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# Esterase and peroxidase isoforms during initial stages of somatic embryogenesis in *Fritillaria meleagris* L. leaf base

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**Abstract:** The aim of this study was to determine the enzymatic profile of esterases and peroxidases during early stages of somatic embryogenesis of *Fritillaria meleagris* L. Somatic embryogenesis was induced using the leaf base as explant on a medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). Zymography showed the presence of different moieties, six isoforms of esterases and peroxidases, during morphogenesis as compared to control explants. One isoform of esterases was detected only during the process of somatic embryogenesis, and one isoform was detected in control explants. Analysis of esterases with 1-naphthyl butyrate proved that esterases, which participate in somatic embryogenesis of *F. meleagris*, belong to the family of aryl esterases. For the first time it was proved that five isoforms of esterases, which are involved in morphogenesis of *F. meleagris*, belong to the family of aryl esterases, while two isoforms are carboxyl esterases. One isoform of carboxyl esterases was visible in control explants. This is also the first description of peroxidases during the morphogenetic process, and of the difference between aryl and carboxyl esterases. More isoforms of esterases during morphogenesis as compared to control explants are probably responsible for some early physiological process during somatic embryogenesis of *F. meleagris*.

**Key words**: aryl esterase; esterase; *F. meleagris*; peroxidase; somatic embryogenesis

## INTRODUCTION

*Fritillaria meleagris* L. (*Liliaceae*) is a rare perennial bulbous plant distributed throughout temperate climates of the northern hemisphere. This valuable ornamental plant has attractive purple-white flowers which produce seeds and subsequently bulbs that remain in the ground in the form of dormant bulbs during the rest of the year. Almost all *Fritillaria* species contain different types of alkaloids that are of interest to the pharmaceutical industry because of their antitussive and expectorant properties. Many of these species have been used in traditional medicine in China [1], Turkey [2] and Japan [3]. Natural propagation of *Fritillaria* is very slow, and bulb cutting or scaling is restricted to 3-5 scales, whereas propagation by seeds takes 5-7 years to produce a plant capable of flower production [4]. *In vitro* propagation and tissue culture techniques offer benefits for the multiplication of this species, as well as many other endangered and difficult to reproduce species [5]. Morphogenesis of *F. meleagris* could be established via organogenesis or somatic embryogenesis. Bulbs are the most frequently used starting material for organogenesis and micropropagation of *Fritillaria* species *in vitro* [6]. Successful *in vitro* organogenesis of *F. meleagris* using bulb scales as explants has been described [7-9]. Despite effective propagation using bulb-scale explants, the leaf base was also used to obtain somatic embryos [10]. It was shown that the best results of somatic embryogenesis were obtained on nutrition medium with 2,4-D at a concentration of 0.1 $mgL^{-1}$ (9.74 somatic embryos per explant-leaf base). It was also shown that direct somatic embryogenesis (without callus interphase) was induced in 93% explants.



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The molecular basis of somatic embryogenesis remains to be elucidated. Biochemical analysis of the total proteins expressed during the entire process has revealed which compounds are markers of somatic embryogenesis [11]. Some of the potential markers of somatic embryogenesis are esterases that were detected in a suspension of *Dactylis glomerata*. Esterases are hydrolytic enzymes that catalyze the decomposition reaction of esters, yielding acid and alcohol. These enzymes are specific to embryogenic cells that can regenerate the whole plant [12]. There is little information about esterases and their role in plant physiology. The most commonly studied esterases in plants are aryl and carboxyl esterases that can be detected according to the protocol of Burlina and Galzigna [13]. The procedure for esterase determination is based on the enzyme's affinity for 1- and 2-naphtyl acetate as a substrates. The enzyme attacks these substrates either on the carboxyl (carboxyl esterases) or on the aryl side (aryl esterases). In this way, the presence of aryl and carboxyl esterases in the sample can be estimated; however, differentiation between these enzymes is impossible without some other substrate.

