

1-1-2014

## Activity of antioxidant enzymes during induction of morphogenesis of *Fritillaria meleagris* in bulb scale culture

MARIJA PETRIC

SLADANA JEVREMOVIC

MILANA TRIFUNOVIC

VOJIN TADIC

SNEZANA MILOSEVIC

*See next page for additional authors*

Follow this and additional works at: <https://journals.tubitak.gov.tr/biology>



Part of the [Biology Commons](#)

---

### Recommended Citation

PETRIC, MARIJA; JEVREMOVIC, SLADANA; TRIFUNOVIC, MILANA; TADIC, VOJIN; MILOSEVIC, SNEZANA; and SUBOTIC, ANGELINA (2014) "Activity of antioxidant enzymes during induction of morphogenesis of *Fritillaria meleagris* in bulb scale culture," *Turkish Journal of Biology*. Vol. 38: No. 3, Article 3.

<https://doi.org/10.3906/biy-1309-45>

Available at: <https://journals.tubitak.gov.tr/biology/vol38/iss3/3>

This Article is brought to you for free and open access by TÜBİTAK Academic Journals. It has been accepted for inclusion in Turkish Journal of Biology by an authorized editor of TÜBİTAK Academic Journals. For more information, please contact [academic.publications@tubitak.gov.tr](mailto:academic.publications@tubitak.gov.tr).

---

## Activity of antioxidant enzymes during induction of morphogenesis of *Fritillaria meleagris* in bulb scale culture

### Authors

MARIJA PETRIC, SLADANA JEVREMOVIC, MILANA TRIFUNOVIC, VOJIN TADIC, SNEZANA MILOSEVIC,  
and ANGELINA SUBOTIC

## Activity of antioxidant enzymes during induction of morphogenesis of *Fritillaria meleagris* in bulb scale culture

Marija PETRIĆ\*, Slađana JEVREMOVIĆ, Milana TRIFUNOVIĆ, Vojin TADIĆ, Snežana MILOŠEVIĆ, Angelina SUBOTIĆ

Department for Plant Physiology, Institute for Biological Research Siniša Stanković, University of Belgrade, Belgrade, Serbia

Received: 19.09.2013

Accepted: 24.12.2013

Published Online: 14.04.2014

Printed: 12.05.2014

**Abstract:** We investigated the activity of 3 antioxidant enzymes: superoxide dismutase, catalase, and peroxidase during morphogenesis of *Fritillaria meleagris* L. in vitro in bulb scale culture. Bulb cultures of *F. meleagris* were established on nutrition medium supplemented with 1.0 mg L<sup>-1</sup> thidiazuron. Bulbs were grown at standard (24 °C) and low temperatures (4 °C) or at an increased sucrose concentration (4.5%) during the first 6 weeks (pretreatments). All bulbs were then grown for 4 weeks at standard temperature on 2, 4-dichlorophenoxyacetic acid and kinetin or thidiazuron (at concentrations of 1 mg L<sup>-1</sup> each) in order to examine the influence of the above-mentioned pretreatments and nutrient medium composition on enzyme activity. Enzyme activity was measured 7, 14, 21, and 28 days after the start of morphogenesis induction. Superoxide dismutase and catalase showed the highest activity at the beginning of morphogenesis in vitro. Peroxidase activity was the highest in bulb segments immediately after isolation following pretreatments and on both nutrition media. We also determined the effect of pretreatment coupled with low temperature (4 °C) on growth and development of *F. meleagris* in vitro and chlorophyll and carotenoids content.

**Key words:** Snake's head fritillary, morphogenesis, superoxide dismutase, catalase, peroxidase

### 1. Introduction

*Fritillaria meleagris* L. (*Liliaceae*), snake's head fritillary, is a very valuable, bulbous plant used for horticultural purposes. The genus *Fritillaria* is mainly distributed throughout various temperature zones of the northern hemisphere and spends a period of the year as a dormant bulb underground. It has a high number of species (41 are natural habitants of Turkey; of these, 36.53% are endemic), and Turkey may be the center of genetic diversity of the genus (Türktaş et al., 2012). Bulbs require a certain period of exposure to low temperature, which happens during winter in natural habitats, to overcome dormancy. Low temperature treatment in vitro can have a positive impact on the percentage of regeneration of new bulbs (Paek et al., 1996). An increased concentration of sucrose in the nutritional medium can also affect bulb regeneration and overcoming dormancy (Langens-Gerrits et al., 2003b). During dormancy, increased amounts of sugar accumulate in bulbs (Miller and Langhans, 1990), because the bulbs are preparing for sprouting and full development in the next vegetation period.

Production of fritillaries by conventional methods (seed germination or bulbs) is very slow and unpredictable, and it takes several years to get a whole plant. In most of the

described protocols in vitro, as initial explants were used parts of bulbs or bulb scale segments (Paek and Murthy, 2002). As a result, there is a great interest in improving protocols for micropropagation of species in this genus (Sun and Wang, 1991). In vitro plant propagation of *F. meleagris* can lead to effective and rapid multiplication of this species through various morphogenetic pathways, while conventional methods are ineffective (Kukulezanka et al., 1998; Subotić et al., 2010).

Bulbs of *F. meleagris* can be induced in a morphogenesis process in vitro via organogenesis or somatic embryogenesis. Through the process of organogenesis unipolar organs are formed de novo; somatic embryogenesis is the process of forming embryos from somatic cells. To date, organogenesis has been described in 17 and somatic embryogenesis in 8 species of the genus *Fritillaria* (Petrić et al., 2012). Both morphogenetic paths lead to the formation of a plant, but it is still unknown why some cells form somatic embryos and others are subject to the process of organogenesis (Karami et al., 2009). Bulb scales, i.e. parts of bulbs, can be used as initial explants for the induction of morphogenesis in many *Fritillaria* species (Witomska and Lukaszewska, 1997). Somatic embryogenesis of *F. meleagris* was achieved on 2 types of

\* Correspondence: marija.nikolic@ibiss.bg.ac.rs

explant: mature zygotic embryo (Petrić et al., 2011) and leaf base (Subotić et al., 2010). Commonly used nutrition media for *F. meleagris* regeneration were supplemented with auxin.

