicated in bold for each species.

PGR content, induction medium[a]	PGR content, maintenance/ subculturing medium[a]	Culture age (days)	Main features of culture	No. of whole plants or shoots/ callus[b]
1. C. scepusiensis shoot tip explants				
NAA(10) + BA(1)	NAA(10) + BA(1)	20–50	organogenic callus	0
NAA(10) + BA(1)	NAA(5) + BA(1)	20–30	organogenic callus, SLS[c], sporadic shoot formation	0.2 ± 0.2
NAA(10) + BA(1)	**NAA(5) + BA(5)**	**20–30**	**whole plant regeneration[d]**	**6 ± 2.44**
NAA(10) + BA(1)	NAA(2) + BA(4)	20–60	SLS	0
2. C. tommasinianus corm explants				
NAA(10) + BA(1)	NAA(10) + BA(1)	30	embryogenic callus, sporadic shoot formation	1.09 ± 0.32
NAA(10) + BA(1)	NAA(5) + BA(1)	25–50	embryogenic callus, shoot regeneration	2.36 ± 0.32
NAA(10) + BA(1)	**IBA(1)[e]**	**35–50**	**embryogenic callus, whole plant regeneration**	**8.28 ± 2.31**
3. C. vittatus, corm explants				
NAA(10) + BA(1)	NAA(10) + BA(1)	30–40	embryogenic callus, sporadic shoot regeneration	0.6 ± 0.2
IBA(1)[e]	**IBA(1)[e]**	**40–90**	**no callus involved; corm explants developed cormlets, then whole plants**	**no callus; 1 corm explant produced 1.71 ± 0.42 cormlets with plants**
4. C. banaticus, corm explants				
NAA(10) + BA(1)	NAA(10) + BA(1)	20–60	embryogenic callus, shoot regeneration	1.71 ± 0.91
NAA(10) + BA(1)	NAA(5) + BA(1)	25–50	embryogenic callus, sporadic shoot regeneration	1.11 ± 0.69
NAA(10) + BA(1)	NAA(5) + BA(5)	25–50	embryogenic callus, shoot regeneration	1.95 ± 0.64
NAA(10) + BA(1)	**NAA(0.5) + BA(2)**	**40–60**	**embryogenic callus, plant regeneration with cormlets**	**8.0 ± 0.77**
NAA(10) + BA(1)	IBA(1)[e]	30–90	embryogenic callus, plant regeneration with cormlets	2.16 ± 0.79

[a]concentrations in mg L^{-1} are given in brackets; [b]mean ± SE values are given; [c]SLS- stigma-like structures; [d]for whole plant regeneration, both root and shoot development occurred; [e]culture medium contained 5% (w/v) sucrose.

a buffer containing 100 mM KH_2PO_4/K_2HPO_4 (VWR International Ltd., Debrecen, Hungary), pH 7.2, 8 mM $MgCl_2$ (Reanal, Budapest, Hungary). After 2 x 10 min centrifugations with a Heraeus Biofuge, protein content of supernatants was determined by the Bradford (1976) method. This was necessary to ensure that protein amounts were sufficient for detecting enzyme activities. Extracts containing equal amounts (FW) of plant samples were loaded onto native 7.5% polyacrylamide gels. POD activity gels (native polyacrylamide gels) were prepared and assayed using a pyrogallol staining method as described previously (Demeter et al., 2014; Garda et al., 2016). In-gel analysis of CAT activity was performed according to the protocol described by Weidert and Cullen, 2010: native gels were incubated in 0.03% H_2O_2, washed and incubated in 0.76 mM $K_3[Fe(CN)_6]$ and 1.5 mM $FeCl_3$.

Enzyme activities (band areas) were quantified with the aid of GelAnalyzer2010® software.

All tissue culture and enzyme assay experiments were repeated at least five times.