An increase in esterase activity was observed during both zygotic and somatic embryogenesis [14]. It is thought that the role of these enzymes is degradation of pectin by the process of demethylation and deacetylation, reactions that are necessary for cell proliferation [15]. Despite the increase in activity of esterases during the process of embryogenesis, increased enzyme activity was observed during organogenesis [16]. The expression of different esterase isoforms in the embryogenic callus of *Mammillaria gracilis* can be explained by their role in the morphogenesis of this species. Petrić et al. [17] described several esterase isoforms (more than six isoforms, depending on treatment) during morphogenesis of *F. meleagris* in bulb-scale culture, in contrast to control explants in which morphogenesis did not begin. During morphogenesis of *F. meleagris* in bulb-scale culture, both somatic embryos and bulblets were observed on the explants. However, in this experiment we could not distinguish aryl from carboxyl esterases, nor specific isoforms related to organogenesis and somatic embryogenesis.

Aside from esterases, we described the isoform profile of peroxidases during morphogenesis of *F. meleagris* in bulb-scale culture [17]. More peroxidase isoforms were observed after the initiation of morphogenesis in comparison to control explants. Like esterases, peroxidases have also been reported as markers of somatic embryogenesis [18,19] or morphogenesis and tumorization [20].

The aim of this study was to identify the isoforms of esterases and peroxidases during the initial stages of somatic embryogenesis of *F. meleagris* in leaf base culture. We also wanted to differentiate between aryl and carboxyl esterases during somatic embryogenesis and to identify the types of isoforms that are involved during different stages of the process.

## MATERIALS AND METHODS

### Plant material

Cultures of *F. meleagris* L. were maintained on Murashige and Skoog (MS) medium [21] supplemented with 3% sucrose, 0.7% agar and 1 $mgL^{-1}$ thidiazuron according to the published procedure [10]. All cultures were maintained at 24±2°C and in a 16 h light/8 h dark photoperiod with an irradiance of 40 $W/m^2$. Leaf base segments (about 1 cm in length) were cut from the mother plant and placed in MS medium with 2,4-D (0.1 $mgL^{-1}$) for induction of somatic embryogenesis. The cultures were observed after 7 days when the first morphogenic changes were detected. Leaf explants were collected 7 days after culture initiation and each day after that for 16 days for further analysis. The control leaf base was the basal part of the leaf that was not subjected to the process of somatic embryogenesis after cutting. The second control was a leaf base cultured on medium without 2,4-D for 7 days.

### Enzyme extraction

To determine peroxidase and esterase isoforms, leaf bases cultured on medium with 0.1 $mgL^{-1}$ 2,4-D were used. The procedure for isoform determination was modified according to the protocol of Petrić et al. [17]. The samples were collected 7 days after morphogenesis induction (when the first signs of morphogenesis were visualized), and then every day for 16 days, in order to establish the different isoforms of enzymes that participate in the initial stages of the morphogenesis.

Frozen (-70°C) leaf bases (300 mg) were homogenized on ice in 4 mL of 0.1 M potassium phosphate extraction buffer (K-P buffer), pH 6.8, containing 200 mg of insoluble polyvinylpyrrolidone (PVP) and phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged (Thermo Electron Corporation, Biofuge Strators Centrifuge) for 5 min at 10000 g at 4°C. For esterase protein determination, the samples were homogenized in 0.2 M Tris-HCl buffer, pH 7.8, and evaluated by the Bradford method [22].

### Separation of peroxidase and esterases isoforms

For the separation of enzyme isoforms, polyacrylamide gel electrophoresis was used as described [17]. After the run, one part of the gel was stained with Coomassie Brilliant Blue (CBB) and the other was processed for enzyme activity staining [23]. Isoelectric focusing was performed using a Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology) according to the manufacturer's instructions. Isoelectric focusing was performed on a gel with ampholytes at a pH range of 3-10 at 7 W constant power for 1.5 h at 10°C. A pI kit (GE Healthcare) was used for the isoelectric point (pI) markers.

