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In vitro kalitev in zorenje peloda črnega bezga (*Sambucus nigra* L.)

Izvleček
Med obetajoče biotehnološke postopke žlahtnjenja rastlin sodi tudi *in vitro* zorenje peloda. Z namenom, da bi vzpostavili uspešen protokol za *in vitro* zorenje črnega bezga (*Sambucus nigra* L.), smo opravili postopno optimizacijo različnih faz samega procesa zorenja in kalitve peloda. V sklopu optimizacije postopka *in vitro* kalitve zrelega peloda z uporabo spremenjenega gojišča po Brewbaker-Kwacku (BK) smo dosegli visoko stopnjo kalivosti (77 %) po samo enourni inkubaciji. Razvili smo tudi učinkovito metodo inokulacije enojedrnih mikrospor za *in vitro* zorenje, pri kateri smo dosegli visoko živost inukuliranih mikrospor in nizko stopnjo kontaminacije kulture. Za gametofitni razvoj mikrospor, nastanek dvoceličnega in troceličnega peloda ter končno kalitev se je kot najbolj primerno izkazalo BK-K gojišče, ki je vsebovalo BK-minerale, glutamin, dva nukleozida, visoko koncentracijo saharoze, pri pH vrednosti 5.1. Pelod je dozorel v 10–12 dneh s 56,1-odstotno končno kalivostjo.

Ključne besede: črni bezeg (*Sambucus nigra*), *in vitro* zorenje mikrospor, kalitev zrelega peloda

In vitro germination and maturation of elderberry (*Sambucus nigra* L.) pollen

Summary
Establishing protocols for in vitro pollen maturation has recently been proposed as a promising biotechnological approach that can be utilized for breeding purposes. In order to establish efficient in vitro pollen maturation of elderberry (Sambucus nigra L.), the protocol was elaborated in several stages. Optimization of germination of mature pollen procedure was well established by modification of Brewbary and Kwak medium (BK). In an optimal treatment, a high pollen germination rate (77.4%) was achieved after only 1 hour incubation. An efficient inoculation procedure was developed for maturation, which resulted in the inoculation of viable unicellular microspores at a low contamination rate. A BK-K medium consisting of BK salts, two nucleosides, glutamine, high sucrose and adjustment of pH to 5.1, was found to be suitable for initiation of gametophytic development of microspores, formation of bicellular and tricellular pollen and final germination within the same medium. The whole maturation process was performed within 10-12 days and the final germination rate of in vitro matured pollen was 56.1%.

Keywords: *elderberry (Sambucus nigra) – in vitro microspore maturation – pollen germination*

1. Introduction

Sambucus nigra L., an important fruit species known as elderberry, black elder or common elder, is a tall, tree-like shrub, that is widespread on sunny locations in most parts of Europe, Asia, North Africa and is also naturalized in the United States. The small, white hermaphrodite flowers in large corymbs appear in early summer. Umbels, consisting of dark purple individual berries, ripen in late summer. An extensive description of black elder was given by Atkinson and Atkinson (2002). Many cultivars of *S. nigra* are planted in Europe for agronomic or ornamental purposes. Elderflower extracts are used as a beverage and food flavouring (Christensen et al. 2007). *S. nigra* berries have been globally utilized as a medicine or a source of dietary supplements (Dawidowicz et al. 2006; Lee and Finn 2007). The juice pressed from black elderberry fruit contains many primary metabolites, including various sugars and organic acids, but is mainly valued for secondary metabolites, particularly high amounts of the anthocyanins (Veberič et al. 2009). Various elder products, such as juice, tea, wine and pies, rich in anthocyanins and flavonols, exhibit antioxidant, anticarcinogenic, immune-stimulating, antibacterial, antiallergic and antiviral properties (reviewed by Charlebois 2007).

Because of its culinary and medicinal value, several improved cultivars of *S. nigra* are grown in dense plantations in Europe and the USA (Strauss and Novak 1971; Müller 1976). However, the agronomic performance of cultivars is still not optimal (Wazbinska and Puczel 2004). Breeding efforts are therefore needed for optimization of the growth habit and fruit characteristics.