Plant tissue violation during introduction of plant material in culture also leads to oxidative stress (Yahraus et al., 1995). Injuries to plant tissue can be a trigger for cell division (Sangwan et al., 1992). Low temperature, which is necessary to overcome dormancy, also represents a kind of oxidative stress for the plant. Synthetic auxin 2, 4-D affects modification of endogenous indol-3-acetic acid (IAA) and as a stress factor has a bigger influence on somatic embryogenesis than IAA, because it cannot be metabolized in the plant cell (Karami and Saidi, 2010). The amount of stress can be measured by measuring the activity of antioxidant enzymes, which are a part of plant antioxidant defense. Oxidative stress has been studied in many plants, but the mechanism of its action is still not completely understood (Sharma, 2012). Factors that negatively affect the growth and development of plants, reducing their productivity level, often lead to the release of large amounts of reactive oxygen species (ROS) (Bowler et al., 1994). ROS can react with DNA, proteins, and lipids, which results in cell damage. Cell damage can lead to loss of cell competence for morphogenesis (Lambe et al., 1997), hyperhydration of plant tissue (Olmos et al., 1997), and formation of somatic embryos (Cassells et al., 2001). Oxidative stress during morphogenesis can also affect change in the cytokinins and auxins ratio (Jia et al., 1996), which directly affects the morphogenesis process. To prevent cell damage, enzymatic and nonenzymatic components of the antioxidant plant defense eliminate ROS (Bowler et al., 1992). Plants have a very efficient enzymatic antioxidant system that catalyzes the removal of ROS (Inzé and Van Montagu, 1995). Enzymes such as catalases (CATs) and peroxidases (POXs) are part of the plant enzymatic antioxidative defense system (Apel and Hirt, 2004).

Superoxide dismutases (SODs) participate in the removal of superoxide anion radical, which can be formed in different parts of the cell where an electron transport chain exists; therefore, different isoforms of SOD can be found in many cell organelles (Alscher et al., 2002). Depending on the metal cofactor, the enzyme can be classified as: Fe/SOD, Mn/SOD, Cu/Zn-SOD, and Ni/SOD (Bannister et al., 1987).

CATs are metalloenzymes that remove oxygen peroxide from plant cells (Mizuno et al., 1998). Large amounts of hydrogen peroxide synthesized in photosynthetic tissues may be removed by CAT, which represents a first line of cell defense against this harmful molecule (Auh and Scandalios, 1997).

POXs are involved in a large number of reactions in the cell, such as the oxidation of phenolic compounds, lignifications, and linking cell wall polysaccharides (Passardi et al., 2004). Their role in the growth and development of plants (Huyste and Carnis, 1982), auxins catabolism (Passardi et al., 2005), and in defending plants against pathogens and oxidative stress (Veljović-Jovanović et al., 2006) was confirmed. POX activity and different isoforms of peroxidases were reported as markers of somatic embryogenesis in a few studies (Krsnik-Rasol et al., 1982; Joersbo et al., 1989). Moreover, POX can be applied as a marker of morphogenesis and tumorization, which has been demonstrated in horseradish tissue culture (Balén et al., 2003).

The amount of chlorophyll and carotenoids also changes when the plant is exposed to stress conditions (Edge et al., 1997).

The goal of the present study was to determine the activity and potential roles of antioxidative enzymes during morphogenesis in vitro of *F. meleagris* as well as possible differences in their activity in relation to bulb pretreatment before the induction of morphogenesis.

## 2. Materials and methods

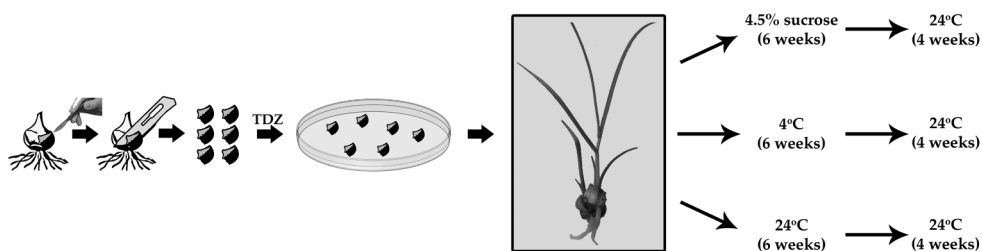
### 2.1. Plant material

Bulb cultures of *F. meleagris* were established according to previously published procedures (Petrić et al., 2011). Cultures were maintained on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.7% agar, 250 mg L<sup>-1</sup> caseine hydrolyzate, 250 mg L<sup>-1</sup> L-proline, and 1.0 mg L<sup>-1</sup> thidiazuron (TDZ) for shoot multiplication. Stock cultures were maintained at 24 ± 2 °C and a 16 h light/8 h dark photoperiod with irradiance of 40 μmol m<sup>-2</sup> s<sup>-1</sup>.

The influence of increased sucrose concentration on the growth and development of bulbs was investigated on hormone-free medium that, apart from the standard sucrose concentration of 3%, contained an elevated concentration of sucrose (4.5%). Bulbs were grown at standard temperature (24 °C) or low temperature (4 °C) during the first 6 weeks, and then all bulbs were grown for 4 weeks at standard temperature (Figure 1) on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) (1.0 mg L<sup>-1</sup>) or TDZ (1.0 mg L<sup>-1</sup>).

### 2.2. Determination of chlorophyll and carotenoids

For isolation of chlorophyll and carotenoids 20 mg of plant material were used. For the extraction 96% ethanol (2 mL) was used. Tubes with plant material and ethanol were heated in a water bath at 70 °C for 10 min. Chlorophyll and carotenoid content was determined spectrophotometrically, and the value was determined as absorbance (A): 470 nm, 648 nm, and 664 nm (Lichtenhaler, 1987). Total chlorophyll and carotenoid



**Figure 1.** Schematic view of the method for obtaining plant material. Segments of bulbs were obtained by cutting the initial bulbs longitudinally. The whole plants developed from bulb segment culture and were transferred to 3 different pretreatments for 6 weeks. Bulb segments obtained in this way were used in further experiments.

content was calculated according to the following formulae and expressed in mg/g fresh weight:

$$C_{(a+b)} = 5.24A_{664} + 22.24A_{648},$$

$$\text{content of chlorophyll a: } C_a = 13.36A_{664} - 5.19A_{648},$$

$$\text{content of chlorophyll b: } C_b = 27.43A_{648} - 8.12A_{664},$$

$$\text{carotenoid content: } C = (1000A_{470} - 2.13C_a - 97.64C_b)/209.$$

The ratio of chlorophyll a and b was calculated according to the formula:  $C_a/C_b$ , all extractions were repeated 3 times.