RESULTS

TISSUE CULTURE AND PLANT REGENERATION

Culture conditions and morphogenetic responses are summarized in Table 1. Surface sterilized explants were transferred to a culture medium containing 10 mg L^{-1} NAA and 1 mg L^{-1} BA as for the initiation of tissue cultures in *C. heuffelianus* (Demeter et al., 2010). As for *C. scepusiensis*, shoot tip explants derived from 7 d old seedlings were the most suitable for callus induction, as compared to corm and leaf explants. Callus formation was characteristic after 25–30 days of explant culture and after 50–60 days these calli were ready for subculture/maintenance (Fig. 1a). This was characteristic of all cultures including the other *Crocus* species presented in this study (see below). When NAA concentration was reduced (5 mg L^{-1} NAA and 1 mg L^{-1} BA) or cytokinin/ auxin ratio was reverted and higher cytokinin concentration was used, calli produced stigma-like structures (SLS, Figs. 1b, c). For the former PGR composition, SLS formation was accompanied by shoot development (Fig. 1c). On culture media containing 5 mg L^{-1} NAA and 5 mg L^{-1} BA, no SLS formation was detected, but whole plants (structures containing both well-developed shoots and roots) regenerated efficiently (Fig.1d).

As for the rest of species belonging to the "*Verni*" series (*C. tommasinianus* and *C. vittatus*) and *C. banaticus*, corm explants were the most suitable for the efficient production of tissue cultures. On 10 mg L^{-1} NAA and 1 mg L^{-1} BA (initiation and maintenance medium) they all produced visibly morphogenic calli (Figs. 1e, i, k). In *C. tommasinianus*, when NAA content of culture medium was reduced, shoot regeneration was stimulated and somatic embryo development became macroscopically visible (Figs. 1f, g; Table 1). When calli were shifted from initiation and maintenance medium to a medium containing 1 mg L^{-1} IBA and high (5%) sucrose concentration, embryogenic calli efficiently produced whole plants (Fig. 1h). In *C. vittatus*, initiation and maintenance medium sporadically induced shoot formation, while the calli were embryogenic (Fig. 1i). If corm explants were initiated and maintained on modified MS medium with 1 mg L^{-1} IBA and 5% sucrose, efficient whole plant regeneration and cormlet formation occurred (Fig. 1j). In the case of *C. banaticus*, initiation and maintenance medium (10 mg L^{-1} NAA and 1 mg L^{-1} BA) did induce somatic embryo formation that developed into shoots with young cormlets (Fig. 1k). Similar results were obtained, when auxin content was decreased, and cytokinin content increased in the medium (Fig. 1l, Table 1). Reverting the auxin/ cytokinin ratio (0.5 mg L^{-1} NAA and 2 mg L^{-1} BA) led to efficient whole plant regeneration and these plants developed cormlets (Fig. 1m).

For *C. scepusiensis* cultures, autofluorescence studies showed that these calli are capable of tracheary element differentiation (Fig. 2a). Histological analysis confirmed differentiation of SLS showing typical stigma and stylar structures including formation of stylar canals (Figs. 2b–d). Microscopical analysis of callus structures capable of shoot or whole plant regeneration showed that first shoot primordia, and not somatic embryos, will develop (Figs. 2e, f), and in the case of whole plant development, this is followed by rooting. Thus, *C. scepusiensis* calli are organogenic. Histological analysis confirmed the formation of typical somatic embryos in *C. banaticus* cultures. Young embryogenic calli had early stage somatic embryos on their surface (Fig. 2g).

Acclimation experiments for tissue culture derived regenerants started. Cormlets of *C. vittatus* and *C. banaticus* regenerated *in vitro* were planted into pots with normal soil with medium nutrient content and pH 6–7 and grown under a 12 h photoperiod with temperature conditions of 15 ± 2°C during the illumination and 6 ± 1°C during the dark period. *C. vittatus* cormlets show the best results with frequent formation of viable shoots (data not shown).

