Plant Cell Tiss Organ Cult (2007) 91:53–60
DOI 10.1007/s11240-007-9277-5

ORIGINAL PAPER

In vitro maturation and germination of *Orychophragmus violaceus* microspores

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Received: 12 December 2006 / Accepted: 23 July 2007 / Published online: 8 August 2007

Abstract Microspores cultured in vitro can be regarded as a system to study gene regulation, cell fate determination and cell differentiation during pollen development as well as an alternative method of genetic transformation in plants. In our study, pollen development and viability in *Orychophragmus violaceus* in vivo were determined and then pollen from the late unicellular stage was cultured in vitro. MS liquid medium + White vitamins + 2% (V/V) coconut milk + 0.5 M maltose, pH = 7.0 was the most appropriate for in vitro culture of *Orychophragmus violaceus* microspores. With this medium, the rates of in maturation and germination were 19.3% and 4.7%, respectively. Liquid medium with 0.6 M maltose + 1.6 mM boric acid + 2.9 mM $Ca(NO_3)_2$ + 29.6 μM vitamin B1, pH = 7.0 was optimal for germination of pollen matured in vivo. The rate of germination was 70.7%. Pollen matured in vitro cultured in similar medium exhibited a rate of germination of 62.7%. Hence, the experimental study showed that in vitro maturation of microspores is feasible and this experimental system can be applied to further theoretical and practical research.

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Keywords Brassicaceae · Pollen germination · Pollen culture · Viability

Abbreviations

6-BA	Benzyladenine
DAPI	4′,6-diamidino-2-phenylindole
FDA	Fluorescein diacetate

Introduction

Microspores of higher plants normally develop in planta into bicellular (e.g., tobacco) or tricellular (e.g., rice) pollen by one or two mitotic divisions following meiosis and mature by substance accumulation accompanying physiological and biochemical changes (Mascarenhas et al. 1984; van Went and Cresti 1989; McCormick 1993). Alternatively, under in vitro culture conditions favoring androgenesis, microspores may develop either directly or indirectly into haploid plants. This altered developmental pathway has been the focus of researchers hoping to derive haploid plants of many important crops species (Hu 1996). In contrast, research of in vitro maturation of microspores or immature pollen into functional pollen grains has received relatively little attention since it was reported in lily (Tanaka et al. 1980), tobacco (Kyo and Harada 1986; Benito et al. 1988; Tupý et al. 1991; Touraev and Heberle-Bors 1999), wheat (Stauffer et al. 1991), maize (Pareddy

and Petolino 1992) and *Antirrhinum majus* (Barinova et al. 2002). However, such an in vitro maturation system can provide possibilities for direct observation of the various events, including morphology, biophysiology and biochemistry, occurring throughout the microspore developmental process and for understanding their molecular regulation. Novel applications of in vitro microspore maturation include in vitro pollination (Zenkteler 1999) and genetic transformation of microspores (Aziz and Machray 2003). Unfortunately, this alternative biotechnological strategy is not currently available.

Orychophragmus violaceus (L.) O. E. Schulz, an annual or biennial brassicaceous plant, has good agricultural characteristics, such as a short growth cycle and high seed yield. Its seeds are rich in oil and have good nutritional value: the seed oil content can be as high as 35.8% and contains palmitic acid, oleic acid, and linoleic acid (Xiao and Luo 1994). As a vegetable, it has high protein content and is palatable (Luo et al. 1994). Therefore, to improve yield and quality of this oil and vegetable resource, and to facilitate crossbreeding with other Brassicaceae (Cheng et al. 2002; Ge and Li 2006), we have investigated in vitro culture of various plant tissues including microspore culture and established plant regeneration systems (Wu and Luo 1996; Jia et al. 1999; Wu et al. 2004). Unfortunately, little is known about *O. violaceus* gametophyte development and its genetic transformation has never been reported. The main objective of our research was to develop methodology for in vitro microspore maturation and germination of *O. violaceus* as prerequisites to pollen transformation.

