

# Somatic embryogenesis from stamen filaments of *Aesculus flava* Sol. and peroxidase activity during the transition from friable to embryogenic callus

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## ABSTRACT

Species of the genus *Aesculus* are among the most attractive ornamental woody plants. Conventional propagation methods of these species are either inefficient (stem cuttings) or unsuitable for clonal propagation (seeds). The aim of the present study was to develop an efficient protocol for clonal propagation of elite specimens of yellow buckeye (*Aesculus flava*) by somatic embryogenesis. For this purpose, stamen filaments of yellow buckeye were cultivated on media supplemented with 1, 5 or 10  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D) combined with 0, 1, 5 or 10  $\mu\text{M}$  6-furfurylaminopurine (Kin), either under light or dark conditions, for 8 weeks, and then subcultivated on plant growth regulator (PGR)-free medium with 400 mg/l of glutamine. The highest somatic embryo (SE) initiation rates were achieved for the explants cultivated in darkness on medium containing 1  $\mu\text{M}$  2,4-D + 10  $\mu\text{M}$  Kin during callus induction (CI) phase. Embryogenic calli (EC) were initiated from friable calli, starting from the 7th week of culture initiation, while SEs appeared two weeks later, following a week of subcultivation of the explants on PGR-free medium. EC and SEs were observed only in the explants grown in darkness during CI phase. Minimal duration of CI phase and darkness necessary for SE induction was four weeks, while the highest embryogenic response was achieved when each lasted for 8–10 weeks. Obtained SEs were efficiently multiplied on medium supplemented with 0.05  $\mu\text{M}$  2,4-D + 5  $\mu\text{M}$  Kin by recurrent somatic embryogenesis. SEs at globular stage of development exhibited the highest capacity for secondary SE regeneration. High germination and conversion rates were attained in cotyledonary-stage SEs cultivated on medium with 0.05  $\mu\text{M}$  2,4-D + 5  $\mu\text{M}$  Kin, but this phase needs to be further optimised, since the obtained plants failed to acclimatize to greenhouse conditions. During the transition of calli from friable to embryogenic state, total peroxidase (POX) activity significantly decreased, indicating their involvement in the acquisition of embryogenic capacity. The presented protocol is suitable for clonal propagation and genetic transformation of this ornamental species, and POX activity may be used as a marker for SE initiation.

## 1. Introduction

Species of the genus *Aesculus* have frequently been used in the urban greening and landscape architecture, as they are among the most attractive ornamental woody trees and shrubs, capable of withstanding high pollution in the urban environment (Aničić et al., 2011; Pavlović et al., 2017). In addition, *Aesculus* species are also valued as medicinal plants, as their seed extracts have been used in traditional medicine since ancient times. Currently, they are widely used in commercial remedies for the treatment of venous insufficiency (Bombardelli et al., 1996) and in cosmetic preparations (Wilkinson and Brown, 1999).

*Aesculus* species have been conventionally propagated mainly by

seeds and stem cuttings. However, seeds are not suitable for clonal propagation, while other techniques for conventional vegetative propagation have been used with quite limited success (Chanon, 2005). Woody plant species are characterized by the long generation time and the prolonged period of juvenility, which make both the breeding process and quality assessment too lengthy. This could be circumvented by the implementation of tissue culture methods, among which somatic embryogenesis has been recognized as the most suitable method for clonal propagation (Merkle and Dean, 2000; von Arnold et al., 2002; Hernández et al., 2011; Guan et al., 2016; Corredoira et al., 2017). In this respect, embryogenic tissue is an ideal material for germplasm cryostorage conservation (Merkle et al., 2014; Barra-Jiménez et al.,

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2015; San José et al., 2015), and encapsulated somatic embryos may be used for artificial seed production and thus for automated large-scale and cost-effective production of the planting material (Merkle and Dean, 2000; Rai et al., 2009). Consequently, somatic embryogenesis is considered as a method of choice for clonal propagation of previously genetically tested and selected elite tree specimens, as well as for the maintenance of juvenile plant material during field-testing of clonal lines (Park et al., 2006; Barra-Jiménez et al., 2014).

As woody plants lose their juvenility, they become more recalcitrant to *de novo* regeneration (Bonga et al., 2010, 2017; Klimaszewska et al., 2011). Accordingly, juvenile tissues, mainly immature zygotic embryos, have been used most frequently for the induction of regeneration in woody species (Park et al., 2006; Bonga, 2017; Corredoira et al., 2013). However, zygotic embryos are not suitable for clonal propagation, since they are not genetically identical to the selected parent specimen due to cross-pollination. Thus, other tissues or organs (meristems, leaves, inflorescences or flower organs) have been used for clonal propagation of woody species (Merkle et al., 2003; Toribio et al., 2004; Lyyra et al., 2006; San-José et al., 2010; Correia et al., 2011; Corredoira et al., 2015; Blasco et al., 2013; Barra-Jiménez et al., 2014; Bonga, 2017; Asthana et al., 2017). Unfortunately, the frequencies of tissue culture response in these explants are still too low in the vast majority of tree species, and, in addition to rather low germination and conversion rates of embryos to green plants (Vieitez et al., 2012), this is the main reason why *in vitro* propagation of woody plants has not yet widely and routinely been used for commercial production of the planting material (Merkle and Dean, 2000; Park et al., 2006; Lelu-Walter et al., 2013; Ballester et al., 2016).

In *Aesculus* species, somatic embryogenesis has been thoroughly studied only in horse chestnut (*A. hippocastanum*). Consequently, many protocols for somatic embryo induction from different explant types are available. In *A. hippocastanum*, somatic embryogenesis has been initiated from: filaments (Jørgensen, 1989; Kiss et al., 1992; Capuana and Debergh, 1997; Troch et al., 2009), stem (Gastaldo et al., 1994), bark (Gastaldo et al., 1996), leaves (Dameri et al., 1986), cotyledons (Profumo et al., 1990), immature zygotic embryos (Radojević, 1988), and anthers (Radojević, 1978). Other *Aesculus* species are far less studied, and there are only a few reports for *A. carnea* (Radojević et al., 1989; Zdravković-Korać et al., 2008) and *A. glabra* (Trick and Finer, 1999) tissue culture response. To date, embryo regeneration in *A. flava* was only induced from anthers (Štajner et al., 2014) using a standard protocol developed for *A. hippocastanum* (Radojević, 1978).

Stamen filaments have been successfully used for clonal propagation of numerous woody plant species, including grapes (Dhekney et al., 2009) and cocoa (Alemanno et al., 1996), even in those genotypes that were recalcitrant to regeneration by other methods (Nakajima and Matsuta, 2003). Unexpanded inflorescences of some mature hardwood tree species have also been used for this purpose (Merkle and Dean, 2000; Merkle et al., 2003; Lyyra et al., 2006). However, among the species of the genus *Aesculus*, stamen filaments were exploited only in *A. hippocastanum* (Jørgensen, 1989; Kiss et al., 1992; Capuana and Debergh, 1997; Troch et al., 2009).

Peroxidases (POX) are involved in the protection of cells from reactive oxygen species (ROS), but they also participate in regulation of many physiological processes (Foreman et al., 2003; Tsukagoshi et al., 2010; Heyman et al., 2013), including *de novo* regeneration (Libik-Konieczny et al., 2015; Tubić et al., 2016; Guo et al., 2017). The best evidence for their diverse functions is the high number of genes encoding POX; 73 genes encoding POX were found in the genome of *Arabidopsis thaliana* (Tognolli et al., 2002). POX also participate in the regulation of some physiological processes through the regulation of local levels of ROS (Tsukagoshi et al., 2010). Considering above mentioned, POX were proposed as a marker for some physiological processes, including embryogenesis, thus allowing distinguishing between non-embryogenic and embryogenic calli (Kormutak and Vookova, 2001; Sun et al., 2013; Varhaníková et al., 2014).