CAT AND POD ACTIVITIES

To avoid PGR dependent variation of antioxidant activities, we produced enzyme extracts of the *Crocus* cultures studied, from calli grown under similar conditions and of similar age (5 mg L^{-1} NAA and 1 mg L^{-1} BA, 45 d old cultures). These conditions were suitable for large callus biomass production for *C. scepusiensis*, *C. tommasinianus* and *C. banaticus*. *C. vittatus* was an exception, where we had to change the conditions, because the former PGR content and age did not allow to produce enough biomass to prepare sufficient amounts of protein extracts (for this species, 65 d old cultures grown on 10 mg L^{-1} NAA and 1 mg L^{-1} BA were used). All cultures showed characteristic isoenzyme activity patterns. For both CAT and POD, quantitative analysis showed low enzyme activities, as compared to positive controls, with the highest activities in *C. scepusiensis* (Fig. 3). As for CAT, different cultures showed different isoenzyme activity patterns (Fig. 3a). Band 1 was detected
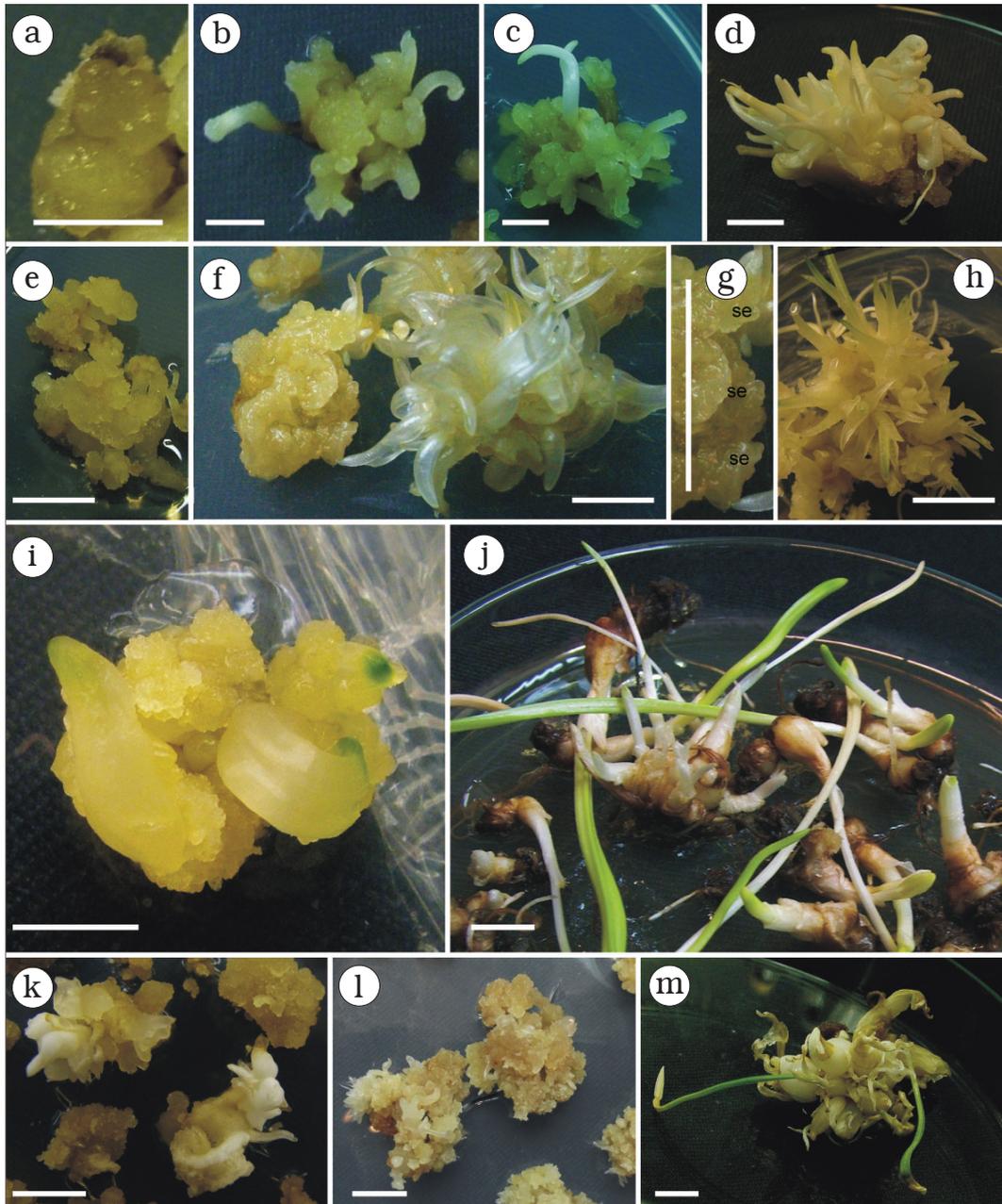
Fig. 1. (a–d) Tissue cultures of *C. scepusiensis*. **(a)** Induction of organogenic callus formation, 60-day-old culture on MS medium with Gamborg's vitamins, 10 mg L^{-1} NAA and 1 mg L^{-1} BA. **(b)** Formation of stigma-like structures (SLSs) on