### 2.3. Enzyme extraction

Frozen ( $-70^\circ\text{C}$ ) bulb scale (500 mg) was homogenized in 4 mL of 0.1 M potassium phosphate (K-P) extraction buffer (pH 6.8 containing 200 mg of insoluble polyvinyl pyrrolidone and phenylmethylsulfonyl fluoride). The homogenate was centrifuged for 5 min at  $12,000 \times g$  at  $4^\circ\text{C}$ . Protein content of supernatants was determined according to Bradford (1976). All measurements were repeated 3 times.

### 2.4. Quantification of SOD activity

SOD activity was determined spectrophotometrically by a modified method of Beyer and Fridovich (1987). The reaction mixture (1 mL) contained 100 mM K-P buffer (pH 7.8), 2 mM ethylenediaminetetraacetic acid (EDTA), 260 mM methionine, 1.5 mM nitroblue tetrazolium chloride (NBT), and 0.04 mM riboflavin. For each sample, 6 dilutions were prepared (sample volume: 0, 5, 10, 15, 20, and 25  $\mu\text{L}$ ; K-P buffer volume: 800, 795, 790, 785, 780, and 775  $\mu\text{L}$ ) and placed in a microtiter plate. The reaction mixture was then illuminated for 30 min at  $25^\circ\text{C}$ . The measurement was done at 540 nm. One unit of SOD activity is the amount of sample required for 50% inhibition of NBT photoreduction and is presented as specific activity (U/mg). All measurements were repeated 3 times.

Proteins were separated by native polyacrylamide gel electrophoresis using 5% stacking and running gels with a buffer consisting of 25 mM Tris and 192 mM glycine (pH 8.3) at 200 V for 6.5 h. The total amount of protein applied per lane was 20  $\mu\text{g}$ . After electrophoresis the gels were

incubated with 1 mM potassium cyanide (inhibitor of Cu/Zn SOD isoforms) and 30% hydrogen peroxide (inhibitor of Cu/Zn SOD and Fe SOD) followed by incubation with a reaction mixture (0.1 M EDTA, 0.098 mM NBT, 0.03 mM riboflavin, and 2 mM tetramethylethylenediamine in K-P buffer, pH 7.8) for 30 min in the dark. The gels were washed in distilled water and visualized with regular light.

### 2.5. Quantification of CAT activity

CAT activity was determined spectrophotometrically by monitoring the kinetics of disappearance of hydrogen peroxide by the method of Aebi (1984), which can be detected by measuring the decrease in absorbance at 240 nm of reaction mixture consisting of 50 mM K-Na-P buffer (pH 7), 20 mM hydrogen peroxide, and enzyme extract. CAT activity was measured at  $20^\circ\text{C}$  every 20 s for 3 min. One unit of catalase activity is defined as the amount of enzyme that degrades 1  $\mu\text{mol}$  of hydrogen peroxide in 1 min and is indicated as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  (U/mg). All measurements were repeated 3 times.

### 2.6. Quantification of POX activity

Activity of POX was determined spectrophotometrically by measuring the change in absorbance at 430 nm (Kukavica and Veljović-Jovanović, 2004). The reaction mixture contained 2.9 mL of 0.05 M K-P buffer (pH 6.5) and 60  $\mu\text{L}$  of 1 M pyrogallol (Sigma) as enzyme substrate. The reaction was started by adding 30  $\mu\text{L}$  of 30% hydrogen peroxide after the first 20 s. The POX-catalyzed oxidation of pyrogallol with hydrogen peroxide to purpurogallin was monitored at 430 nm. Enzyme activity is indicated as  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  (U/mg). All measurements were repeated 3 times.

### 2.7. Statistical analysis of data

The results of all experiments are presented as mean values  $\pm$  standard errors. Statistical analyses were performed using StatGraphics software version 4.2. Data were subjected to analysis of variance (ANOVA), and comparisons between the mean values of treatments were made by least significant difference (LSD) test calculated at the confidence level of  $P \leq 0.05$ .

### 3. Results

Fully developed plants grown continuously for 10 weeks at 24 °C showed statistically significant differences in length compared to plants obtained from bulbs grown for 6 weeks at 4 °C (Table 1). The average length of plants obtained after cooling increased 42.92% compared to plants grown at standard conditions.

The content of photosynthetic pigments was determined in bulbs after cold treatment (6 weeks) and then after 1 week at standard conditions (Table 2). Results showed increased amounts of chlorophyll and reduced amounts of carotenoids compared to bulbs exposed to low temperature for 6 weeks.

The highest SOD activity was observed 7 days after the beginning of induction of morphogenesis in vitro at 24 °C, on both culture media (Figure 2a). The lowest activity was observed in bulb segments immediately after isolation ( $6.33 \pm 0.69$  U/mg). Bulbs previously grown at 4 °C showed maximum SOD activity ( $30.16 \pm 0.33$  U/mg) 7 days after the morphogenesis induction on medium with 2,4-D and KIN (Figure 2b). The bulb segments grown on medium with TDZ had an increase in SOD activity for the first 14 days after induction, and then it decreased. SOD activity was higher than in bulbs continuously grown under standard conditions (constant 24 °C). Growing bulbs in medium with increasing concentrations of sucrose (4.5%) leads to increased SOD activity (Figure 2c) compared to bulbs grown under standard conditions (24 °C). As in bulbs that were previously grown at 24 °C, the highest SOD activity was 7 days after induction on both nutrient media. Moreover, the lowest SOD activity ( $6.33 \pm 0.68$

U/mg) was observed in bulb segments immediately after isolation on both culture media. SOD activity decreased after 7 days from the start of induction of morphogenesis, and that level of activity was maintained until the end of the fourth week of culture on both culture media.

Native electrophoresis showed the presence of 5 SOD isoforms including 2 Cu/Zn SOD and 3 Fe SOD (Figure 3a). All 5 isoforms were active during induction of morphogenesis in vitro on both nutrition media, but the activity of 1 Fe/SOD isoform in bulbs on medium with TDZ was very weak. Electrophoresis also showed the presence of all 5 SOD isoforms (Figure 3b), and isoforms are generally stronger in bulbs grown on medium containing 2,4-D and KIN than medium with TDZ, in particular Fe/SOD isoforms. Five SOD isoforms were detected in bulbs grown on medium with TDZ (Figure 3c). Four weeks after the induction of morphogenesis in vitro 1 Fe/SOD isoform was lacking.