Materials and methods

Seeds of *O. violaceus* were provided by the Laboratory of Bio-resources and Eco-environment (College of Life Science, Sichuan University, China). Plants were grown in a greenhouse at 22°C, with a daylength of 14 h.

Observation of flower bud size

For observations of in planta pollen development, we labelled 50 flower buds with microspores at the tetrad stage and followed their development into mature pollen. Lengths of young flower buds were measured at 10:00 am every day. At the same time, developing pollen samples were taken and the stages of pollen development were observed after samples were stained with DAPI (Kapuscinski 1995).

Determination of pollen development duration and viability

Androecia were removed from flower buds and put onto a slide with a drop of 15% sucrose. Pollen grains were released by crushing with a glass rod and cytological observations were made under a microscope (OLYMPUS BH2, Japan). Pollen size measurements were taken on ten samples; at each sampling 50 pollen grains were counted. Developing pollen was stained with DAPI (Kapuscinski 1995) for about 10 min and examined under a fluorescent microscope (LEICA DMR, Germany). Pollen viability was estimated under a fluorescent microscope after FDA staining (Heslop-Harrison et al. 1984). The experiments were repeated three times; each time 300 pollen grains were counted.

Preparation and culture of isolated microspores

Flower buds of *O. violaceus* at the uninucleate stage of pollen development were surface-sterilized in 70% (V/V) ethanol for 3 min. Then they were washed five times with sterile distilled water. The anthers from 30 flower buds were released into a glass vial (5 ml) and gently pressed with a glass rod in 1 ml 0.4 M maltose. The suspension was filtered though nylon mesh (50 μm pore size). The flow-through was collected in a sterile tube and centrifuged at 500 rpm for 5 min. The pellet was washed with 0.4 M maltose and centrifuged three times. After determining density with a haemacytometer, we diluted and cultured purified microspores in medium A [MS + White vitamins (White 1963) +2% (V/V) coconut milk + 0.5 M maltose, pH = 7.0] at a density of 2–3×10^5 ml^{-1} at 25–30°C in the dark. A drop of fresh medium A was added every 2 days. The developmental stages and viability of pollen cultured in vitro were determined under a fluorescent microscope every other day. The experiments were

repeated three times; each time 500 pollen grains were counted.

In vitro germination of matured pollen

Pollen taken from anthers before anthesis was cultured in germination medium B (0.6 M maltose + 1.6 mM boric acid + 2.9 mM $Ca(NO_3)_2$ + 29.6 μM vitamin B1, pH = 7.0) at 25 ± 1°C in the dark. About 2 h later, pollen germination was observed under a microscope. Pollen with tubes that were double the length of pollen diameter, were considered to have germinated and the number of germinated pollen grains was counted every 2 h for 24 h. The experiment was repeated three times and each time 500 pollen grains were counted.

In vitro-matured microspores were collected by centrifuging at 500 rpm for 5 min and then released into germination medium B. The other experimental procedures and conditions were the same as the germination of matured pollen in vivo.

Results

O. violaceus pollen development in vivo

Immature pollen at the tetrad stage from *O. violaceus* anthers during the spring season required 1–2 d for development into microspores in vivo, an additional 2–3 d for development into bicellular pollen, and a further 2–3 d into tricellular pollen. The total time was 7–11 d, from tetrad to tricellular mature pollen (Table 1). Tetrad diameters were 11.5–12.5 μm and microspores were 12.5–19.6 μm. The nucleus of pollen at the early unicellular stage was in the center of the cell and gradually moved to the edge because of vacuole accretion. The diameters of bicellular pollen were 19.6–25.4 μm. Tricellular pollen was spherical at first gradually becoming mostly ovoid. The diameters of tricellular pollen were 25.4–27.3/35.6 μm (short axis/long axis). Pollen was already tricellular when the flower buds were 10 mm long. However, pollination and fertilization did not occur until flower buds were greater than 12 mm, about another 2 d later.