Among biotechnological methods, *in vitro* manipulation of pollen grains have been used for haploid induction, *in vitro* pollination and, more recently, for genetic transformation attempts. Increasing interest has been focused on *in vitro* microspore maturation protocols, particularly due to their novel utilization as an alternative genetic transformation protocol. Since transformation of mature pollen was found to result only in transient, not stable transformation (Twell et al. 1989; Keller and Hamilton 1998), an innovative approach has been developed based on biolistic treatment of microspores. Following bombardment, microspores are subjected to *in vitro* maturation and pollen formation, which is germinated and used for fertilization (Touraev et al. 1997). When successfully applied, this approach has many advantages, including the absence of *in vitro* plant regeneration, a short *in vitro* period, an absence of chimerism, stability of transformation and low genotype influence (Heberle-Bors 1998). The prerequisite for this protocol is a well developed *in vitro* maturation assay. Attempts at *in vitro* germination of mature *in situ* formed pollen grains are often studied as the initial step. *In vitro* germination of mature pollen itself has been utilized as a breeding technique, since it can be utilized for gametophytic selection (Hormaza and Herrero 1996). For microspore maturation attempts, *in vitro* germination of mature pollen is frequently used in order to pre-select the basic components of media that are later used for maturation. Other important factors influencing *in vitro* pollen maturation and germination include a sterilization and isolation method, a selection of carbohydrates, boron and calcium, temperature, various supplements, pH and pollen density (Adaniya 2001; Lee 2009). Optimal conditions vary considerably among species. In *S. nigra*, mature pollen is tricellular (Charzynska and Lewandowska 1990). According to Shivanna and Johri (1985), nutritional media used for germinating tricellular species need to be carefully supplemented. In elderberry, successful germination of isolated pollen has already been reported by Muccifora et al. (2003) using BK nutritional medium, but pollen sterilization and *in vitro* growth has not been attempted.

The development of biotechnological methodologies based on *in vitro S. nigra* pollen treatments would be of particular interest for plant breeding approaches, in order to influence its pharmacological properties and/or culinary value. The main objective of our research was therefore to develop techniques for *vitro* maturation and germination of *S. nigra* pollen. We attempted to establish optimal *in vitro* germination conditions, to identify suitable pollen development monitoring, to establish a microspore maturation technique and to study the pollen developmental process with daily monitoring.

2. Materials and methods

2.1 Plant material

Selected *S. nigra* flower buds of suitable size, growing in optimal conditions, were collected. For experiments on mature pollen, opened flowers were collected in the morning, immediately after flower anthesis.

2.2 *In vitro* germination media

For i*n vitro* germination of mature pollen grains, a modified BK medium (Brewbaker and Kwack, 1963) was used. For the optimization process, BK medium consisting of 300 mg/l Ca $(NO_3)_2 \cdot 4H_2O$, 200 mg/l $MgSO_4 \cdot 7H_2O$, 100 mg/l KNO_3 and 100 mg/l H_3BO_3 was supplemented with 150 g/l sugar and 500 mg/l MES. Variations of this medium, including different pH values (4.0 - 7.0) and sources of carbohydrates (maltose, sucrose and glucose), were tested.

2.3 Determination of pollen development stage and viability

Acetocarmine staining techniques (1g carmine in 45% acetic acid) were used to select flower buds containing unicellular microspore. Determination of microspore and further developmental stages during *in vitro* cultivation was done by fluorescent staining. Propidium iodide (PI) was added to the staining solution, as described by Custers (2003). A drop of cultured microspores was added to a drop of staining solution on the microscope slide. Slides were analyzed after overnight incubation at 4 ℃ on a Nikon Eclipse 80i microscope equipped by UV. Pictures were taken using a VDS Vossküler digital camera, analysis was done using image analysis software (Lucia Cytogenetics 1.5.7 Software, Laboratory Imaging, s.r.o., Prague).
Pollen viability was assessed using tetrazolium salts prepared according to Rodriguez–Riano & Dafni, 2000. Pollen was considered viable if it turned deep pink. This technique was used to test the viability of mature pollen before germination tests and for microspores after *in vitro* cultivation.

2.4 Pollen (microspores) isolation

Fresh mature pollen was obtained by gentle squeezing of anthers from 15 - 20 flower buds directly into the germination media inside Petri dishes (35 x 10 mm). Incubation was performed in humidity chambers at 24℃, with stirri ng at 43 r.p.m. on an orbital shaker for 60 minutes. After incubation, at least 100 pollen grains from 3 different petri dishes per treatment were analyzed under a light microscope (Nikon Eclipse 80i). The measurement of pollen tube length was done by Lucia Cytogenetics 1.5.7 Software (Laboratory Imaging s.r.o., Prague).
Unicellular microspores were obtained from immature 1.8 -2.0 mm flower buds. The optimized inoculation protocol consisted of sterilization of flower buds in 16.6 g/l dichloroisocyanuric acid supplemented with of a few drops of surfactant Tween 20 for 10 min, followed by three rinses in sterile water. Microspores were released into the maturation medium by gently chopping the flower buds with a razor blade and manual stirring for a few minutes. Microspores were then passed through a 100 µm stainless-steel mesh to remove the larger debris. The microspore suspension was pelleted by centrifugation at 700 r.p.m. for 5 minutes and the pellet was resuspended in maturation media to achieve a final density of 1-2 x 10^4 microspores per ml. Isolated microspores were cultured on a rotary shaker at 43 r.p.m., in the darkness at 25 ℃ and dispensed into at least 15 petri dishes per isolation. During the incubation time of each maturation experiment, progress was analyzed daily by

measuring increased microspore size and monitoring the developmental stage by fluorescent staining.