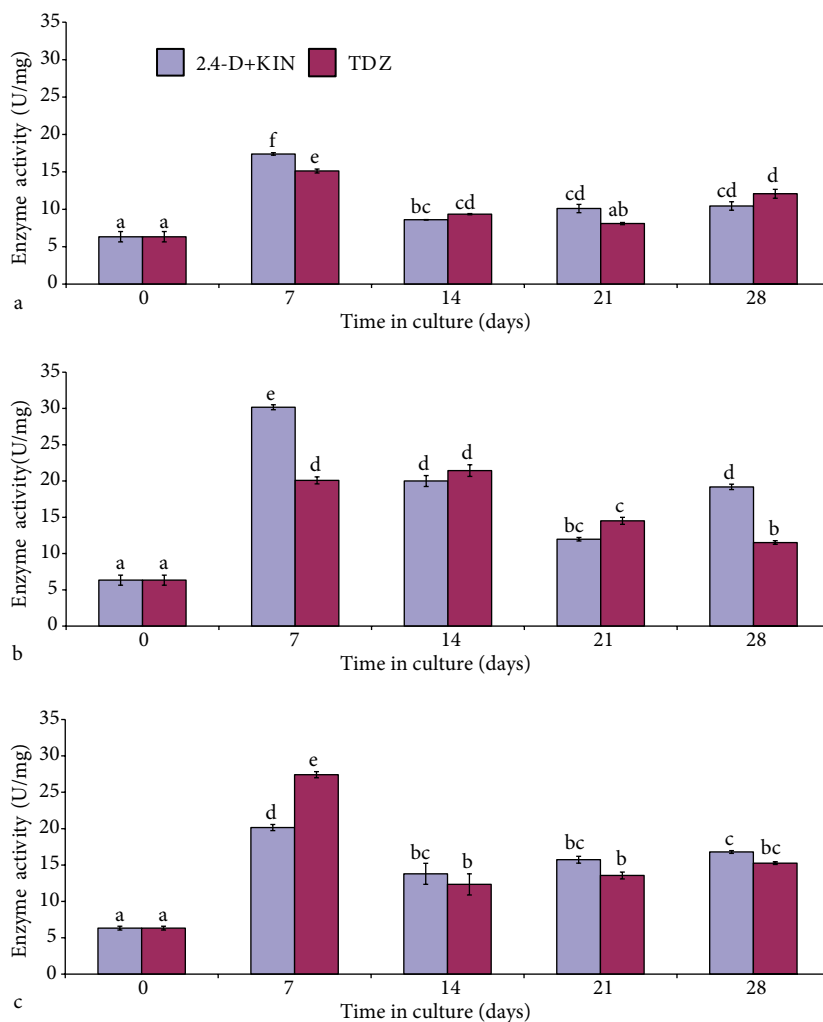
CAT activity rapidly increases 7 days after the induction of morphogenesis in the bulbs segments grown at standard and low temperature in pretreatment and on both culture media (Figure 4). CAT activity was the lowest ( $196.08 \pm 18.4$  U/mg) immediately after isolation. CAT activity decreased after 28 days from the start of induction in bulbs grown under standard conditions (24 °C) on both nutrition media (Figure 4a). Bulb segments previously grown at 4 °C for 6 weeks showed an increase in CAT activity (Figure 4b) after 14 days on media with TDZ, and this remained constant until the end of the culture. CAT activity in bulbs grown on media with 2,4-D and KIN showed an even greater decrease in activity after 14 days,

**Table 1.** The effect of pretreatment with low temperature (4 °C) on growth and development of *F. meleagris* in vitro.

Treatment (°C)	Length of plant (mm)	Roots	
		Number	Length (mm)
24 (10 weeks)	$39.93 \pm 5.45^a$	$2.7 \pm 0.62^a$	$15.81 \pm 2.68^a$
4 (6 weeks) + 24 (4 weeks)	$57.07 \pm 6.16^b$	$3.03 \pm 0.94^a$	$20.11 \pm 4.12^{ab}$

**Table 2.** The effect of a 6 week duration of low temperature (4 °C) on chlorophyll and carotenoids content in *F. meleagris* bulbs (values are given as mg/L of fresh weight).

Treatment (°C)	Chlorophyll (Chl)			Carotenoids	Total pigments
	Chla	Chlb	Chla/Chlb		
24	$0.218 \pm 0.01^c$	$0.418 \pm 0.03^c$	$0.535^b$	$0.034 \pm 0.01^a$	$0.677 \pm 0.04^c$
4 (6 weeks)	$0.029 \pm 0.02^a$	$0.067 \pm 0.03^a$	$0.339^a$	$0.072 \pm 0.01^b$	$0.169 \pm 0.05^a$
4 (6 weeks) + 24 (1 week)	$0.140 \pm 0.01^b$	$0.264 \pm 0.03^b$	$0.535^b$	$0.036 \pm 0.01^{ab}$	$0.444 \pm 0.04^b$



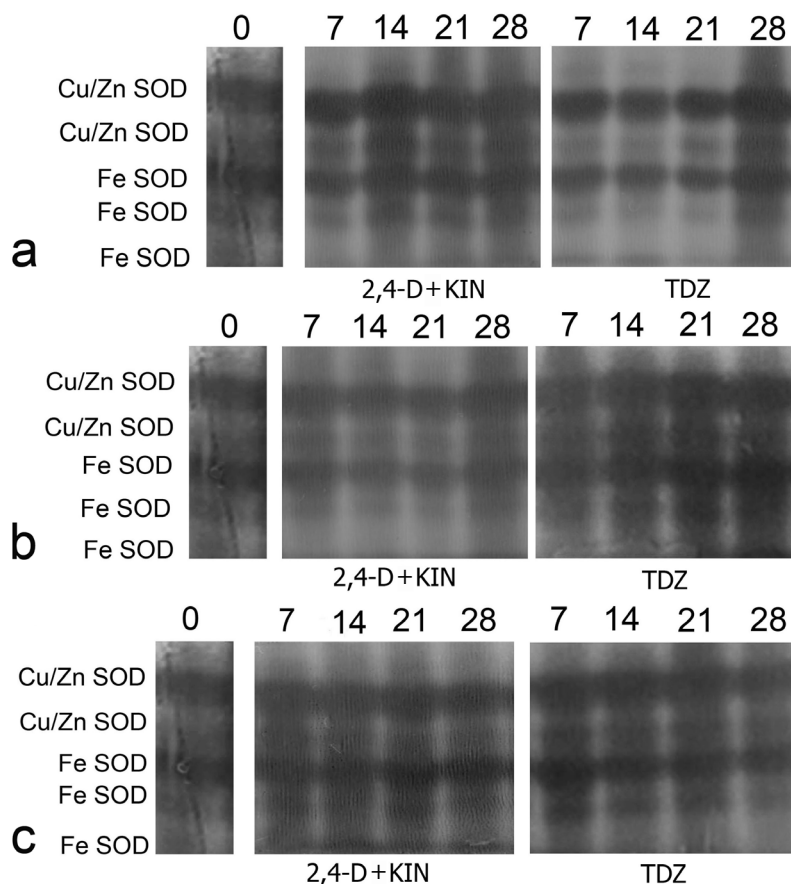
**Figure 2.** SOD activity during induction of morphogenesis in vitro in bulb segment culture after a pretreatment of 6 weeks at 24 °C (a), 4 °C (b), and with an increase in sucrose concentration (c).

but increased at the end of culture. CAT activity increased in bulb segments that were previously grown in increased concentrations of sucrose (Figure 4c). Unlike previous pretreatments, an increase in CAT activity was detected after 14 days of induction in the case of culture medium with 2,4-D and KIN and after 21 days in the case of culture medium with TDZ.