In vitro culture of *O. violaceus* microspores

The viability and germination rates of microspores were used as criteria for successful pollen development in vitro. Three different liquid media were used for microspore culture: MS (Murashige and Skoog 1962), BK (Brewbaker and Kwack 1963) and AT_3 (Touraev and Heberle-Bors 1999), all supplemented with White vitamins and 0.4 M sucrose. These media were assayed in preliminary experiments for optimizing components to promote microspore maturation. After uninucleate microspores had been cultured for 8 d, the greatest subsequent pollen viability estimated by FDA staining was observed in MS medium (9.3 ± 1.2% compared with 5.0 ± 2.1% and 6.0 ± 1.7% in BK and AT_3, respectively). Therefore, MS medium was used for further optimization experiments to evaluate the effect of various carbohydrates on microspore culture. First, four combinations of sucrose and maltose plus White vitamins and MS medium were tested. After 8 d of incubation, the greatest viability (12.4%) was observed in MS supplemented with 0.4 M maltose and the four combinations had no obvious effect on microspore germination (Table 2). The effect of various concentrations of maltose (0.3 M, 0.4 M and 0.5 M) at different pH values (5.8, 6.5, 7.0, 7.5) on the maturation of *O. violaceus* microspores was tested. Viability increased gradually with increasing maltose concentration at each pH value and peaked at pH 7.0 with a slight decline at pH 7.5 (Fig. 1).

Coconut milk at 1%, 2% or 3% was added to the medium to determine its effects on microspore maturation and germination. While coconut milk did not impact the viability of microspores, it did

Table 1 Association between flower bud length, pollen diameter and stage of pollen development

Flower bud length (mm)	3–4	4–8	8–10	10 ≥
Stage of pollen	Tetrad	Unicellular	Bicellular	Tricellular
Diameter of pollen (μm)	11.5–12.5	12.5–19.6	19.6–25.4	25.4–27.3/35.6 (short axes/long axes)
Progress of pollen (day)	1–2	2–3	2–3	2–3

Table 2 Viability and germination rate of *O. violaceus* microspores cultured in medium with different carbohydrates

Carbohydrates	Concentration	Viability (%)	Germination frequency (%)
Sucrose	0.4 M	9.3 ± 1.2 A[a]	0.5 ± 0.2 A
Maltose	0.4 M	12.4 ± 2.3 B	0.7 ± 0.3 A
Maltose + sucrose	0.4 M + 0.05 M	7.7 ± 1.8 A	0.3 ± 0.1 A
Sucrose + maltose	0.4 M + 0.0.5 M	5.2 ± 1.5 C	0.2 ± 0.1 A

[a] Values sharing the same letter in each column are not significantly different from each other at $P < 0.05$

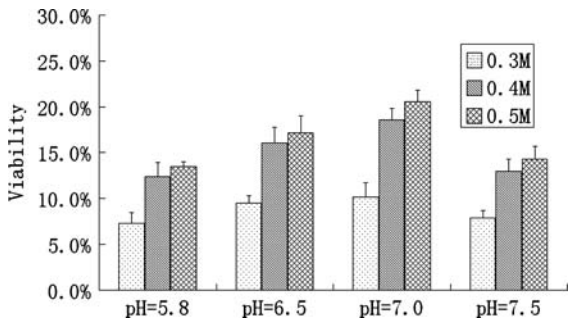

Fig. 1 Viability of *O. violaceus* microspores cultured in medium with different maltose concentrations and pH values

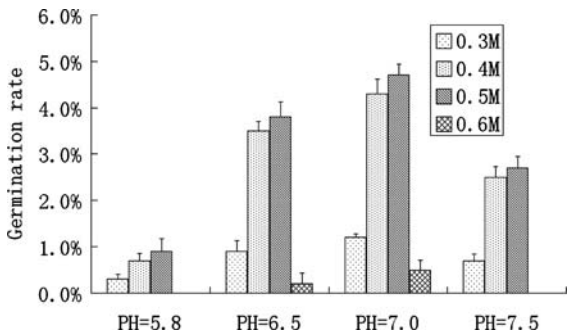

Fig. 2 Germination of *O. violaceus* microspores cultured in vitro in medium MS supplemented with different concentrations of maltose at various pH values

affect maturation and germination. The germination of pollen was highest (4.7%) at 2% coconut milk (Table 3). The effect of different concentrations of maltose and pH on the germination rate of microspores was also evaluated. As with viability, the rate of germination peaked when microspores were cultured in medium supplemented with 0.5 M maltose at pH 7.0. Therefore, the optimal media for in vitro microspore maturation was medium A (MS + White vitamins + 2% (V/V) coconut milk + 0.5 M maltose, pH = 7.0), yielding 19.3% maturation rate and 4.7% germination rate (Fig. 2).