In order to develop an efficient protocol for somatic embryo induction from stamen filaments of yellow buckeye, the 2,4-D/Kin balance, light conditions, and duration of the induction treatment were examined and optimized in the present study. To test a relationship between POX activity and the induction of somatic embryogenesis, the activity of POX was studied during the course of transition of friable to embryogenic callus and somatic embryo regeneration. To the best of our knowledge, there are no other protocols specifically developed for *in vitro* propagation of yellow buckeye.

## 2. Materials and methods

### 2.1. Plant material

The elongated inflorescences (12–15 cm) of *A. flava* were harvested from a tree growing in the Botanical garden “Jevremovac”, University of Belgrade, Belgrade, Serbia, at the beginning of April. Completely closed flower buds (7 mm) were separated, washed in running water with a few drops of household detergent (Fairy, Procter & Gamble), and then surface sterilized in 95% ethanol for 5 min, followed by 70% ethanol for 5 min, rinsed three times with sterile distilled water and blotted dry on a piece of sterile tissue paper. The perianth was removed and stamen filaments (4 mm) dissected out and placed on a callus induction medium.

### 2.2. Basal medium and culture conditions

The basal medium contained MS (Murashige and Skoog, 1962) macro and micro mineral salts (Lachner, Brno, Czech Republic), 2% sucrose, 100 mg/l myo-inositol, 200 mg/l casein hydrolysate, 2 mg/l thiamine, 2 mg/l adenine, 5 mg/l nicotinic acid, 10 mg/l pantothenic acid (all purchased from Sigma-Aldrich, St. Louis, MO, USA). The medium pH was adjusted to 5.5 before sterilization. The media were solidified with 0.7% (w/v) agar (Torlak, Belgrade, Serbia) and sterilized in an autoclave at 114 °C (80 kPa) for 25 min.

All cultures were maintained under cool white fluorescent light with a specified photosynthetic photon flux density (PPFD), as measured by an LI-1400 DataLogger equipped with an LI-190SA Quantum sensor, LI-COR Biosciences, for 16 h per day at 25 ± 2 °C, unless otherwise stated.

### 2.3. Callus induction and somatic embryo regeneration

In experiment 1, isolated filaments were placed in 90-mm plastic Petri-dishes containing callus induction medium (CIM) consisting of basal medium supplemented with 1, 5 or 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) combined with 0, 1, 5 or 10 μM 6-furfurylaminopurine (Kinetin, Kin). For each treatment, an equal number of filaments was kept either in darkness or exposed to 16 h photoperiod with a PPFD of approximately 100 μmol m<sup>-2</sup> s<sup>-1</sup> for 8 weeks (two subcultures, 4 weeks each). Thereafter, all explants were transferred to embryo regeneration medium (ERM) consisting of basal medium without plant growth regulators (PGRs) and supplemented with 400 mg/l of filter-sterilized glutamine (Glu, Sigma-Aldrich), and maintained under 16 h photoperiod with a PPFD of 100 μmol m<sup>-2</sup> s<sup>-1</sup> for an additional 8 weeks.

Four replications (Petri-dishes), each with 15 samples (filaments), were used per treatment (n = 60). Explant weight and the frequency of callus formation were recorded following 8 weeks of culture on CIM, while SE regeneration was recorded following an additional 8-week period on ERM using a stereomicroscope.

In experiment 2, the effect of culture duration on CIM containing 1 μM 2,4D + 10 μM Kin for different periods of time (1–10 weeks), followed by subcultivation on ERM until the end of a 16-week period in total was tested. The explants were kept in darkness for 8 weeks and then exposed to 16 h photoperiod with a PPFD of 100 μmol m<sup>-2</sup> s<sup>-1</sup> for

an additional 8 weeks.

To optimize the duration of dark treatment, in a last experiment, the explants were kept in darkness for different periods of time (1–10 weeks), and then exposed to 16 h photoperiod with a PPF of  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  until the end of a 16-week period in total. The explants were cultivated on CIM containing  $1 \mu\text{M}$  2,4-D +  $10 \mu\text{M}$  Kin for 8 weeks, and then subcultivated on ERM for an additional 8 weeks.

For experiments 2 and 3, four replications (Petri-dishes), each with 15 filaments, were used per treatment ( $n = 60$ ). In these experiments, somatic embryo (SE) regeneration was recorded weekly and the recordings were conducted at the end of a 16-week time period.

#### 2.4. Somatic embryo proliferation

By the end of both CI and embryo regeneration (ER) phases, SEs were isolated and cultured horizontally on embryo proliferation medium (EPM) consisting of basal medium supplemented with  $0.05 \mu\text{M}$  2,4-D +  $5 \mu\text{M}$  Kin +  $400 \text{ mg/l}$  Glu. Glutamine was previously shown to be beneficial for SE multiplication and maintenance in *A. hippocastanum* (Radojević, 1978, 1988) and *A. carnea* (Radojević et al., 1989; Zdravković-Korać et al., 2008). The cultures were exposed to 16 h photoperiod with a PPF of  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . To determine the ability of primary somatic embryos (PSE) to generate secondary somatic embryos (SSE), PSEs at different developmental stages (1–2 mm-long globular and heart-shaped PSEs, 3–4 mm-long torpedo PSEs, 5–10 mm-long cotyledonary PSEs and germinating PSEs  $\geq 10 \text{ mm}$ ) were cultivated on EPM for 4 weeks. The experiment was conducted in 8–25 replicates (Petri-dishes), with 10–20 PSEs per Petri-dish ( $n = 160$ –500). SSE formation was recorded after 4 weeks of culture initiation, with the aid of a stereomicroscope.

SEs originating from one filament were maintained as a single embryogenic line. The capacity of SEs of the acquired lines for recurrent somatic embryogenesis was tested by cultivating globular and heart-shaped PSEs on EPM (experiment 5). Six replicates (Petri-dishes) with 5 SEs in each replicate were used per embryogenic line ( $n = 30$ ). Number of SSEs per PSE was recorded after 4 weeks of cultivation using a stereomicroscope.

#### 2.5. Somatic embryo germination and plant acclimatization

For germination experiments, cotyledonary-stage SEs of different size (5 mm, 7 mm or 10 mm), with two cotyledons, were cultivated on basal medium containing 3% sucrose,  $0.05 \mu\text{M}$  2,4-D and  $5 \mu\text{M}$  Kin or  $5 \mu\text{M}$  BA (Radojević et al., 1989), designated as embryo germination medium (EGM). The radicle pole of SEs was inserted into the EGM. The cultures were maintained in light, as described above. Four to six replications (Petri-dishes) with 10–20 SEs in each replicate, were used per treatment ( $n = 60$ –80). The length of roots and epicotyls was measured after 4 weeks of cultivation.

The obtained plantlets were potted in plastic pots, containing a mixture of soil and perlite (1:1) pretreated with fungicide Previcur® (Bayer CropScience, Monheim, Germany), according to the manufacturer instructions. Plants were kept in a greenhouse, protected from direct sunlight. Humidity was preserved by covering the pots with transparent foil for 2 weeks.

#### 2.6. Total proteins isolation and spectrophotometric determination of POX activity

To assess the activity of peroxidases (POX), total proteins were extracted from friable calli with or without necrotic zones and embryogenic calli with or without somatic embryos at the globular or heart-shaped stage of development. All these calli were maintained in darkness on medium supplemented with  $1 \mu\text{M}$  2,4-D +  $10 \mu\text{M}$  Kin.