POX activity was the highest ( $72.72 \pm 0.68$  U/mg) in bulb segments immediately after isolation after all pretreatments and on both nutrition media (Figures 5a–c). In bulb segments grown at elevated sucrose concentrations, no significant trend of increase or decrease in POX activity was observed, except in bulb segments previously grown at low temperatures and 21 days from the start of induction (Figure 5b).

#### 4. Discussion

Growing bulbs at 4 °C for 4 weeks increased rooting of bulbs (60.4%) compared to the bulbs grown at standard temperature (32.4%) (Nikolić et al., 2008). Number of roots and plant length were also significantly higher in plants grown at 4 °C. After the low temperature treatment plants were grown for another 4 weeks at standard temperature. Length and number of roots were higher in plants previously grown at 4 °C. Low temperature increases the percentage of sprouting and rooting bulbs, as was shown in other geophytes such as lily (Langens-Gerrits et al., 2003a). The positive influence of growing bulbs at low temperatures has been observed in *Dioscorea polystachya*, where the percentage of rooted bulbs is between 71% and 99% compared to noncooled bulbs in which the rooting percentage was only 9% (Walck et al., 2010).



**Figure 3.** Isoforms of SOD during induction of morphogenesis in vitro in bulb segment culture after a pretreatment of 6 weeks at 24 °C (a), 4 °C (b), and with an increase in sucrose concentration (c).

The concentration of chlorophyll decreases in bulbs of *F. meleagris* that are grown at low temperature, while the carotenoid content increases. Chlorophyll content decreases when red pepper is exposed to different types of stress (Kim et al., 2004). Similar results were obtained in this study; after rapid decline in the activity of photosynthetic pigments during low temperature, the amount of pigments increased again when the bulbs were grown at standard temperature. Carotenoids are involved in a number of antioxidant reactions that occur under stressful conditions (Edge et al., 1997). They participated in the defense of photosystem II from the harmful effects of free radicals (Gilmore, 1997); therefore, their concentration increases during cooling.

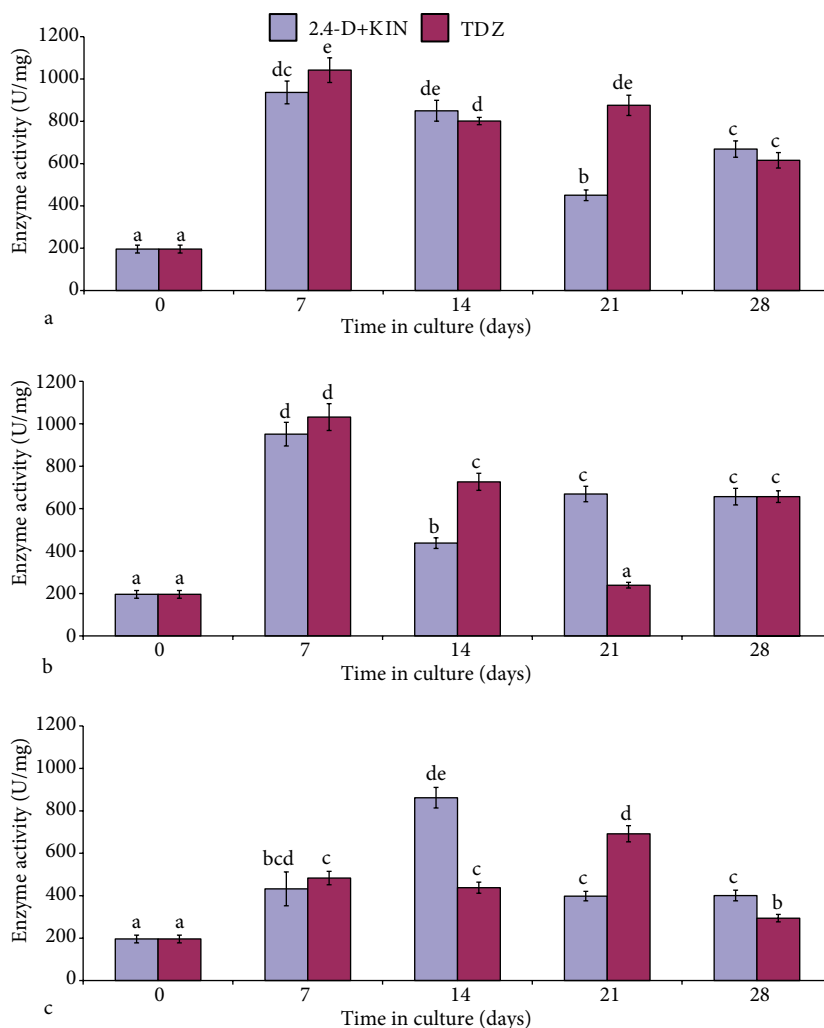
Five isoforms of SOD were found: 2 Cu/Zn and 3 Fe/SOD. All 5 isoforms occurred during morphogenesis only in bulbs that were continuously grown at 24 and 4 °C. Four isoforms (2 Cu/Zn and 2 Fe/SOD) can occur in bulb segments grown on medium with TDZ and 4.5% sucrose after 28 days from the start of induction of morphogenesis. One Fe/SOD isoform is lost at certain

stages of morphogenesis. Similar to these results, Fe/SOD appeared successively at some stages during somatic embryogenesis of chestnut (Bagnoli, 1998). Fe/SOD in this case was the isoform activated under conditions of stress, in order to protect chloroplasts from the harmful effects of ROS, which are rapidly accumulating during electron transport chain activity.