### Determination of peroxidase isoforms

Peroxidase isoforms were determined by gel incubation for 30 min in 50 mM monopotassium phosphate buffer (pH 5.8) that contained guaiacol (10 µL of guaiacol was dissolved in 10 mL of 20 mM Tris, pH 7.0), and 10 µL of hydrogen peroxide, according to the modified method of Siegel and Galston [24]. Gel analysis was performed using the TotalLab TL 120 graphics package. Twenty mg of 1-naphtylacetate and 2-naphtylacetate (substrates for esterases) was dissolved in 2 mL of 50% (v/v) acetone and mixed with 100 mL of 50 mM K-P buffer (pH 7.2). The gel was incubated for 30 min in this solution at room temperature, rinsed in tap water and stained for 25 min at 37°C in 0.2% Fast Blue RR Salt (Sigma Chemical Co., New York). The staining solution was prepared by dissolving 50 mg of Fast Blue RR Salt in 10 mL of distilled water and added to the gel. By staining the gel with only one substrate, 1-naphtyl acetate gave a dark brown color, while the 2-naphtyl acetate gave a pink color. The staining procedure with 1-naphthyl butyrate (third substrate for esterases) was the same as in the previous case, except that 1-naphthyl butyrate was in the liquid state (20 µL of 1-naphthyl butyrate was mixed with 2 ml 50% acetone).

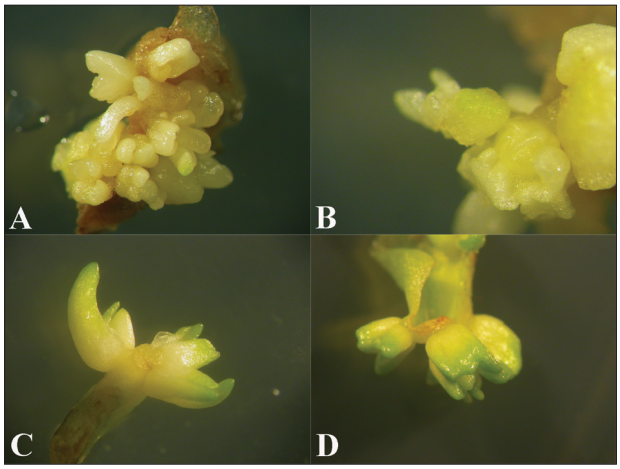


**Fig. 1.** Initial stages of somatic embryo formation in *F. meleagris* leaf-base culture. Somatic embryogenesis was achieved on a medium supplemented with 0.1 $mgL^{-1}$ 2,4-D. **A** – early formed globular somatic embryos on the surface of the leaf explant after 7 days in culture; **B** – late globular embryos together with early globular embryos on the same explant; **C** – differentiated, cotyledonary somatic embryos formed at the edge of the leaf base after 14 days; **D** – mature somatic embryos with clearly defined polarity after 28 days. Embryos at this stage can be separated from the explant and grown independently.

## RESULTS

The first morphological changes in the form of early globular somatic embryos were observed only after 7 days in culture (Fig. 1A). Late globular somatic embryos were detected after 14 days (Fig. 1B). All detected stages of somatic embryo development were observed directly on the surface of the leaf base explant without a callus interphase. The somatic embryos were regenerated on the edge of the leaf base. The early cotyledonary stadium of somatic embryos was visible after 14 days in culture (Fig. 1C). After 28 days, the first mature somatic embryos were observed (Fig. 1D). The embryos at the cotiledonary stages were fully developed, with a clearly visible shoot apex and an opposite root meristem attached to the surface of the explant. Somatic embryos at the mature cotyledonary stage can be removed from the explant and

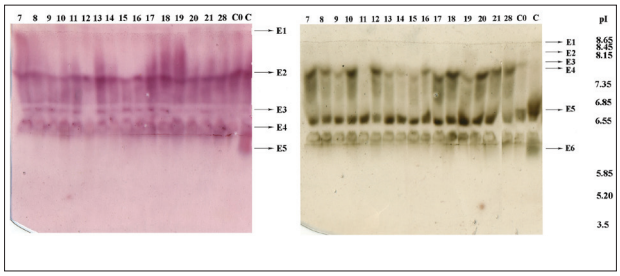


**Fig. 2.** Esterase isoforms detected with 2-naphtyl acetate (**A**) and 1-naphtyl acetate (**B**) in *F. meleagris* leaf-base culture grown on medium supplemented with 0.1 $mgL^{-1}$ 2,4-D during the first 28 days in culture. Numbers at the top of the image – days after which the different esterase isoforms were detected; C0 – control leaf base at the beginning of the experiment; C – leaf base after 7 days of induction on medium without 2,4-D; E1-E6 – different esterase isoforms.