For each treatment, three biological replicates, consisting of calli cultivated in separate Petri dishes, were analyzed. Total proteins were

extracted after the method described by Tubić et al. (2016) by grinding material to fine powder in liquid nitrogen with addition of ice-cold  $50 \text{ mM}$  Tris-HCl pH 7.6 buffer containing  $10 \text{ mM}$  ethylenediaminetetraacetic acid (EDTA) pH 8.0,  $1 \text{ mM}$  dithiothreitol (DTT),  $1 \text{ mM}$  phenylmethylsulfonyl fluoride (PMSF),  $10\%$  (v/v) glycerol and  $5\%$  (w/v) insoluble polyvinylpyrrolidone (PVP), in 2:1 (v:w) proportion. After subsequent centrifugation at  $12,000 \times g$  for 10 min at  $4^\circ\text{C}$ , total soluble protein content in crude extracts was determined by fluorometric assay using Qubit Protein assay kit (ThermoFisher, Waltham, Massachusetts, USA) on the Qubit Fluorometer 3.0 (ThermoFisher).

Total POX activity was determined spectrophotometrically according to a modified method of Flatmark (1964) in a 1.5-ml reaction mixture consisting of  $50 \text{ mM}$  K-phosphate buffer pH 6.5,  $10 \text{ mM}$  pyrogallol and aliquots of  $10 \mu\text{l}$  of crude protein extract. The reaction was started by the addition of  $0.07\%$   $\text{H}_2\text{O}_2$  (v/v), and an increase in absorbance at  $430 \text{ nm}$  was monitored using Agilent 8453 spectrophotometer (Life Sciences, USA). Specific activity (U) was calculated as the rate of the reaction product purpurogallin formation (mmol) per min and presented per mg of total soluble proteins.

#### 2.7. Data analysis

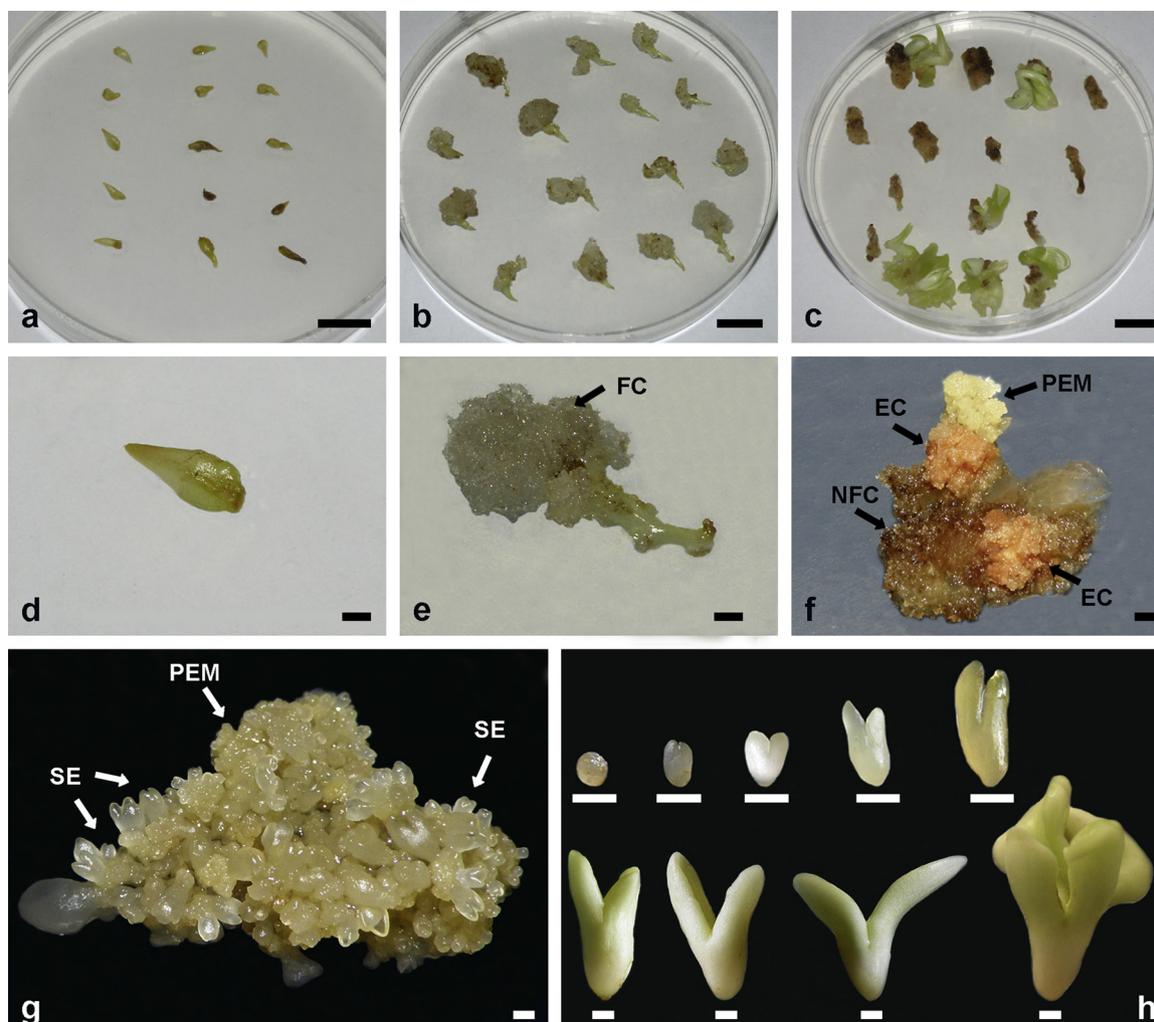
A completely randomized design was used for culture placement. Percentage data were subjected to angular transformation and SE number data to square root transformation prior to analysis, followed by inverse transformation for presentation. To analyze the main effects and interaction effects, the data were subjected to analysis of variance (ANOVA); three-way ANOVA for experiment 1, two-way ANOVA for experiment 5 and one-way ANOVA for experiments 2, 3, 4 and 6, and the means were separated using LSD test at  $p \leq 0.05$ . Results are expressed as the frequency of explants regenerating SEs, the mean SE number per explant and an index of somatic embryo-forming capacity (EFC), calculated as follows:  $\text{EFC} = (\text{mean SE number per explant}) \times (\% \text{ of regenerating explants})$ . For secondary somatic embryo (SSE) formation, an index of secondary somatic embryo-forming capacity (SSEFC) was used, calculated as follows:  $\text{SSEFC} = (\text{mean SSE number per PSE}) \times (\% \text{ of PSEs regenerating SSEs})$ . The indices of EFC and SSEFC were calculated per Petri-dish using appropriately transformed data, and then the means were calculated and presented without inverse transformation. Multiple Regression test was used for testing a correlation between SSEFC and overall SSE number. All data obtained for total POX activity were subjected to ANOVA, and the means were separated using the LSD test at  $p \leq 0.05$ .

### 3. Results

#### 3.1. Plant material and callus formation

Stamen filaments of yellow buckeye were easily and efficiently disinfected, with only up to 0.5% contaminated explants recorded in several consecutive years. In order to determine suitable conditions for efficient callus induction, the explants were exposed to a range of 2,4-D/Kin combinations, and grown either under light or dark conditions. Under the light, the explants slightly enlarged and usually displayed red pigmentation (Fig. 1a, d), but only a few of them formed small, greenish, granulated and solid calli. In the darkness, calli were friable, watery, pale-ocher, without solid zones (Fig. 1b, e). The proximal part of the filaments was the most responsive, so less efficient 2,4-D/Kin combinations induced callus formation only from the proximal part of the explants, while the most effective 2,4-D/Kin combinations triggered callus formation all over the explants, including the distal part (Fig. 1e).