Table 3 Viability and germination rate of *O. violaceus* microspores cultured in medium with different concentrations of coconut milk

Coconut milk (V/V)	Viability (%)	Germination frequency (%)
0%	20.7 ± 1.2 A[a]	1.7 ± 0.3 A
1%	20.3 ± 1.3 A	2.1 ± 0.2 AC
2%	20.5 ± 1.2 A	4.7 ± 0.2 B
3%	20.4 ± 1.5 A	2.6 ± 0.4 C

[a] Values sharing the same letter in each column are not significantly different from each other at $P < 0.05$

Cytology of *O. violaceus* microspores in culture

Microspores developed into bicellular pollen after 3 d of in vitro culture (Fig. 3C). Another 2–3 d later, some bicelluar pollen developed into tricellular pollen. Sparse starch grains were observed after microspores were cultured for 2 d, but then increased rapidly filling the entire pollen grain within 7 d (Fig. 3E). A few mature pollen grains germinated after 7 d (Fig. 3H). A change in microspore size was obvious during culture. Microspores were about 15 μm at the beginning of culture and grew to 28 μm after 7 d. Diameters of some bicellular pollen grains elongated to 36 μm in in vivo-matured pollen (Table 1). Furthermore, in vitro-matured pollen was nearly spherical, compared with the ellipsoid shape of most in vivo-matured pollen grains.

Viability change of microspores and bicellular pollen cultured in vitro

Both microspores and bicellular pollen cultured in media A declined in viability with culture duration

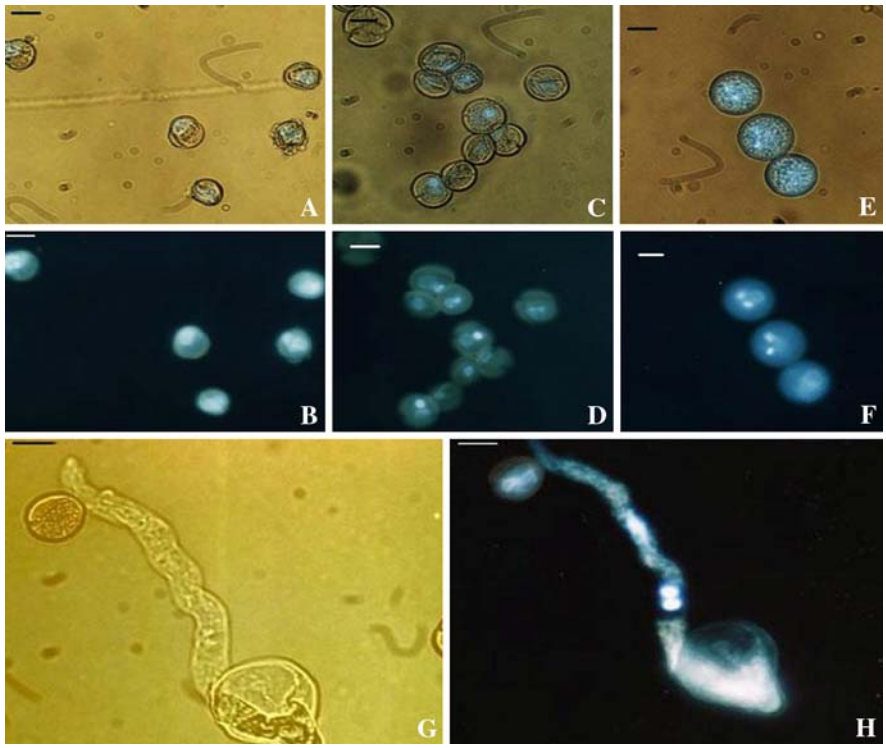

Fig. 3 In vitro maturation of *O. violaceus* microspores. (**A–B**) microspores in vitro cultured for 1 day under the light and fluorescence microscope; (**C–D**) microspores in vitro cultured for about 3 days under the light and fluorescence microscope; (**E–F**): in vitro matured pollen grains under the light and fluorescence microscope; (**G–H**): germination of in vitro matured pollen under the light and fluorescence microscope. Bars : 20μm