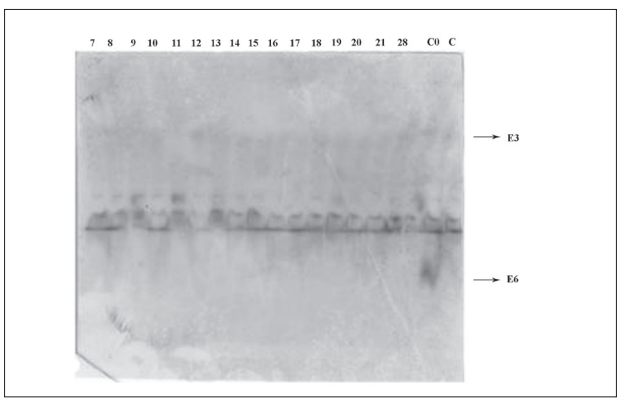


**Fig. 3.** Esterase isoforms detected with 1-naphthyl butyrate in *F. meleagris* leaf-base culture grown on medium supplemented with 0.1 $mgL^{-1}$ 2,4-D during the first 28 days of culture. Numbers at the top of the image – days after which different esterase isoforms were detected; C0 – control leaf base at the beginning of the experiment; C – leaf base after 7 days of induction on medium without 2,4-D; E3, E6 – different esterase isoforms.

transferred to the medium to grow separately and form the whole plant. Different developmental stages of somatic embryo regeneration were observed on the same explant at the same time, thus mature cotyledonary and globular embryos were observed together at the same leaf base, indicating asynchronous somatic embryogenesis.

Five isoforms with 2-naphtyl acetate and six isoforms with 1-naphtyl acetate were detected in the leaf bases that were grown on medium with 0.1 $mgL^{-1}$ of 2,4-D during the first 28 days of somatic embryogenesis (Fig. 2). Esterases were detected in acidic and basic pH regions. Larger numbers of esterases (isoforms E1, E2 and E3 with 1-naphtyl acetate and E1, E2, E3,

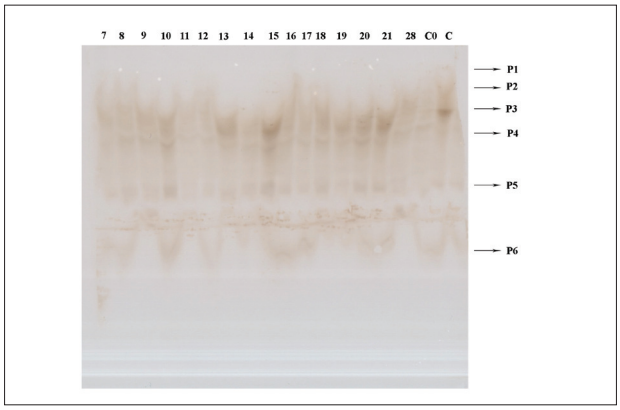


**Fig. 4.** Peroxidase isoforms in *F. meleagris* leaf-base culture grown on medium supplemented with 0.1 $mgL^{-1}$ 2,4-D during the first 28 days of culture. Numbers at the top of the image – days after which different esterase isoforms were detected; C0 – control leaf base at the beginning of the experiment; C – leaf base after 7 days of induction on medium without 2,4-D; P1-P6 – different peroxidase isoforms.