The SOD activity in *F. meleagris* bulbs grown at standard temperature (24 °C) and bulbs grown in medium with 4.5% sucrose was highest after 7 days on both inductive nutrient media. After pretreatment at low temperature (4 °C), high SOD activity was detected after 7 and 14 days. CAT also had the highest activity after 7 days in bulbs grown at standard temperature and in bulbs that were at low temperature. After growing bulbs in a medium with 4.5% sucrose the activity of CAT was the greatest after 14 days of cultivation on medium containing 2,4-D and KIN or after 21 days of cultivation on medium with TDZ.

Increased activity of these antioxidant enzymes during the first weeks of induction of morphogenesis in vitro can be associated with growth regulators to which

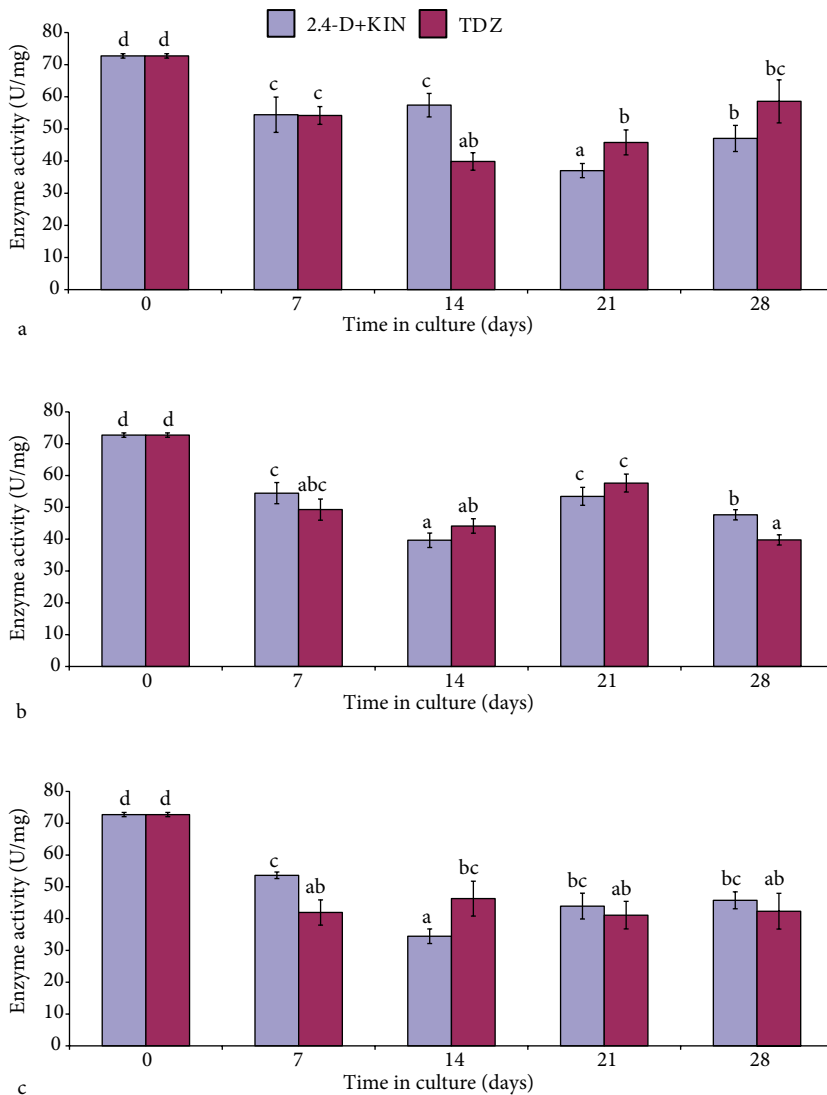




**Figure 4.** CAT activity during induction of morphogenesis in vitro in bulb segments culture after a pretreatment of 6 weeks at 24 °C (a), 4 °C (b), and with an increase in sucrose concentration (c).

bulb segments were subjected, as well as the stress caused by cutting the bulbs. During oxidative stress, cells can dedifferentiate and move towards the process of somatic embryogenesis (Pasternak, 2002). Synthetic growth regulators per se can lead to oxidative stress, which is similar to the oxidative stress caused by other stress factors (Grossmann, 2000). Glutathione reductase (GR) activity in the leaves of 2 tobacco varieties subjected to salinity gradually increased with rising NaCl concentrations, while other measured antioxidant enzymes did not show a similar trend. Therefore, GR is a key element in the evaluation of salinity tolerance of tobacco (Çelik and Atak, 2012). The early stages of somatic embryogenesis are characterized by induction of the expression of many genes related to plant response to stressful stimuli (Davletova et al., 2001), supporting the hypothesis of Dudits et al.

(1995) that somatic embryogenesis is a response of plants to stressful conditions to which they are exposed in vitro. Oxidative stress and increased production of ROS lead to the induction of somatic embryogenesis by increasing the level of endogenous auxin and accelerating the process of dedifferentiation (Correa-Aragunde et al., 2006). Kairong et al. (2002) found a correlation between increased levels of hydrogen peroxide and induction of somatic embryogenesis. Their results showed that SOD activity increased in the first days of induction of morphogenesis. It happened with the induction of morphogenesis in vitro of *F. meleagris*, where the highest SOD activity was found after 7 days of cultivation in all treatments. Increased activity of SOD at the beginning of somatic embryogenesis leads to increased concentrations of hydrogen peroxide. Thus, there is a change in the activity



**Figure 5.** POX activity during induction of morphogenesis in vitro in bulb segments culture after pretreatment of 6 weeks at 24 °C (a), 4 °C (b), and with an increase in sucrose concentration (c).

of catalases and peroxidases, which are in competition for the same substrate, i.e. hydrogen peroxide. The increase in catalase activity was followed by increased activity of SOD, so that the activity of catalase was highest 7 and 14 days from the start of morphogenesis induction. Peroxidase activity was highest in control bulbs where the concentration of hydrogen peroxide was the lowest, because they have a higher affinity for hydrogen peroxide than catalase (Passardi et al., 2005). Hydrogen peroxide accumulates under the influence of stress factors and SOD activity and acts as a signaling molecule that can activate certain genes and, thus, affects the synthesis of proteins involved in the initiation of somatic embryogenesis (Apel and Hirt, 2004). Vranová et al. (2002) reported that when

the reproduction and growth of cells was under negative ROS control, hydrogen peroxide stimulated somatic embryogenesis. Ganesan and Jayabalan (2004) confirmed the hypothesis that oxidative stress was connected with somatic embryogenesis. They added hemoglobin to nutritional medium in order to increase the concentration of oxygen and induce oxidative stress in cotton cell culture, where there had been observed increases in concentrations of SOD and POX at the first stages of somatic embryogenesis. There was evidence that hydrogen peroxide activated a specific protein kinase in *Arabidopsis* that started the phosphorylation of mitogen-activated protein kinase (MAPK), thus activating genes specific to the defense of the plant from stress factors (Kovtun et

al., 2000). Antioxidant enzymes play an important role in the organogenesis of plants as confirmed by analysis of POX, CAT, SOD, and esterase during morphogenesis of saffron (Sharifi and Ebrahimzadeh, 2010). In addition to esterase and POX, which are mentioned as potential markers of somatic embryogenesis, Bagnoli et al. (1998) suggest that superoxide dismutase and CAT may also be appropriate for monitoring the stage of somatic and zygotic embryogenesis in chestnut.