Light/dark conditions and Kin concentration significantly affected both the frequency of callus formation and callus yield ( $p \leq 0.001$  each), while the concentration of 2,4-D only affected callus yield ( $p \leq 0.001$ ) (Supplementary Table 1). Filaments cultured on the media containing 2,4-D as a sole PGR elongated and seldom formed calli. In



**Fig. 1.** Somatic embryo (SE) induction from stamen filaments of yellow buckeye. Filaments were cultured on callus induction medium supplemented with  $1\ \mu\text{M}$  2,4D +  $10\ \mu\text{M}$  Kin, either in the presence of light (a, d) or in darkness (b, e). (c, f) Embryogenic calli, proembryonic masses (PEM), and SEs emerged from the explants during subculture on plant growth regulator-free medium for an additional 8 weeks. (g) PEM was maintained and SEs multiplied by recurrent somatic embryogenesis on medium supplemented with  $0.05\ \mu\text{M}$  2,4-D +  $5\ \mu\text{M}$  Kin +  $400\ \text{mg/l}$  Glu. (h) SEs at all distinctive stages of development were observed. EC – embryogenic callus; FC – friable callus; NFC – necrotic friable callus; PEM – proembryonic masses; SE – somatic embryos. Bar a-c: 1 cm; d-h: 1 mm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the presence of light, only  $10\ \mu\text{M}$  2,4-D was sufficient for callus induction from these explants, but with low frequency (12.6%). In the darkness, the frequency of callusing reached 36% in explants cultivated on medium with  $10\ \mu\text{M}$  2,4-D, but 2,4-D at lower levels was also capable of callus induction at low frequency (Fig. 2a).

However, when 2,4-D was combined with Kin, filaments did not elongate at all and callusing started from the proximal part of filaments following one week of culture. Under the light, calli were formed on up to 15.4% of the explants, while in darkness all explants responded, irrespective of 2,4-D/Kin combination (Fig. 2a). The highest callus yield was achieved in explants grown in darkness on medium supplemented with  $1\ \mu\text{M}$  2,4-D +  $10\ \mu\text{M}$  Kin (treatment designated as 1/10), followed by 1/5, 5/10 and 10/10 (Fig. 2b).

Following 4 weeks of culture initiation, the appearance of necrotic zones was observed all over the surface of friable calli cultivated on CIM, regardless of 2,4D/Kin combination used for callus induction. Subcultivation of these calli on fresh media or in a liquid medium did not prevent their necrosis. The embryogenic tissue appeared as nodular structures protruding from the necrotic calli (Fig. 1c, f), and were visible starting from the 7<sup>th</sup> week of cultivation on CIM. They were conspicuous by color and morphotype, scattered over the surface of necrotic friable calli (Fig. 1f). Embryogenic callus was solid, beige, and

it turned to pale yellow proembryonic masses (PEM, Fig. 1f). Neither embryogenic callus, nor PEM or SEs were observed on the explants which were grown under light during CI phase. So, the darkness was indispensable factor not only for callus yield, but also for SE induction.

All explants obtained in the above described experiment were transferred to PGR-free medium following an 8-week culture period and exposed to light. Regeneration of SEs was recorded after an additional 8-week period.

### 3.2. Somatic embryo regeneration

SEs started regenerating after a week of subcultivation of the explants on PGR-free medium. Light/dark conditions and 2,4-D/Kin combination, to which the explants were exposed during CI phase, affected subsequent SE regeneration during ER phase. The frequency of SE regeneration, the mean SE number per explant and the index of embryo-forming capacity (EFC) were all significantly affected by the light regime, Kin level and their interaction ( $p \leq 0.001$  each, Table 1). Although 2,4-D was indispensable for the induction of embryogenic calli, its level in CIM did not significantly impact the efficiency of SE regeneration. The presence of 2,4-D alone in CIM, as well as its combinations with  $1\ \mu\text{M}$  Kin, did not yield SEs at all (Table 1). Only 2,4-D/

**Table 1**Effect of 2,4-D/Kin ( $\mu\text{M}/\mu\text{M}$ ) concentration/combination and dark/light treatment during callus induction phase on subsequent somatic embryo induction from stamen filaments of *A. flava*.

2,4-D $\mu\text{M}$	Kin $\mu\text{M}$	Light (L) Dark (D)	Regeneration frequency (%)	Mean SE number	EFC
1	0	L	0 e	0 e	0 e
		D	0 e	0 e	0 e
	1	L	0 e	0 e	0 e
		D	0 e	0 e	0 e
	5	L	0 e	0 e	0 e
		D	4.93 $\pm$ 0.62 d	0.21 $\pm$ 0.05 de	0.14 $\pm$ 0.06 e
10	L	0 e	0 e	0 e	
	D	26.32 $\pm$ 0.27 a	10.93 $\pm$ 0.52 a	1.87 $\pm$ 0.50 a	
5	0	L	0 e	0 e	0 e
		D	0 e	0 e	0 e
	1	L	0 e	0 e	0 e
		D	0 e	0 e	0 e
	5	L	0 e	0 e	0 e
		D	17.59 $\pm$ 0.36 bc	1.75 $\pm$ 0.14 c	0.63 $\pm$ 0.21 cd
10	L	0 e	0 e	0 e	
	D	21.24 $\pm$ 0.25 ab	6.99 $\pm$ 0.44 ab	1.33 $\pm$ 0.36 b	
10	0	L	0 e	0 e	0 e
		D	0 e	0 e	0 e
	1	L	0 e	0 e	0 e
		D	0 e	0 e	0 e
	5	L	0 e	0 e	0 e
		D	12.93 $\pm$ 0.17 c	1.18 $\pm$ 0.14 cd	0.42 $\pm$ 0.11 de
10	L	0 e	0 e	0 e	
	D	17.26 $\pm$ 0.56 bc	4.34 $\pm$ 0.33 b	1.03 $\pm$ 0.44 bc	
<b>Source of variation</b>					
2,4-D concentration (A)			ns	ns	ns
Kin concentration (B)			$p \leq 0.0001$	$p \leq 0.0001$	$p \leq 0.0001$
Light/Dark (C)			$p \leq 0.0001$	$p \leq 0.0001$	$p \leq 0.0001$
A x B			$p \leq 0.05$	ns	ns
A x C			ns	ns	ns
B x C			$p \leq 0.0001$	$p \leq 0.0001$	$p \leq 0.0001$
A x B x C			$p \leq 0.05$	ns	ns

The explants were cultured on callus induction medium either under dark or light conditions for 8 weeks and then on embryo regeneration medium under light for an additional 8 weeks, when somatic embryo number was recorded. Data, obtained in experiment 1, indicate the mean  $\pm$  standard error. Four repetitions with 15 explants in each repetition, were used per treatment ( $n = 60$ ). Treatments denoted by the same letter within a column are not significantly different ( $p \leq 0.05$ ) according to LSD test. L - light; D - dark; EFC - embryo-forming capacity; ns - not significant.

Kin combinations containing Kin at 5 or 10  $\mu\text{M}$  induced SE regeneration, regardless of 2,4-D level. The highest frequency of SE regeneration (26%) and the highest mean SE number per explant (10.93) were obtained with 1/10 combination, and then dropped with increasing levels of 2,4-D (Table 1). The index of EFC also clearly indicated 1/10 as the most effective treatment during CI phase for subsequent SE regeneration from stamen filaments of yellow buckeye (Table 1).

To optimize regeneration conditions in the present study, two factors that most significantly impacted SE induction in a preliminary study - duration of darkness and CI phase, were further tested.

### 3.3. Effect of culture duration on CIM and dark treatment duration on SE initiation

The culture duration on CIM significantly affected the frequency of SE regeneration ( $p \leq 0.01$ ), the mean SE number per explant ( $p \leq 0.0001$ ) and the index of EFC ( $p \leq 0.0001$ , Table 2). Minimal duration of CI phase necessary for SE induction was four weeks, while the highest embryogenic response was achieved when CI phase lasted 8–9 weeks (Table 2).

The effect of dark treatment duration during CI phase on subsequent SE regeneration during ER phase was also analyzed. As expected, the duration of darkness significantly influenced the frequency of embryogenic explants, the mean SE number per explant and the index of EFC ( $p \leq 0.001$  all, Table 3). A minimum of four weeks of cultivation of the explants in darkness was necessary for SE initiation, while the highest embryogenic response required 8–10 weeks (Table 3).