E4 and E5 with 2-napthyl acetate) were observed in the acidic region. Also, a more pronounced staining intensity was identified in the acidic part when both substrates were used. Isoform 3 was less intense after 11 days in culture in the case of 1-naphtyl acetate, while in the case of 2-naphtyl acetate it was missing. Immediately after, on the 12$^{th}$ day, isoform 3 had a very strong staining intensity that was much more pronounced with 1-naphtyl acetate (Fig. 2B). Isoform 3 was once again undetectable after 13, 14 and 15 days post induction and remained unchanged from the 16$^{th}$ day to the end of the experiment on the 28$^{th}$ day. The control leaf base and the leaf base after seven days of incubation on the medium without growth regulators showed very pale strips in the acid pH region with 1-naphtyl acetate. The leaf base after 7 days without 2,4-D exhibited the presence of one esterase isoform that was absent from all the other samples: isoform 5 with 1-naphtyl acetate and isoform 6 with 2-naphtyl acetate. Isoforms ranging from E1 to E2 were detected in all samples treated with 2-napthyl acetate; however, the isoforms were the most pronounced after 17, 18 and 19 days (Fig. 2A). Isoforms E1 and E2 were absent when the samples were treated with 1-naphtyl acetate (Fig. 2B).

Isoform E3 was only detectable with 1-naphthyl butyrate in the acidic pH region (Fig. 3). This isoform was visible after 12 days in culture until the end of the experiment (28$^{th}$ day). In the control samples, a

new isoform, which was absent at all others stages of somatic embryogenesis, was detected in the basic pH region.

During somatic embryogenesis in leaf-base culture, six peroxidase isoforms were observed (Fig. 4). All isoforms of peroxidases were present in all samples, but their staining intensity was different depending on the duration of somatic embryogenesis. Isoform 3 was the most intense moiety after 13, 15 and 21 days post initiation of the process, and also in the control explants. Leaf bases which were cultured on medium without 2,4-D, had a very strong band which originated from isoform 3 on day seven. Isoform 5 displayed a very stable staining intensity during the entire process of somatic embryogenesis, except in control leaf bases (Fig. 4).

## DISCUSSION

The process of morphogenesis was investigated using leaf-base culture on a medium containing 0.1 $mgL^{-1}$ 2,4-D. We investigated esterase and peroxidase isoforms during the initial stages of somatic embryogenesis of *F. meleagris*. Considering the that the first changes related to somatic embryogenesis were detected after 7 days, the isoform profiles were examined from the $7^{th}$ until the $28^{th}$ day. Direct somatic embryogenesis of *F. meleagris* in leaf-base culture and inducted by auxin, was described in detail in our paper [10]. It is very important to identify the stages of the process by morphological as well as physiological observations. According to the differences and changes in isoform profiles, a histological examination was performed. It was confirmed that early changes occurred after only 7 days in culture. At this stage, very pronounced stripes of esterases (isoform E4) on gels incubated with 2-naphtyl acetate were visible. This is in contrast with control leaf bases in which morphogenesis did not begin (isoform E4 was missing). Previously we observed that during morphogenesis, more esterase isoforms were observed than in control bulbs [17]. Similar results were obtained in [25], when a larger number of esterase isoforms were detected in embryogenic than in non-embryogenic tissue. A larger number of esterases was visible in the acidic pH region, which is in accordance with our previous results in bulb scale culture morphogenesis [17]. Using 1-naphthyl acetate, it was also observed that isoform E4 was absent in leaf bases that were cultured for 7 days in the medium without 2,4-D. Therefore, it can be assumed that E4 played a potential role during the initial stages of somatic embryogenesis of *F. meleagris*. Somatic embryogenesis was delayed in the medium without 2,4-D, and E4 was not detected after 7 days. This isoform was constant throughout the entire period, but its staining intensity was variable. After about 17 days and until the end of experiment, isoform E4 exhibited a very intense staining intensity, detected with 2-naphthyl acetate. This period was in correlation with the early cotyledonary stage of development of somatic embryo. This isoform could be associated with intensive cell divisions during the early globular and cotyledonary stages. During periods of growth (late globular stage) its intensity is lower. Enzyme isoforms that are specific to early stages of somatic embryogenesis can serve as markers of the first stages of the process [12,25,26]. In bulb scale culture of *F. meleagris*, certain esterase isoforms appeared at some later stages of morphogenesis (after 21 and 28 days, depending on treatment), but they were not present at the very beginning of the process. In leaf-base culture, all isoforms were visible at all stages, but their staining intensities were different. The difference between bulb and leaf-base culture can be due to different physiological paths of morphogenesis initiated from the two types of explants. Esterase isoforms in the leaf-base culture described in this manuscript are more visible than in the bulb scale culture [17]. This can be explained by the type of plant tissue and its sensitivity to the method used in the protocol for enzyme detection. More than six esterase isoforms were detected in the bulb scale culture [17]. The higher number of esterases in our previous experiment can be explained by the different types of explants and applied treatments, as well as by cessation of dormancy. Further experiments will be useful in determining the specific role of isoform E4, which was the most interesting in our experiment. The role of certain enzymes in the process of somatic embryogenesis has already been described, but in this case, the presence of different esterase isoforms during the early stages is only noted.