organogenic calli, 60-day-old culture in the presence of 4 mg L^{-1} BA and 2 mg L^{-1} NAA. **(c, d)** Organogenic calli with shoot development together with SLS formation on 5 mg L^{-1} NAA and 1 mg L^{-1} BA (c) or intensive plant development in the presence of 5 mg L^{-1} NAA and 5 mg L^{-1} BA (d), 40-day-old cultures. **(e–h)** Tissue cultures of *C. tommasinianus*, **(e)** 29-day-old embryogenic callus of *C. tommasinianus* on a callus initiation and maintenance medium containing 10 mg L^{-1} NAA and 1 mg L^{-1} BA. **(f)** 43-day-old culture after transfer of calli from initiation medium to a culture medium with 5 mg L^{-1} NAA plus 1 mg L^{-1} BA. Shoot regeneration can be observed. **(g)** Detail from the culture presented on **(f)**, showing somatic embryos (se). **(h)** Mass plant regeneration in the presence of 1 mg L^{-1} IBA and 5% (w/v) sucrose, 50-day-old culture. **(i, j)** Tissue cultures of *C. vittatus*. **(i)** 42-day-old culture of *C. vittatus* grown in the presence of 10 mg L^{-1} NAA and 1 mg L^{-1} BA. **(j)** High-frequency of (cormlet containing) plant regeneration in 90-day-old cultures that were transferred to 1 mg L^{-1} IBA and 5% (w/v) sucrose. **(k–m)** Tissue cultures of *C. banaticus*. **(k)** 55-day-old culture of *C. banaticus* grown on 10 mg L^{-1} NAA and 