(Fig. 4). The slope of bicellular pollen viability was less than that of microspore viability over time. After 7 d of culture, the viability of cultured bicellular pollen decreased from 74% to 32%, with a maturation rate of 27.5%, whereas viability of cultured microspores decreased from 70% to 21%, with maturation rate of 19.3%.

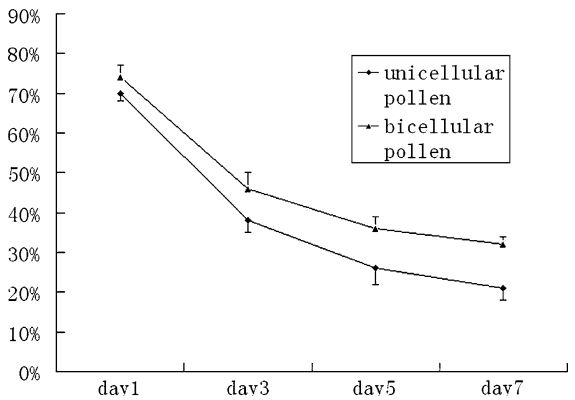

Fig. 4 Viability of in vitro cultured microspores and bicellular pollen in *O. violaceus* over 7 days of culture

In vitro germination of *O. violaceus*

To develop a protocol for in vitro pollen germination of *O. violaceus*, we examined eight combinations of maltose and boric acid in combination with 2 .9 mM $Ca(NO_3)_2$ and 29.6 μM vitamin B1, pH = 7.0 (Wang et al. 2000; Wang and Zhang 2004). A combination of 0.6 M maltose plus 1.6 mM boric acid yielded the greatest germination rate (Table 4). Subsequently, 5% (w/v) or 10% (w/v) polyethylene glycol (PEG) was added to evaluate its impact on germination; pollen did not germinate in these media. Therefore, medium B (0.6 M maltose + 1.6 mM boric acid + 2.9 mM $Ca(NO_3)_2$ + 29.6 μM vitamin B1, pH = 7.0) was optimal for *O. violaceus* pollen germination, with a 70.7% germination rate. When pollen grains that had matured in vitro were transferred to medium B, the pollen germination rate was 12.1%. Pollen matured in vivo began to germinate after 3 h. The lengths of pollen tubes were twice the pollen diameter after 10 h, with a 45% germination rate. They were three times the diameter after 15 h with a 65% germination rate and eight times after 20 h where the rate of germination exceeded 70%. The

Table 4 Germination frequency of *O. violaceus* in vivo matured pollen in germination medium supplemented with different concentrations of maltose and boric acid

Maltose (M)	Boric acid (mM)	Germination frequency (%)[a]
0.5	0	10.4 ± 5.2 A[b]
0.5	0.8	28.4 ± 3.6 B
0.5	1.6	61.9 ± 5.4 C
0.5	2.4	42.7 ± 3.5 D
0.6	0	6.9 ± 2.6 E
0.6	0.8	34.8 ± 5.9 F
0.6	1.6	70.7 ± 3.4 G
0.6	2.4	46.7 ± 6.3 H

[a] Rates of germination were accounted after 20 h

[b] Values sharing the same letter in each column are not significantly different from each other at $P < 0.05$

viability of pollen was 92% initially and 90% after 12 h and then sharply declined after 1 d, with no viability after 2 d.

Discussion

To be best of our knowledge, successful in vitro maturation of microspores has been reported only in a few species: wheat (Stauffer et al. 1991), maize (Pareddy and Petolino 1992), snapdragon (Barinova et al. 2002), and tobacco (Aziz and Machray 2003). The maturation rate of microspores in our study was 19.3% in optimal medium, an improvement over wheat (1%: Stauffer et al. 1991) and maize (14%: Pareddy and Petolino 1992).