All detected esterase isoforms, with both substrates (1- and 2-naphtyl acetate), belong to one of the carboxy or aryl esterases. Only one esterase isoform (E3)

was visible when 1-naphthyl butyrate was used. This provides very good evidence that isoform E3 belongs to the family of carboxyl esterase, i.e. esterases that attack the substrate from the carboxyl side. All other isoforms belong to the family of aryl esterases, and this is the first time that esterase isoforms were identified as aryl or carboxyl esterases. In control explants (C0), carboxyl esterase E6 was detected. This carboxy isoform was absent during somatic embryogenesis. We concluded that the family of aryl esterases participated in somatic embryogenesis in *F. meleagris* leaf-base culture. Six isoforms of aryl esterases appeared during different early stages of the morphogenesis, and their potential role in the mentioned process could be attributed to the decomposition of polysaccharides in the cell wall. This physiological process can be considered as a starting point of morphogenesis [27]. Esterases play a very important role in the degradation of tapetum cells during pollen grain germination [28], a process involving multiple cell divisions, similar as in other morphogenetic pathways.

Six peroxidase isoforms were observed during somatic embryogenesis of *F. meleagris*. Isoform P3 was the most intense during the entire process, especially after 15 days, the time when the early cotyledonary stage was observed. This isoform was not visible in control leaf bases but was present in leaf bases cultured on medium without 2,4-D after 7 days. Therefore, isoform P3 was involved in the early stages of somatic embryogenesis. Isoforms of peroxidases (three isoforms) were not detected in control explants in *F. meleagris* bulb scale culture [17]; however, these isoforms were detected at all other stages of morphogenesis. Compared to the control, the number of peroxidase isoforms increased during the initial stages of somatic embryogenesis. Plant class III peroxidases can affect the levels of endogenous auxins and influence somatic embryogenesis, especially during its early stages [29]. Certain peroxidase isoforms are detected only in embryogenic tissue [30], which is indirect proof of their involvement in early embryogenic events. Increased peroxidase activities can be detected after bulb explant injury, after cutting, [17,31], and certain isoforms that were noted during early stages of somatic embryogenesis can be attributed to this phenomenon. This result may be connected to their role in the morphogenetic process; however, this role needs to be examined further.

To the best of our knowledge, this is the first paper describing the physiological aspect of the early stages of *F. meleagris* somatic embryogenesis in leaf-base culture with regard to esterase and peroxidase isoforms. This is the first description of five isoforms of esterases that are involved in *F. meleagris* morphogenesis, proving they belong to the family of aryl esterases, while two isoforms are described as carboxyl esterases.

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**Authors' contribution:** MP produced and maintained the *in vitro* cultures, designed and supervised the entire experiment. SJ provided the plant material. AS helped with the interpretation of data and supervised the writing of the manuscript. MT performed the statistical analysis and helped with the experimental design. VT, MG and ZV were involved in the detection and interpretation of antioxidant enzyme activity.

**Conflict of interest disclosure:** The authors claim no conflict of interest

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