Plants of *Stevia rebaudiana* propagated in vitro and adapted to field conditions possess higher antioxidant capacity, expressed as equivalents of ascorbic acid, and accumulate more antioxidant metabolites during micropropagation (Zayova et al., 2013). Antioxidant enzymes involved in the morphogenesis of *F. meleagris*

in vitro showed no significant changes in relation to the applied growth regulators and pretreatments, indicating that the process of morphogenesis is strictly defined regardless of the growth regulators used. Small differences in the activity of these enzymes in bulbs subjected to different pretreatments and growth regulators can be attributed to the different stress levels different factors can cause.

### Acknowledgments

This work was supported by the Serbian Ministry of Education, Science, and Technological Development (project nos.: ON173015 and TR31019). Special thanks are due to Jovan Tadić (California, USA) for English language correction.

### References

- Aebi H (1984). Catalase in vitro. *Method Enzymol* 105: 121–126.
- Alscher RG, Erturk N, Heath LS (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J Exp Bot* 53: 1331–1341.
- Apel K, Hirt H (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Ann Rev Plant Biol* 55: 373–399.
- Auh CK, Scandalios JG (1997). Spatial and temporal responses of the maize catalases to low temperature. *Physiol Plantarum* 101: 149–156.
- Bagnoli F, Capuana M, Racchi M (1998). Developmental changes of catalase and superoxide dismutase isoenzymes in zygotic and somatic embryos of horse chestnut. *Aust J Plant Physiol* 25: 909–913.
- Balen B, Krsnik-Rasol M, Simeon-Rudolf M (2003). Isoenzymes of peroxidase and esterase related to morphogenesis in *Mammillaria gracillis* Pfeiff. tissue culture. *J Plant Physiol* 160: 1401–1406.
- Bannister JV, Bannister WH, Rotilio G (1987). Aspects of the structure, function and application of superoxide dismutase. *Crit Rev Biochem* 22: 111–118.
- Beyer WF, Fridowich I (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal Biochem* 161: 559–566.
- Bowler C, Van Montagu M, Inzé D (1992). Superoxide dismutase and stress tolerance. *Ann Rev Phys Chem* 43: 83–116.
- Bowler C, Van Camp W, Van Montagu M, Inzé D (1994). Superoxide dismutases in plants. *Crit Rev Plant Sci* 13: 199–218.
- Bradford MM (1976). A rapid sensitive method for the quantification of microgram quantities of proteins using the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
- Cassells AC, Curry RF (2001). Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. *Plant Cell Tiss Org* 64: 145–157.
- Çelik Ö, Atak Ç (2012). The effect of salt stress on antioxidative enzymes and proline content of two Turkish tobacco varieties. *Turk J Biol* 36: 339–356.
- Correa-Aragunde N, Graziano M, Chevalier C, Lamattina L (2006). Nitric oxide modulates the expression of cell cycle regulatory genes during lateral root formation in tomato. *J Exp Bot* 57: 581–588.
- Davletova S, Meszaros T, Miskolczi P, Oberschall A, Torok K, Magyar Z, Dudits D, Daek M (2001). Auxin and heat shock activation of a novel member of calmodulin like domain protein kinase gene family alfalfa cells. *J Exp Bot* 52: 215–221.
- Dudits D, Gyorgyey J, Bogre L, Bako L (1995). Molecular biology of somatic embryogenesis. In: Thorpe TA, editor. *In Vitro Embryogenesis in Plants*. Dordrecht: Kluwer Academic Publishers, pp. 267–308.
- Edge R, McCarvey DJ, Truscott TJ (1997). The carotenoids as antioxidants: a review. *J Photoch Photobio* 41: 189–200.
- Ganesan M, Jayabalan N (2004). Evaluation of hemoglobin (erythrocyte): for improved somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L. cv. SVPR 2). *Plant Cell Rep* 23: 181–187.
- Gilamore AM (1997). Mechanistic aspects of xanthophyll cycle-dependent photoprotection of higher plant chloroplasts and leaves. *Physiol Plantarum* 99: 197–209.
- Grossmann K (2000). Mode of action of auxinic herbicides: a new ending to a long, drawn out story. *Trends Plant Sci* 5: 506–508.
- Huyste VRB, Carnis WL (1982). Progress and prospects in the use of peroxidase to study cell development. *Photochemistry* 21: 1843–1847.