### 3.4. Somatic embryo proliferation

As soon as yellow buckeye SEs regenerated from embryogenic callus, they started forming SSEs, thus it was not possible to distinguish SEs formed by *de novo* regeneration from PEM, from those newly formed by recurrent somatic embryogenesis from PSE (Fig. 1g). Somatic embryos at all stages of development were observed (Fig. 1h). The embryogenic cultures of yellow buckeye have been maintained through repetitive somatic embryogenesis, with sequential subculture at 4-week intervals on EPM medium for up to 2 years. The stage of development of PSE significantly affected the frequency of SSE regeneration ( $p \leq 0.001$ ), the mean SSE number per PSE ( $p \leq 0.01$ ) and the index of SSEFC ( $p \leq 0.001$ , Table 4). The youngest PSEs, at the globular and heart-shaped stage of development, exhibited the highest capacity for SSE regeneration. They responded with the highest frequency (74%), the highest mean SSE number per PSE (4.83) and the highest index of SSEFC (2.35, Table 4). Some of these PSEs formed up to 45 SSEs for 4 weeks. However, PSEs at more advanced stages of development exhibited significantly lower embryogenic response. SEs at the cotyledonary stage of development usually formed SSEs at the base and the tip of the radicle (Fig. 3a), most likely by direct somatic embryogenesis, as no callus formation was observed (Fig. 3a). The process of recurrent somatic embryogenesis allowed for a quick and massive multiplication of SEs (Fig. 3b).

Since the smallest SEs exhibited the highest capacity for SSE formation, the capacity of different lines, obtained from independent regeneration events, was further estimated by comparison of the capacity of globular and heart-shaped PSEs for recurrent somatic embryogenesis.

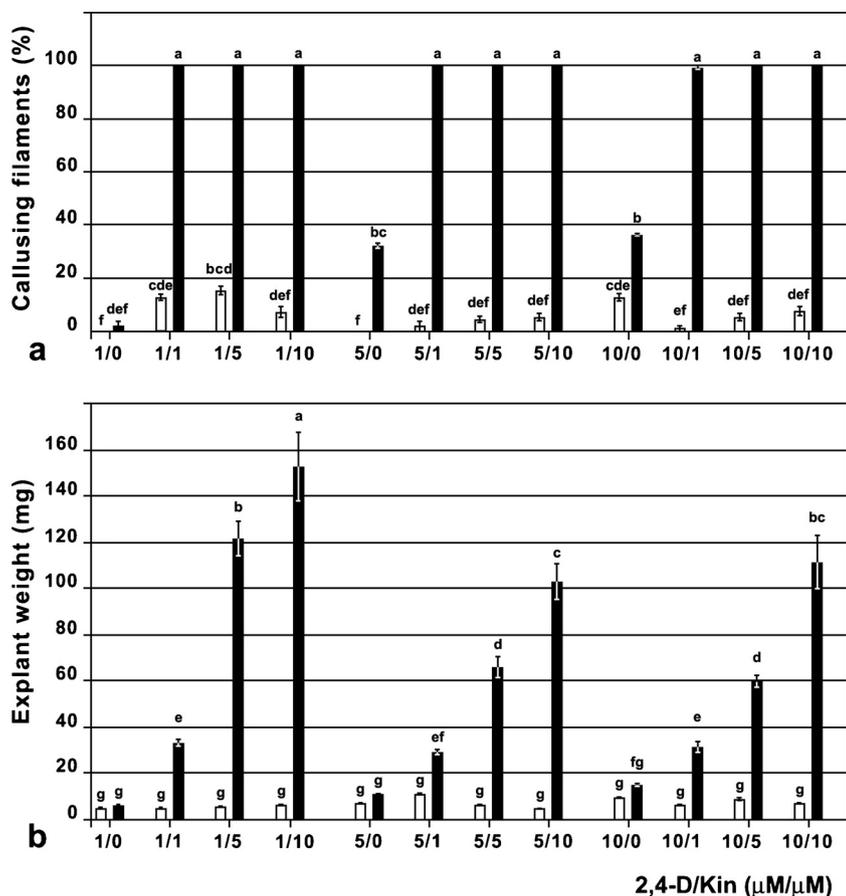


Fig. 2. The effect of 2,4D/Kin (μM/μM) concentration/combination and dark/light treatment on callus formation from stamen filaments of yellow buckeye. (a) Callus formation frequency. (b) Filament weight. Filaments were cultivated on medium supplemented with 1, 5 or 10 μM 2,4-D in combination with 0, 1, 5 or 10 μM Kin, either under light (white bars) or dark (black bars) conditions for 8 weeks. Data indicate the mean ± standard error of four repetitions with 15 explants in each repetition (n = 60). Values marked with the same letter were not significantly different (p ≤ 0.05) according to LSD test.

Table 2

The effect of callus induction (CI) phase duration on somatic embryo (SE) induction from stamen filaments of *A. flava*.

Duration of CI (weeks)	SE initiation frequency (%)	Mean SE number	EFC
1	0 f	0 e	0 f
2	0 f	0 e	0 f
3	0 f	0 e	0 f
4	9.73 ± 0.10 e	0.56 ± 0.10 de	0.25 ± 0.09 ef
5	16.51 ± 0.07 cd	1.61 ± 0.17 cd	0.55 ± 0.13 def
6	14.30 ± 0.34 de	2.07 ± 0.20 bcd	0.64 ± 0.31 de
7	23.03 ± 0.18 bc	4.54 ± 0.28 abc	1.09 ± 0.23 cd
8	31.55 ± 0.11 a	9.93 ± 0.43 a	1.92 ± 0.37 ab
9	34.89 ± 0.11 a	11.02 ± 0.41 a	2.12 ± 0.30 a
10	28.31 ± 0.03 ab	6.22 ± 0.30 ab	1.41 ± 0.17 bc
<b>Source of variation</b>			
Duration of CI	p ≤ 0.01	p ≤ 0.0001	p ≤ 0.0001

The explants were cultured on callus induction medium containing 1 μM 2,4-D + 1 μM Kin for different periods of time (1–10 weeks), and then transferred to embryo regeneration medium and grown until the end of a 16-week period. The explants were cultivated in darkness for 8 weeks and then under light for another 8 weeks. Data, obtained in experiment 2, indicate the mean ± standard error. Four repetitions with 15 explants in each repetition, were used per treatment (n = 60). Treatments denoted by the same letter within a column are not significantly different (p ≤ 0.05) according to LSD test. EFC - embryo-forming capacity.

As shown in Supplementary Table 2, the tested lines exhibited quite a different capacity for recurrent somatic embryogenesis. The frequency of SSE regeneration ranged from 53.5% to 100%, mean SSE number per PSE ranged from 0.50 to 12.74, and the index of SSEFC varied from 0.60 to 4.94. Overall SSE number, obtained from 30 globular PSEs for 4 weeks, ranged from 30 to 514. A high correlation was observed

Table 3

The effect of dark treatment duration on somatic embryo (SE) regeneration from stamen filaments of *A. flava*.