1 mg L^{-1} BA, initiation of shoot regeneration. **(l)** 30-day-old embryogenic calli grown on 5 mg L^{-1} NAA and 5 mg L^{-1} BA. **(m)** Regeneration of whole plants with cormlets, 60-day-old culture grown on 2 mg L^{-1} BA and 0.5 mg L^{-1} NAA. Scalebars: 5 mm.
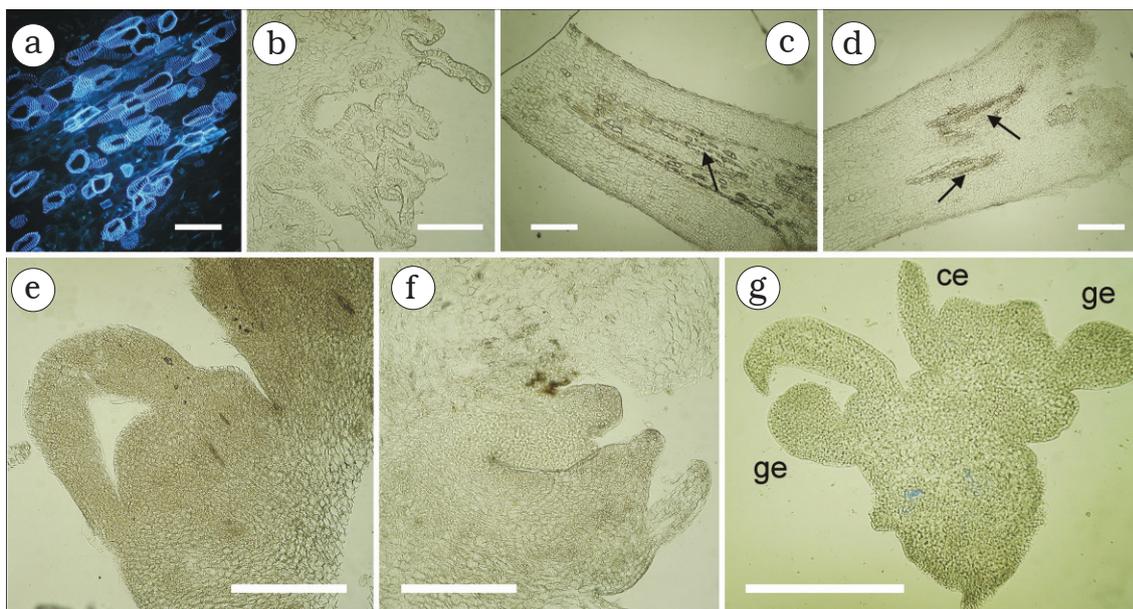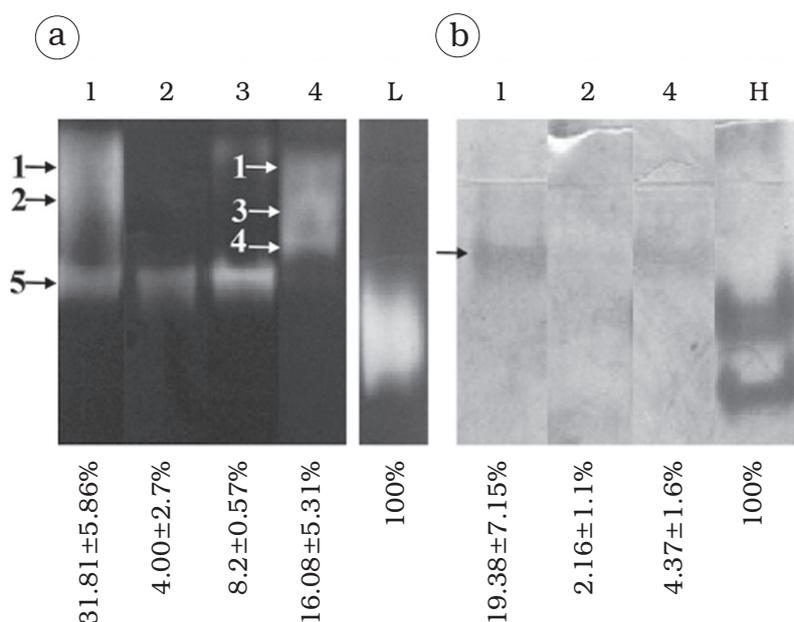
Fig. 2. Histological analysis of *C. scepusiensis* (**a–f**) and *C. banaticus* (**g**) tissue cultures. (**a**) Autofluorescence of *C. scepusiensis* tracheary elements differentiated within calli in the presence of 2 mg L^{-1} NAA and 2 mg L^{-1} BA, 13-day-old culture. (**b–d**) Formation of stigma-like structures (SLSs) on organogenic calli. (**b**) 30-day-old cultures in the presence of 5 mg L^{-1} NAA plus 1 mg L^{-1} BA. (**c, d**) Longitudinal histological sections showing SLSs formed on 4 mg L^{-1} BA and 2 mg L^{-1} NAA after 40–45 days of culture Arrows: stylar canal. (**e, f**) Histological images of shoot primordia in the presence of 5 mg L^{-1} NAA and 1 mg L^{-1} BA, 40-day-old cultures. (**g**) Histological section of an embryogenic callus of *C. banaticus* (25-day-old culture grown on 5 mg L^{-1} NAA and 1 mg L^{-1} BA) showing somatic embryos. ge- globular stage embryo; ce- cotyledonary stage embryo. Scalebars: 100 μm (a), 500 μm (b–g)