- Inzé D, Van Montagu M (1995). Oxidative stress in plants. *Curr Opin Biotech* 6: 153–158.
- Jia SR, Kumar PP, Kush A (1996). Oxidative stress in *Agrobacterium*-induced tumors on *Kalanchoe* plants. *Plant J* 10: 545–551.
- Joersbo M, Andersen JM, Okkels FT, Rajagopal R (1989). Isoperoxidases as markers of somatic embryogenesis in carrot suspension cultures. *Physiol Plantarum* 76: 10–16.
- Kairong C, Ji L, Gengmei X, Jianlong L, Lihong W, Yafu W (2002). Effect of hydrogen peroxide on synthesis of proteins during somatic embryogenesis in *Lycium barbarum*. *Plant Cell Tiss Org* 68: 187–193.
- Karami O, Aghavaisi B, Pour AM (2009). Molecular aspects of somatic-to-embryogenic transition in plants. *J Chem Biol* 2: 177–190.
- Karami O, Saidi A (2010). The molecular basis for stress-induced acquisition of somatic embryogenesis. *Mol Biol Rep* 37: 2493–2507.
- Kim JH, Baek MH, Chung BY, Wi SG, Kim JS (2004). Alternations in the photosynthesis pigments and antioxidant machineries of red pepper (*Capsicum annuum* L.) seedlings from gamma-irradiated seeds. *J Plant Biotech* 47: 314–321.
- Krsnik-Rasol M, Jelaska S, Šerman D (1982). Isoperoxidases—early indicators of somatic embryoid differentiation in pumpkin tissue. *Acta Bot Croat* 41: 33–39.
- Kukavica B, Veljović-Jovanović S (2004). Senescence-related changes in the antioxidant status of ginkgo and birch leaves during autumn yellowing. *Physiol Plantarum* 122: 321–327.
- Kukulczanka K, Kromer K, Czastka B (1989). Propagation of *Fritillaria meleagris* L. through tissue culture. *Acta Hort* 251: 147–153.
- Kovtun Y, Chiu WL, Tena G, Sheen J (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade. *P Natl Acad Sci USA* 97: 2940–2945.
- Lambe P, Mutambel HSN, Fouche JG, Deltour R, Foidart JM, Gaspar T (1997). DNA methylation of organogenesis totipotency and plant neoplastic progression? *In Vitro Cell Dev Pl* 33: 155–162.
- Langens-Gerrits MM, Miller WBM, Croes AF, Klerk GJ (2003a). Effect of low temperature on dormancy breaking and growth after planting in lily bulblets regarded in vitro. *Plant Growth Regul* 40: 267–275.
- Langens-Gerrits MM, Klerk GJ, Croes A (2003b). Phase change in lily bulblets regenerated in vitro. *Physiol Plantarum* 119: 590–597.
- Lichtenhaler HK (1987). Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol* 148: 350–382.
- Miller WBM, Langhans RW (1990). Low temperatures alters carbohydrate metabolism in Easter lily bulbs. *Hort Sci* 25: 463–465.
- Mizuno M, Kamei M, Tsuchida H (1998). Ascorbate peroxidase and catalase cooperate for protection against hydrogen peroxide generated in potato tubers during low-temperature storage. *Biochem Mol Biol Int* 44: 717–726.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plantarum* 15: 473–497.
- Nikolić M, Mišić D, Maksimović V, Jevremović S, Trifunović M, Subotić A (2008). Effect of low temperature on rooting rate and carbohydrate content of *Fritillaria meleagris* bulbs formed in culture *in vitro*. *Arch Biol Sci* 60: 5–6.
- Olmos E, Piqueras A, Martinez-Solano JR, Hellin E (1997). The subcellular localization of peroxidase and implication of oxidative stress in hyperhydrated leaves of regenerated carnation plants. *Plant Sci* 130: 97–105.
- Paek KY, Murthy HN (2002). High frequency of bulblet regeneration from bulb scale section of *Fritillaria thunbergii*. *Plant Cell Tiss Org* 68: 247–252.
- Paek KY, Sung NS, Park CH (1996). Several factors affecting bulblet regeneration from the culture of scale segment and node-bud in fritillary as medicinal bulbous plant. *Acta Hort* 440: 499–503.
- Passardi F, Cosio C, Penel C, Dunand C (2005). Peroxidases have more function than a Swiss army knife. *Plant Cell Rep* 24: 255–265.
- Passardi F, Penel C, Dunand C (2004). Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends Plant Sci* 9: 534–540.
- Pasternak TP, Prinsen E, Ayaydin F, Miskolczi P, Potters G, Asard H, Van Onckelen HA, Dudits D, Fehér A (2002). The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. *Plant Physiol* 129: 1807–1819.
- Petrić M, Subotić A, Jevremović S, Trifunović M (2011). Somatic embryogenesis and bulblet regeneration in snakehead fritillary (*Fritillaria meleagris* L.). *Afr J Biotechnol* 10: 16181–16188.
- Petrić M, Subotić A, Trifunović M, Jevremović S (2012). Morphogenesis *in vitro* of *Fritillaria* spp. In: Tuyl J, Arens P, editors. *Floriculture and Ornamental Biotechnology 6, Bulbous Ornamentals (Special Issue)*. London, UK: Global Science Books, pp. 78–89.
- Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel B (1992). Characterization of competent cells and early events of *Agrobacterium* mediated genetic transformation in *Arabidopsis thaliana*. *Planta* 188: 439–456.
- Sharifi G, Ebrahimzadeh H (2010). Changes of antioxidant enzyme activity and isoenzyme profiles during *in vitro* shoot formation in saffron (*Crocus sativus* L.). *Acta Biol Hung* 61: 73–89.
- Sharma P, Jha AB, Dubey RS, Pessaraki M (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J Bot* 2012: 1–26.

- Subotić A, Trifunović M, Jevremović S, Petrić M (2010). Morpho-histological study of direct somatic embryogenesis in endangered species *Fritillaria meleagris*. *Biol Plantarum* 54: 592–596.
- Sun CS, Wang DY (1991). *Fritillaria* spp. (*Fritillary*): in vitro culture and the regeneration of plants. In: Bajaj YPS, editor. *Biotechnology in Agriculture and Forestry: Medicinal and Aromatic Plants III*, 1 ed., Vol. XV. Berlin, Germany: Springer, pp. 258–269.
- Türktaş M, Aslay M, Kaya E, Ertuğrul F (2012). Molecular characterization of phylogenetic relationships in *Fritillaria* species inferred from chloroplast trnL-trnF sequences. *Turk J Biol* 36: 552–560.
- Veljović-Jovanović S, Kukavica B, Stevanović B, Navari-Izzo F (2006). Senescence and drought related changes in peroxidase and superoxide dismutase isoforms in leaves of *Ramonda serbica*. *J Exp Bot* 57: 1769–1778.
- Vranová E, Van Breusegem F, Dat J, Belles-Boix E, Inzé D (2002). The role of active oxygen species in plant signal transduction. In: Scheel D, Wasternack C, editors. *Plant Signal Transduction: Frontiers in Molecular Biology*. Oxford, UK: Oxford University Press, pp. 45–73.
- Walck JL, Cofer MS, Hidayati SN (2010). Understanding the germination of bulbils from an ecological perspective: a case study on Chinese yam (*Dioscorea polystachya*). *Ann Bot-London* 106: 945–95.
- Witomska M, Lukaszewska A (1997). Bulblet regeneration in vitro from different explants of *Fritillaria imperialis*. *Acta Hort* 430: 331–338.
- Yahraus T, Chandra S, Legendre L, Low PS (1995). Evidence for a mechanically induced oxidative burst. *Plant Physiol* 109: 1259–1266.
- Zayova E, Stancheva I, Geneva M, Petrova M, Dimitrova L (2013). Antioxidant activity of in vitro propagated *Stevia rebaudiana* Bertoni plants of different origins. *Turk J Biol* 37: 106–113.