Duration of darkness (weeks)	Regeneration frequency (%)	Mean SE number	EFC
1	0 f	0 c	0 c
2	0 f	0 c	0 c
3	0 f	0 c	0 c
4	1.70 ± 0.57 ef	0.02 ± 0.01 c	0.04 ± 0.02 c
5	4.94 ± 0.63 de	0.20 ± 0.05 bc	0.15 ± 0.08 bc
6	8.16 ± 0.08 cd	0.33 ± 0.07 bc	0.17 ± 0.05 bc
7	14.30 ± 0.34 bc	2.24 ± 0.23 b	0.64 ± 0.24 b
8	28.31 ± 0.03 a	11.50 ± 0.52 a	1.91 ± 0.12 a
9	23.73 ± 0.70 ab	8.20 ± 0.44 a	1.62 ± 0.47 a
10	29.95 ± 0.04 a	10.78 ± 0.46 a	1.91 ± 0.22 a
<b>Source of variation</b>			
Duration of darkness	p ≤ 0.0001	p ≤ 0.0001	p ≤ 0.0001

The explants were cultured on callus induction medium containing 1 μM 2,4-D + 1 μM Kin for 8 weeks and then on embryo regeneration medium for another 8 weeks. The explants were kept in darkness for different periods of time (1–10 weeks), and then transferred to light and grown until the end of a 16-week period. Data, obtained in experiment 3, indicate the mean ± standard error. Four repetitions with 15 explants in each repetition, were used per treatment (n = 60). Treatments denoted by the same letter within a column are not significantly different (p ≤ 0.05) according to LSD test. EFC - embryo-forming capacity.

**Table 4**  
The impact of stage of development of primary somatic embryos (PSE) on secondary somatic embryo (SSE) regeneration.

PSE size (mm)	Frequency of SSE regeneration (%)	SSE mean number	SSEFC
1-2	74.38 ± 0.08 a	4.83 ± 0.006 a	2.35 ± 0.16 a
3-4	42.37 ± 0.33 b	0.55 ± 0.003 b	0.60 ± 0.12 b
5-10	20.18 ± 0.15 c	0.07 ± 0.001 c	0.14 ± 0.03 c
> 10	18.68 ± 0.19 c	0.08 ± 0.003 c	0.14 ± 0.04 c
<b>Source of variation</b>			
PSE size	$p \leq 0.0001$	$p \leq 0.0001$	$p \leq 0.01$

PSEs were cultured on medium supplemented with 0.05  $\mu\text{M}$  2,4-D + 5  $\mu\text{M}$  Kin + 400 mg/l Glu. SSE formation was recorded following 4 weeks of culture initiation. Data indicate the mean  $\pm$  standard error. Eight to twenty-five repetitions with 10–20 PSE in each repetition, were used per treatment ( $n = 160$ –500). Treatments denoted by the same letter within a column are not significantly different ( $p \leq 0.05$ ) according to LSD test. SSEFC - secondary somatic embryo-forming capacity.

between SSEFC and overall SSE number ( $r^2 = 0.94$ ), indicating that the index of SSEFC allowed reliable estimation of embryogenic capacity.

### 3.5. Somatic embryo germination and plant acclimatization

For germination, cotyledonary-stage SEs of different sizes were cultivated on EGM. Type of cytokinin in EGM did not significantly influence the frequencies of root formation, conversion of SEs to plantlets and SEs with shoots longer than 2 cm, but it significantly influenced root and shoot length ( $p \leq 0.0001$  and  $p \leq 0.05$ , respectively, Table 5). However, SE size significantly impacted all parameters: the frequency of root formation ( $p \leq 0.0001$ ), conversion rate ( $p \leq 0.0001$ ), root length ( $p \leq 0.0001$ ), shoot length ( $p \leq 0.05$ ) and the frequency of SEs with shoots longer than 2 cm ( $p \leq 0.05$ , Table 5). High germination

rates of above 80% were attained in all treatments, and nearly all 7–10 mm long SEs developed roots (Table 5, Fig. 3c). Some of these embryos (up to 65.3%) also developed epicotyls (Fig. 3d) and converted into plantlets (Fig. 3e). SEs forming only shoots were not observed. In addition, the frequency of formation of shoots longer than 2 cm was the highest in 10 mm-long SEs cultivated on EGM with 5  $\mu\text{M}$  Kin (28.7%, Table 5). Shoots and roots were longer in plants cultivated on EGM supplemented with Kin compared to those cultivated on EGM with BA (Table 5).

However, further development of plantlets was somehow arrested, as plants did not preserve active growth after conversion. During acclimatization phase, these plants survived for a few weeks only and then died mostly due to fungal infection, despite the treatment of the substrate with a fungicide. Consequently, no plantlets were established in the soil for longer periods of time.

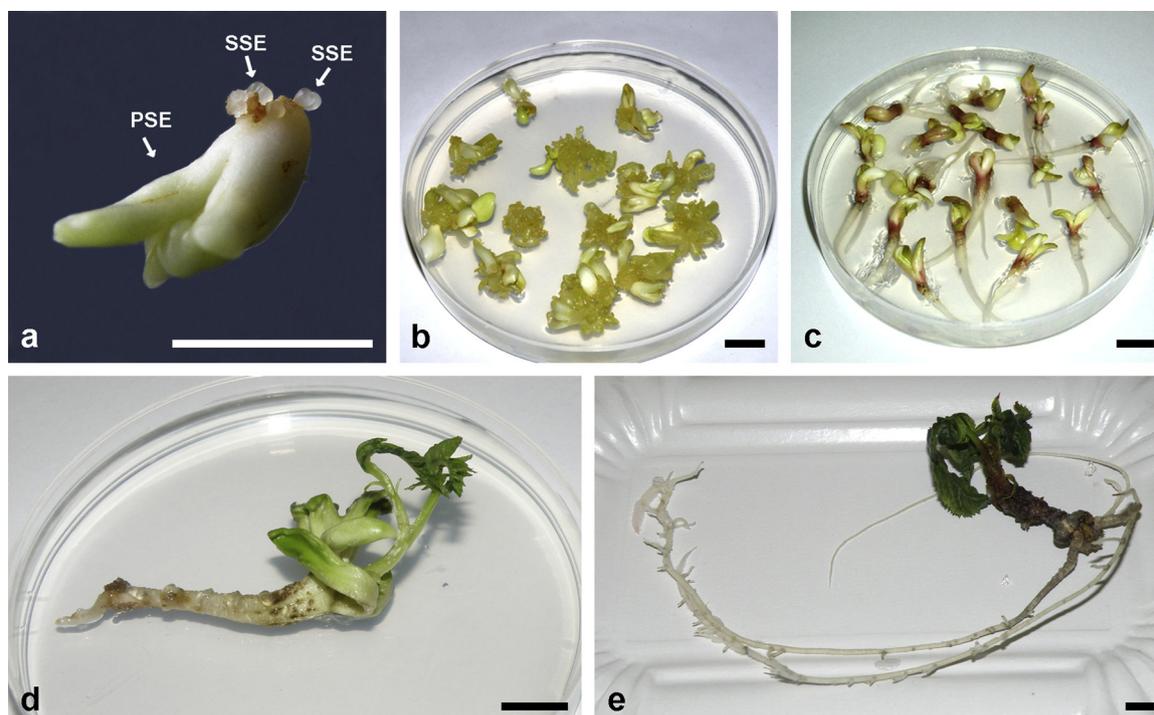
### 3.6. POX activity and somatic embryo regeneration

To test the possible involvement of POX in embryogenic callus initiation, its activity was recorded in calli during the course of their transition from friable to embryogenic state and SE formation. Measurement of total POX activity revealed the highest values in non-necrotic friable callus maintained in darkness for 4 weeks (Fig. 4). A significant decrease in activity was observed with the appearance of the first signs of necrosis, which continued to decrease with the progression of necrosis. The lowest POX activity was measured in embryogenic calli with or without SEs, and was at least seven-fold lower than in non-necrotic friable callus (Fig. 4).