3. Results

3.1 Optimization of *in vitro* germination of S. nigra mature pollen

Effect of stirring on a rotary shaker during incubation was found to be highly efficient. Using this method, a similar germination rate as obtained in 20 hours without stirring was achieved in just one hour. In these conditions, the germination test was done to optimize the pH value and evaluate the choice of carbohydrates. Data (Table 1) showed that using stirring, a high germination (Fig. 1a-b) was achieved on all media combinations, but sucrose was found to be superior to maltose and glucose. In terms of germination efficiency, lower pH values (pH 5.1 and 5.5) were superior to pH 6.0, but longer pollen tube lengths were recorded on media with higher pH values. The optimized BK-A medium (BK supplemented with sucrose as a carbohydrate source and pH value 5.5) were further tested also for optimization of *in vitro* maturation protocol.

Table 1: Germination rate and pollen tube length of *S. nigra* mature pollen cultivated by a stirring method, scored after one hour in BK-A medium with different carbohydrates and pH values

Medium sources of carbohydrates	Pollen germination (%)	Pollen tube length (µm)
sucrose	66.7 ± 3.5	90.6 ± 25.7
maltose	41.5 ± 17.5	103.3 ± 28.8
glucose	48.4 ± 20.7	73.6 ± 18.6

Medium pH values	Pollen germination (%)	Pollen tube length (µm)
pH 5.1	77.4 ± 9.5	84.7 ± 13.4
pH 5.5	65.7 ± 4.0	91.1 ± 24.7
pH 6.0	58.4 ± 6,3	176.1 ± 33.7

3.2 *In vitro* microspore maturation of *S. nigra*

Microspore isolation and inoculation conditions were based on our previous positive results achieved for *in vitro* maturation attempts in cucumber microspores (Vižintin and Bohanec 2004). Flower buds of 1.8-2.0 mm size, which were used for experiments, contained predominantly unicellular microspores. Following sterilization and isolation treatment, the viability of microspores was determined (data not shown). Based on these viability tests and contamination scoring during *in vitro* cultivation, the appropriate isolation procedure was chosen for a further test of *in vitro* maturation media composition. The optimized BK-K medium consisting of 300 mg/l Ca (NO3)2•4H2O, 200 mg/l MgSO4•7H2O, 100 mg/l KNO3, 100 mg/l H3BO3, 150 g/l sucrose, 500 mg/l uridine, 260 mg/l cytidine, 200 mg/l glutamine and 500 mg/l MES at pH 5.1 were used as maturation and germination medium for final experiments.

Maturation was monitored by periodic nuclear staining of microspores in the culture and final scoring of the germination rate in the same medium in which maturation occurred. For the determination of the microspore development stage, bright field (acetocarmine) and fluorescent (DAPI or PI staining) staining were tested. Bicellular and tricellular pollen stages were clearly visible only by fluorescent staining. Furthermore, using overnight staining in a complex buffer, PI was superior to DAPI. Typical unicellular, bicellular and tricellular and germinated pollen grains are presented in Fig. 1 d, e, f and c respectively.

First germinations of *in vitro* matured pollen on optimized medium were noted around the 9th day in culture, but a sudden burst of germination was noted around the 10th -12th day of culture, with a maximum rate of 56.10 % ±10.04 at pH 5.1.