in *C. scepusiensis* and *C. banaticus*, band 2 in *C. scepusiensis* only, bands 3-4 in *C. banaticus* only and band 5 was present in all cultures belonging to the "Verni" series (*C. scepusiensis*, *C. tommasinianus* and *C. vittatus*). As for pyrogallol POD, all cultures were characterized by a common unique activity band (Fig. 3b).

DISCUSSION

There are various culture conditions, including PGR content of media, for the successful induction and maintenance of *Crocus* tissue cultures. One of them – high PGR (especially auxin) – requirement was frequently described both for *C. sativus* and its wild relatives. This includes not only cultures initiated from explants of floral organs, but also for corm explants. For the induction of calli capable of plant regeneration, this rule should be taken into account (Bhagyalakshmi, 1999; Plessner and Ziv, 1999; Demeter et al., 2010; Verma et al., 2016). Indeed, nearly all *Crocus* cultures involved in this study were initiated on a medium with 10 mg L^{-1} NAA and 1 mg L^{-1} BA. When the formation and stable subculture of such calli is established, different strategies/culture conditions are

available for the induction of shoot or whole plant regeneration in the genus *Crocus* (see Plessner and Ziv, 1999; Karamian and Ebrahimzadeh, 2001; Ascough et al., 2009 for reviews of cultures for *C. sativus* and wild relatives). These culture conditions involve mainly somatic embryogenesis and include cultures initiated from corm explants: (i) decrease of PGR content in culture media together with changing of auxin/cytokinin ratio in favor of cytokinins and decrease of medium strength (Karamian, 2007; Demeter et al., 2010; Karamian and Ebrahimzadeh, 2010); (ii) high cytokinin content of media, with or without the use of auxin(s) (this strategy was used mainly for *C. sativus* and can be used for micropropagation purposes as well, where callus induction is not involved. It works well with both corm and shoot tip explants and can lead to cormlet formation, too – Devi et al., 2014); (iii) high organic carbon source (sucrose) content (up to 8%) with or without the use of IBA was reported to promote rooting of regenerated *Crocus* shoots and cormlet formation (Sharma et al., 2008; Verma et al., 2016). Besides, cormlet development in *Crocus* tissue cultures occurs under various PGR treatments. Several papers report the importance of IBA (Zeybek et al., 2012), while others underline the use of

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Fig. 3. Catalase **(a)** and pyrogallol peroxidase **(b)** activities of *Crocus* tissue cultures grown for 45 days on 5 mg L^{-1} NAA plus 1 mg L^{-1} BA (except *C. vittatus*, cultured for 65 days on 10 mg L^{-1} NAA plus 1 mg L^{-1} BA). Activity gels were used for the assay. Lane 1 – *C. scepusiensis*; 2 – *C. tommasinianus*; 3 – *C. vittatus*; 4 – *C. banaticus*. Below gel images, mean ± SE values of enzyme activities (expressed as band intensities) are shown. These values are the percentage of activities for positive controls known for high enzyme activities: *Lemna* whole plant extract (L) for catalase and horseradish root extract (H) for pyrogallol peroxidase, 100% values. *C. vittatus* peroxidase activity (8.33 ± 0.57% of positive control) is not shown in Fig. 3b. Arrows and numbers (numbers on the catalase gels only) indicate characteristic activity bands.

cytokinins without IBA (Sharma et al., 2008) for *C. sativus*. The use of high sucrose concentration and high cytokinin/auxin ratios was proven to be beneficial for plant regeneration and cormlet production from corm-derived somatic embryos in *C. vernus* (Sivanesan et al., 2012, 2014).

For the present study, concerning whole plant regeneration, culture condition (i) (as shown above, but without reducing medium strength) was used for *C. tommasinianus* and *C. banaticus*. Culture condition (ii) was the most efficient for *C. scepusiensis*, where organogenesis instead of embryogenesis occurred (however, for this species, SLSs were also formed under such conditions). Culture condition (iii) involving the use of IBA was used for *C. tommasinianus*, *C. vittatus* and *C. banaticus*. Efficient shoot or whole plant regeneration could be achieved for all *Crocus* species involved (Figs. 1–2 and Table 1).

Plant regeneration from somatic embryos requires particular PGR content in culture media. It is possible that under certain culture conditions somatic embryos show unipolar development with the differentiation of shoots only in *Crocus* and bipolar embryo development occurs only at well-defined PGR combinations (Blazquez et al., 2009). This phenomenon could be observed for two of

our cultures showing somatic embryogenesis (*C. tommasinianus* and *C. banaticus*). The best example is *C. banaticus*, where for most PGR combinations studied, somatic embryos developed shoots. However, when auxin content was decreased, while cytokinin increased dramatically, whole plants with simultaneous shoot, root and cormlet differentiation occurred (Fig. 2h).