## 4. Discussion

### 4.1. Plant material, callus formation and somatic embryo regeneration

In the present work, stamen filaments of yellow buckeye proved to

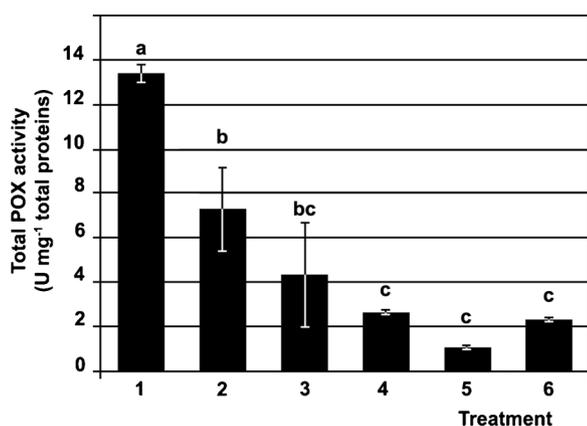


**Fig. 3.** Somatic embryo proliferation and germination in yellow buckeye. (a) A primary somatic embryo (PSE) formed secondary somatic embryos (SSEs) after culture on embryo proliferation medium containing 0.05  $\mu\text{M}$  2,4-D + 5  $\mu\text{M}$  Kin + 400 mg/l Glu. (b) Somatic embryos (SE) multiplied and developed further, passing through the cotyledonary stage of development. (c) Cotyledonary stage SEs (1 cm) developed roots after a week of cultivation on EGM supplemented with 0.05  $\mu\text{M}$  2,4-D + 5  $\mu\text{M}$  Kin. (d) SE (1 cm) cultivated on EGM with 0.05  $\mu\text{M}$  2,4-D + 5  $\mu\text{M}$  Kin for 4 weeks converted to plantlet. (e) Plantlet with well-developed root and shoot, ready for acclimatization. Bar a-e: 1 cm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

**Table 5**  
Effect of cytokinin type and the cotyledonary-stage embryo size on germination response.

Type of cytokinin	SE size (mm)	Root only (%)	Conversion (root + shoot) (%)	Root length (mm)	Shoot length (mm)	Shoots > 2 cm (%)
Kin	5	81.61 ± 0.18 b	39.36 ± 0.53 c	16.32 ± 0.88 c	11.12 ± 1.26 b	5.41 ± 1.11 b
	7	97.17 ± 0.32 a	51.68 ± 0.10 abc	25.29 ± 0.57 a	11.16 ± 1.16 b	1.30 ± 1.30 b
	10	99.68 ± 0.32 a	65.33 ± 0.30 a	26.53 ± 0.66 a	14.64 ± 1.42 a	28.67 ± 0.17 a
BA	5	86.50 ± 0.17 b	41.32 ± 0.26 c	10.62 ± 0.31 d	8.52 ± 0.83 b	0
	7	96.31 ± 0.46 a	50.00 ± 0.13 bc	12.50 ± 0.47 d	9.83 ± 1.17 b	4.81 ± 1.99 b
	10	99.68 ± 0.32 a	56.84 ± 0.25 ab	20.83 ± 0.97 b	11.53 ± 1.20ab	5.12 ± 1.27 b
<b>Source of variation</b>						
Type of cytokinin (A)		ns	ns	$p \leq 0.0001$	$p \leq 0.05$	ns
SE size (B)		$p \leq 0.0001$	$p \leq 0.001$	$p \leq 0.0001$	$p \leq 0.05$	$p \leq 0.05$
A x B		ns	ns	$p \leq 0.0001$	ns	ns

Somatic embryos (SEs) of different sizes (5 mm, 7 mm or 10 mm) at the cotyledonary stage of development were cultured on embryo germination medium supplemented with 0.05  $\mu\text{M}$  2,4-D and 5  $\mu\text{M}$  Kin or 5  $\mu\text{M}$  BA. The length of radicles and epicotyls was measured after 4 weeks of cultivation. Data indicate the mean  $\pm$  standard error. Four to six repetitions with 10–20 SEs in each repetition were used per treatment (n = 60–80). Treatments denoted by the same letter within a column are not significantly different ( $p \leq 0.05$ ) according to LSD test. ns - not significant.



**Fig. 4.** Changes in the activity of peroxidases (POX) during the course of friable callus transition to embryogenic callus. POX activity was measured at distinct phases of friable to embryogenic callus transition: 1- friable callus without necrosis, 2 - friable callus with mild necrosis, 3 - friable callus with large regions of necrosis, 4 - embryogenic callus isolated prior to somatic embryo (SE) formation, 5 - embryogenic callus with globular SEs, 6 - embryogenic callus with globular and early heart-shaped SEs. Results are presented as an average of three independent biological repetitions (n = 3). Means were subjected to analysis of variance (ANOVA) and values marked with the same letter were not significantly different ( $p \leq 0.05$ ) according to LSD test.

be an excellent starting plant material for SE induction with almost no loss of the explants due to contamination. However, to date, in *Aesculus* species stamen filaments have been utilized for SE initiation only in horse chestnut (Jørgensen, 1989; Kiss et al., 1992; Capuana and Debergh, 1997; Troch et al., 2009).

In the present study, 2,4-D/Kin combination and light/dark conditions significantly affected both callus initiation frequency and callus yield, as well as the subsequent SE regeneration from the calli. The highest response was achieved in the explants grown in darkness on medium supplemented with 1  $\mu\text{M}$  2,4D + 10  $\mu\text{M}$  Kin, while auxin alone was not sufficient for embryogenic callus initiation. Accordingly, Jørgensen (1989) reported that both 2,4-D and BA (at concentrations above 1  $\mu\text{M}$ ) were indispensable for embryogenic callus initiation from stamen filaments of horse chestnut. By contrast, Kiss et al. (1992); Capuana and Debergh (1997) and Troch et al. (2009) used auxins as a sole PGR for initiation of embryogenic callus from filaments of horse chestnut; the former group used 2,4-D (4.4–8.8  $\mu\text{M}$ ) + NAA (5.4  $\mu\text{M}$ ), while the two latter groups used 9  $\mu\text{M}$  2,4-D for this purpose. However, 10  $\mu\text{M}$  2,4-D was not sufficient for the induction of embryogenic callus in yellow buckeye.

Darkness was an essential factor for SE initiation in the present study, as the explants exposed to light during CI phase never formed SEs. In previous studies on *A. hippocastanum*, filaments were always kept in darkness during CI phase and the effect of light on subsequent SE regeneration was not known (Kiss et al., 1992; Capuana and Debergh, 1997; Troch et al., 2009). In some plant species light was essential for embryogenic callus induction and maintenance (Cabrera-Ponce et al., 2015), while in others it was inhibitory (Nameth et al., 2013). In the present study, light inhibited proliferation of cells within the explants and prevented the formation of massive calli, and consequently tissue for SE regeneration. Hence, the light probably acted as a stress factor, since it is well known that light can induce an oxidative burst in explants by generating reactive oxygen species (Pfeiffer and Höftberger, 2001; Shohalet et al., 2006). In line with this, red pigmentation of the light-cultivated explants, observed in the present study, was probably a stress-induced photoprotection response of the explants. Similarly, anthocyanin accumulation was observed in cotyledon explants of *A. thaliana* exposed to light (Nameth et al., 2013) and in calli during SE initiation in hybrid larch (von Aderkas et al., 2015).

Necrosis of friable callus preceded the formation of embryogenic callus in the present study. Similar phenomenon was observed in *Eucalyptus globulus* (Corredoira et al., 2015) and *Quercus ilex* (Blasco et al., 2013), in which also only necrotic dark brown calli produced nodular embryogenic tissue. This may be in line with the notion that physical and physiological isolation of cells from other cells could be a triggering signal for the embryogenic program (Kurzcyńska et al., 2007). Also, as these cells lost contact with the medium, and were thus lacking nutrients, starvation may also contributed to the triggering of the embryogenic pathway (von Aderkas and Bonga, 2000; Lee et al., 2001).

For the highest SE initiation rate in yellow buckeye, an eight-week culture period on CIM in darkness was optimal. Culture duration on CIM was investigated as to assure efficient induction of SE regeneration while keeping the CI treatment as short as possible. It is well known that prolonged cultivation of plant material on media containing auxins, particularly 2,4-D, might cause chromosome aberrations (Mukhopadhyay et al., 2005; Acanda et al., 2013).