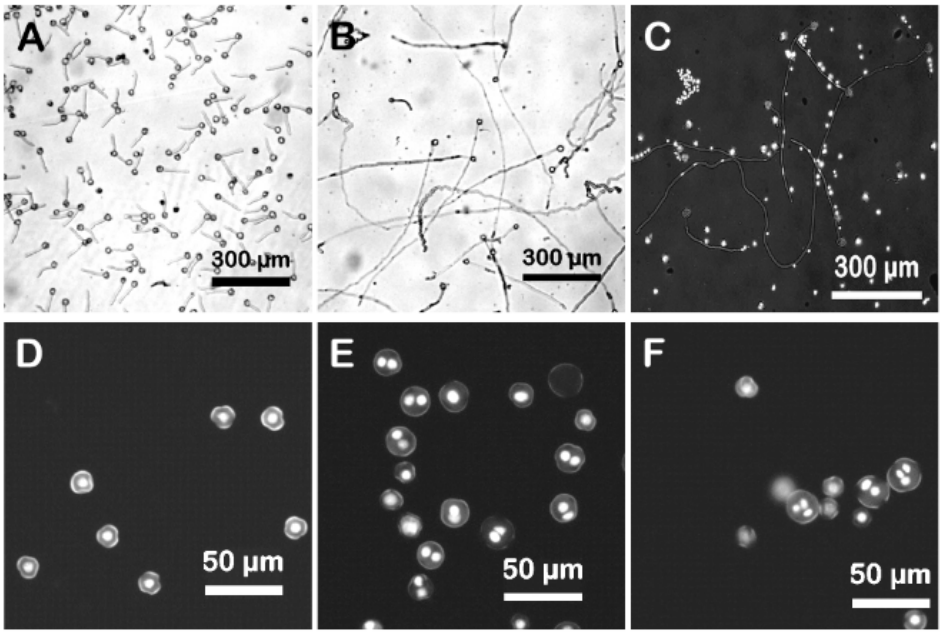

Figure 1: In vitro germination and maturation of S. nigra pollen: (a-b) in vitro germination of in situ matured pollen under light microscope, (c) in vitro germination of isolated, in vitro matured pollen under fluorescence microscope, (d) unicellular microspores after isolation, under fluorescent microscope (e) bicellular immature pollen in vitro cultured for about 3 days, under fluorescence microscope; (f) tricellular immature pollen in vitro cultured for about 11 days, under fluorescence microscope.

4. Discussion

The results of this study demonstrate that an *in vitro* pollen maturation protocol can be efficiently achieved in *S. nigra*. Despite the thick elderberry pollen exine, the appropriate staining protocol used in this study allowed clear visualization of all development stages of

pollen grains and, therefore, enabled monitoring of microspore *in vitro* maturation. Although DAPI stained nuclei have been used more frequently in other species (Rihova and Tupy 1999; Zhao et al. 2007), the main advantage of PI staining is its orange/red fluorescence, which does not interfere with the pollen exine blue fluorescence.

Studies related to the optimization of the mature pollen *in vitro* germination procedure were found to be useful for identifying an appropriate maturation medium. The finding that sucrose is superior to maltose and glucose for *S. nigra* pollen germination suggests a similarity with the carbohydrate requirements of lily maturation media (Clement et al. 1996). However, it is in contrast to results in other species, such as snapdragon (Barinova et al. 2002), with which maturation and germination were achieved only on media supplemented with maltose. pH requirements are also known to be species specific. Optimal values for *in vitro* germination media differ from pH 5.0 in buckwheat (Adhikari and Campbell 1998) to pH 8.0 in Brassica rapa (Sato et al. 1998). In previous experiments in *S. nigra* mature pollen germination, the pH values of the germination media were not reported (Muccifora et al. 2003). Our finding that the pH value is an important factor in *S. nigra* microspore *in vitro* maturation, accord with similar reports in tobacco and snapdragon (Barinova et al. 2004), but a neutral pH value (6.5 – 7.0) was more efficient in these species.

BK medium Brewbaker and Kwak (1963) has been reported as an efficient pollen germination medium for 79 genera (Brewbaker and Kwack 1963; Vasil 1964). The composition of the medium in these studies was relatively simple and has been used extensively thereafter as a basic medium for species specific optimization (Rosell et al. 1999; Gurusamy 2007). Despite the general suitability of Brewbaker and Kwack's (1963) medium (BK) for a large range of species with bicellular pollen, a more complex nutritional medium might be required for species possessing tricellular pollen. For example, germination of *Capsella bursa-pastoris* (Brassicaceae), a species with tricellular pollen, on BK medium was low (16%), and pollen tubes were abnormally short (Leduc et al. 1990), so other more complex media were preferred. The germination requirements of tricellular *S. nigra* pollen are less common, since a high germination rate was achieved on simple BK medium but at a high sucrose concentration (Muccifora et al. 2003).

The microspore isolation and inoculation procedure is often as import as the medium composition for establishing an efficient maturation protocol. Contamination remains a persistent problem in plant tissue culture, mainly due to the difficulty of growing donor plants in a controlled environment (Cassells 2001). The isolation method used in these studies and previously optimized in cucumbers (Vižintin and Bohanec 2004) was found appropriate, since the viability of microspores was highly preserved.

In spite of evident limitations relating to *S. nigra* morphological properties, such as annual flowering, short flowering period and inability of donor plants to grow in controlled conditions, an efficient *in vitro* pollen manipulation of S. nigra was developed. The established protocols can serve as basis for further biotechnological investigations for basic or applied breeding purposes.

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