Pollen development in vitro was similar to in vivo development in this study. It required 5–7 d for late microspores to develop into mature pollen in planta and another 2–3 d for pollination and germination. This indicates the need of after-ripening processes prior to germination to accumulate nutrients for continued development (Mascarenhas et al. 1984), as with fruit ripening (Wang et al. 1992). In contrast, there was no visible after-ripening during in vitro microspore maturation, since the in vitro-matured pollen germinated as soon as they matured, after 7–9 d.

Sucrose, as an osmotic regulator and carbon resource (Bai et al. 2006), has been used widely in tissue culture. However, viability of microspores in medium supplemented with sucrose was much lower than in medium with maltose in our study (Table 2). Similar results have been reported for barley (Scott and Lyne 1994) and snapdragon (Barinova et al. 2002). The most appropriate maltose concentration was 0.5 M. Low concentrations would result in microspore bleaching and high concentrations accelerated development. Plant growth regulators such as kinetin and benzyladenine had no obvious effect on microspore maturation and were unnecessary for culturing *O. violaceus* microspores. Coconut milk, containing a mixture of many activators such as amino acids, hormones, and enzymes to stimulate multiplication and differentiation of cells and tissues (Cao and Liu 1996), was beneficial in microspore culture for stimulating germination. The pH value was also an important factor in microspore culture. Barinova et al. (2004) demonstrated that a pH of 7.0 was equivalent to that in microspores of tobacco and snapdragon. When the pH value was higher or lower than 7.0, gene expression was blocked and the accumulation of starch grains was restricted (Barinova et al. 2004). A similar result was obtained in our study. The maturation medium should allow for normal microspore development or the viability of microspores may decline, rapidly blocking maturation.

Boron and calcium ions are involved in the growth of pollen tubes, protoplasm circumfluence, ion transport, and organelle flow (Guo et al. 2002; Yao and Zhao 2004; Bal and Abak 2005). Medium supplemented with 0.6 M maltose and boric acid enhanced the germination of pollen in our study. Temperature and illumination were also important for germination. PEG has been widely used for the germination of pollen (Wang et al. 2000; Barinova et al. 2002; Wang and Zhang 2004); however, it had no impact on *O. violaceus*.

In vitro maturation of isolated microspores represents an important experimental system for biotechnology, both in understanding cell biology and for utilization in crop improvement. First, pollen is the germ cell carrier responsible for fertilization. Its normal development is key to plant reproduction. However, it is difficult to study pollen cytology, molecular biology and transformation potential while microspores are encased within the anther. Hence, microspore or immature pollen development in vitro may facilitate such studies. Second, microspores are single haploid cells within thin cell walls compared to mature pollen; hence, cultured microspores may take

up exogenous DNA more readily than pollen in culture (Lin et al. 2005). Therefore, transformed zygotes that result from fertilization by in vitro-matured transgenic microspores can produce transgenic plants without chimerism (Shi 1998). This transformation strategy also avoids some negative impacts on transgenic plants that may result from somaclonal variation induced during a long in vitro passage. Alternatively, haploid transgenic plants that result from androgenetic development of transformed microspores may double spontaneously in culture yielding homozygous diploids, thus accelerating a breeding program.

In conclusion, this experiment demonstrated that cultured microspores of *O. violaceus*, an important oil resource and ornamental, could mature into germinable pollen. This method may become an important method to transform microspores of *O. violaceus*. Until now, the derivation of transgenic plants by transforming microspores in culture has been successful only in tobacco (Aziz and Machray 2003). The maturation and germination procedures in this study can be considered a first step in overcoming the recalcitrance of *O. violaceus* to transformation.

Acknowledgements This research was supported jointly by a Grant from the National Natural Science Foundation of China (No. 30270090) and the Fund for Promoting Scientific Research from Sichuan University. The authors would like to thank Drs E. H. King and SC Yan for their critical reading of the manuscript.

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