The inhibitory effect of light on callus induction and subsequent SE regeneration observed in the present study was long-term, as a minimum of a 4-week-long cultivation of the explants in darkness was needed for SE initiation. By contrast, light inhibition of shoot regeneration from cotyledons of *A. thaliana* was transient and short-term, and it was easily overcome just by keeping the explants in darkness for 2–6 h following isolation (Nameth et al., 2013).

The SE initiation frequencies attained in the present study are moderate. However, they are not comparable with those obtained in

horse chestnut, since the effectiveness of protocols for SE initiation from stamen filaments was not reported in these studies (Jørgensen, 1989; Kiss et al., 1992; Capuana and Debergh, 1997; Troch et al., 2009). Generally, initiation frequencies reported for woody species were very variable, ranging from a few percents, e.g. 0.5% in *Kalopanax septemlobus* (Lee et al., 2017), 3–11% in *Quercus ilex* (Blasco et al., 2013; Barra-Jiménez et al., 2014; Martínez et al., 2017) through 26% in *Q. suber* (Hernández et al., 2003) and 52% in *Tsuga canadensis* (Merkle et al., 2014), up to 93.3% in *Picea asperata* (Xia et al., 2017).

#### 4.2. Somatic embryo proliferation

In the present study, embryogenic cultures of yellow buckeye have been maintained through recurrent somatic embryogenesis. This method is widely recognized as a very efficient mean for SE multiplication and maintenance of embryogenic lines (von Arnold et al., 2002; Cabrera-Ponce et al., 2015), especially in plant species with low SE initiation rates (Barra-Jiménez et al., 2014). In some plant species, such as those of the genus *Aesculus*, the process of recurrent somatic embryogenesis was easily achieved (Radojević, 1978, 1988; Radojević et al., 1989; Kiss et al., 1992; Čalić et al., 2005, 2012, 2013; Zdravković-Korać et al., 2008), as well as in *Q. ilex* (Blasco et al., 2013). However, in some other species, low embryo proliferation rates were reported as a limiting factor for embryogenic cultures maintenance (Barra-Jiménez et al., 2014).

SEs at the early stage of development exhibited the highest capacity for SSE formation in the present study. The same was reported for *A. hippocastanum* (Kiss et al., 1992), *A. carnea* (Zdravković-Korać et al., 2008), and *Q. alba* (Martínez et al., 2015). By contrast, zygotic embryos of *Alnus glutinosa* at the early cotyledonary stage of development exhibited much higher capacity for SE regeneration than globular SEs, but they also lost embryogenic capacity beyond the early cotyledonary stage of development (Corredoira et al., 2013).

Lines with poor capacity for SSE regeneration usually formed only a few SSEs that quickly reached the cotyledonary stage of development and thus lost embryogenic capacity. As a result, the embryogenic capacity of these lines was exhausted after only several cycles of recurrent embryogenesis, as was also observed in *Q. ilex* (Martínez et al., 2017). For long-term maintenance of embryogenic lines, only those lines with high capacity for recurrent somatic embryogenesis are suitable, as was also pointed out by Kiss et al. (1992).

#### 4.3. Somatic embryo germination and plant acclimatization

In the present study, quite high germination and conversion rates of SEs were achieved. By contrast, germination and conversion of horse chestnut SEs were much more difficult to achieve. In the first attempts of horse chestnut propagation by somatic embryogenesis, very low frequencies (0.5–1%) of SE germination and conversion were reported (Kiss et al., 1992; Gastaldo et al., 1994), while the acclimatization of the plants was not mentioned. In these studies, a PGR-free medium was used for SE germination. In subsequent studies, medium supplemented with 9.3 μM Kin + 4.6 μM IBA was used for germination, but the attained frequencies of germination were still less than 1% (Capuana and Debergh, 1997). Further improvement of maturation and germination phases raised conversion rates to 88.9% and frequencies of SEs with shoots longer than 2 cm to 43.8% (Capuana and Debergh, 1997; Troch et al., 2009). However, according to the authors, the overall quality of regenerated plantlets was still poor. Moreover, no acclimatization of these plantlets was reported. To the best of our knowledge, there are no reports on mass acclimatization of SE-derived plants for any *Aesculus* species. The best acclimatization rates to date (up to 100%) were achieved in plantlets of *A. hippocastanum*, obtained by shoot organogenesis (Šedivá et al., 2013). These plantlets survived for at least a year, but the establishment of plants in the field was not reported yet. Therefore, maturation and germination phases of SEs in *Aesculus*

species need further attention to improve plant quality and to produce plants well established in the soil.

*Aesculus* species are not the only example of unsuccessful acclimatization of SE-derived plants; poor SE germination, and conversion rates were also reported for some other hardwood species (Corredoira et al., 2002; Blasco et al., 2013). In holm oak and elm, the failure of SE conversion into plantlets was attributed to the retarded development of the shoot apical meristem (Corredoira et al., 2002; Blasco et al., 2013). However, this was not the case for SE-derived horse chestnut plantlets, which had well-developed shoot meristem (Troch et al., 2009). Therefore, further research is needed to reveal the cause of this physiological disorder. Fortunately, somatic seedlings of some hardwood species, such as *Q. suber*, were successfully acclimatized and plants established in the soil performed well during the field trials, without significant differences comparing to zygotic embryo-derived plants (Hernández et al., 2011). These results are encouraging, implying that the establishment of SE-derived plants in the soil is feasible for any plant species and justifying the choice of somatic embryogenesis as a superior in vitro technique for clonal propagation of woody species.

#### 4.4. POX activity and somatic embryo regeneration

A significant decrease in POX activity was observed during the transition from friable to embryogenic callus in the present study. These findings are in line with the previously published study of Kormutak and Vookova (2001), which presented a several-fold reduction of POX activity in embryogenic callus of white fir, as compared to non-embryogenic callus, and therefore considered the activity of these enzymes as a suitable indication of altered biochemical nature of the callus. A similar decrease in POX activity was observed during the induction of regeneration in gladiolus (Gupta and Datta, 2003/4) and *Avena nuda* (Hao et al., 2006). However, there are opposite examples, where increased POX activity correlated with the acquisition of the regeneration capacity (Xu et al., 2013; Sun et al., 2013; Varšančiková et al., 2014) and even new POX isoforms were found in regenerating explants (Tubić et al., 2016). Considering the results obtained in the present study, POX level could be used as a biochemical marker for the acquisition of competency of cells for somatic embryogenesis in yellow buckeye.

## 5. Conclusions

In the present study, a protocol for SE regeneration from stamen filaments of yellow buckeye was developed for the first time. Filament explants were efficiently disinfected by a simple method with almost no loss due to contamination. Darkness, 2,4-D and Kin were all indispensable for SE initiation. Although obtained regeneration frequencies were moderate (about 30%), the SE number was further dramatically increased by a very efficient process of recurrent somatic embryogenesis, which allowed for long-term maintenance of embryogenic lines for up to 2 years. Despite high germination and conversion rates attained in SEs cultivated on EGM, the obtained plants failed to acclimatize to greenhouse conditions. Therefore, further optimization of the maturation/germination phase is needed. A decrease in POX activity during the course of friable to embryogenic callus transition can be used as a marker for SE initiation and a starting point for understanding the biochemical mechanisms underlying somatic embryogenesis from stamen filaments of *A. flava*. The presented protocol is suitable for both clonal propagation of elite specimens of yellow buckeye, and for transgenic plants recovery following genetic modification.

#### Author contributions

SZK designed the study; SZK, DĆ, ND, JM and MM performed tissue culture experiments; LjT, ND and JS performed POX activity measurements; SZK and JS performed statistical analyses and prepared tables and figures; SZK wrote the manuscript and JS contributed in the writing

of the manuscript. All authors read and approved the manuscript.

### Conflict of interest

The authors declare that they have no conflict of interest.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.scienta.2018.12.021>.

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