Concerning SLS formation, there are various PGR combinations inducing this type of morphogenesis. The explant type is also important (Plessner and Ziv, 1999). High cytokinin content favored SLS development, when half-ovary explants of *C. sativus* were used (Loskutov et al., 1999). Several studies report that high cytokinin and auxin content (higher than 4.5 μM) in media favor SLS formation from floral explants (see, e.g., Ebrahimzadeh et al., 2000). It should be noted that the above statements are valid only for *C. sativus* flower organs, while for corm explants there is a limited number of studies reporting SLS formation (Gantait and Vahedi, 2015). In the light of this, it is of particular interest that we could obtain SLS from corm explants in *C. scepusiensis*. It is worth mentioning here, that the anatomy of SLSs formed under tissue culture conditions (Figs. 2b–d) shows the general histological characteristics of

native *Crocus* stigmas as reported by Weryszko-Chmielewska and Chwil (2011), except that stigmatal papillae are absent in SLSs.

In *C. sativus*, different stages of development of callus structures capable of further shoot or whole plant regeneration are accompanied by specific changes in the pattern of antioxidant activity of enzymes like superoxide dismutase (SOD) or CAT (Blazquez et al., 2009). In this study we demonstrate that the isoenzyme pattern of CAT depends on the type of callus developed during tissue culture. Even though *C. scepusiensis*, *C. tommasinianus* and *C. vittatus* taxonomically belong to the same group (series *Verni*), morphogenetic events shown by *C. scepusiensis* cultures were clearly distinct from the other "*C. vernus*" species in that they were organogenic. Meanwhile, they were characterized by two additional CAT activity bands and showed higher (yet still low, as compared to the positive controls) CAT and POD activities, as compared to *C. tommasinianus* and *C. vittatus* (Fig. 3). *C. banaticus* had low antioxidant enzyme activity, as compared to *C. scepusiensis*. Meanwhile – although taxonomical analysis is beyond the scope of this paper – with respect to CAT isoenzyme pattern, cultures of *C. banaticus* were distinct from all other three species involved in the present tissue culture studies (Fig. 3b), confirming that the species is evolutionarily distinct from species belonging to the "*Verni*" section (see, e.g., DNA fingerprinting studies of Mosolygó et al., 2016). Although CAT/POD isoenzyme patterns can vary between different tissues in the same plant, the patterns of tissue cultures were identical to the leaves of the corresponding native plants at least for *C. tommasinianus* and *C. vittatus*, where plant materials collected from the field (1-2 leaves) were available (Supplementary Fig. S1).

In conclusion, we established novel tissue cultures of four wild, rare/endangered *Crocus* species. Since all of them are capable of whole plant regeneration, they have a germplasm preservation value. *C. vittatus* and *C. banaticus* cultures produced cormlets on plant regeneration media (Table 1). These underground modified organs are suitable for *ex vitro* acclimatization, increasing their species conservation value (Gantait and Vahedi, 2015). The distinct callus types (organogenic vs. embryogenic) and taxonomical differences were reflected by differences in CAT activity patterns.

What could be the biotechnological importance of the present study? Apocarotenoids produced in many types of saffron tissue cultures are not applicable here, because wild relatives of saffron are not likely to efficiently produce apocarotenoids in their SLSs produced *in vitro*.

However, the *in vitro* SLS production strategy for *C. scepusiensis* presented in the Results section (Table 1) could be beneficial for those who are interested in the production of SLS in *C. sativus* in order to efficiently produce the characteristic saffron apocarotenoids. Moreover, corm-derived *Crocus* cultures have the potential of producing compounds with cytostatic (potential antitumor) properties, such as proteoglycans (Escribano et al., 1999; Fernández, 2006; Sharifi and Ebrahimzadeh, 2010).

AUTHORS' CONTRIBUTIONS

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