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IN VITRO MANIPULATION OF CUCUMBER (*CUCUMIS SATIVUS* L.) POLLEN AND MICROSPORES: ISOLATION PROCEDURES, VIABILITY TESTS, GERMINATION, MATURATION

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In vitro germination of isolated pollen grains and maturation of microspores are needed for several biotechnological manipulations of cucumber pollen. The present study was focused on establishing optimal conditions for monitoring isolation, sterilization and viability. Germination of mature cucumber pollen and maturation of microspores were attempted. Five protocols for testing pollen and microspore viability were tested and three were studied in detail. The viability of fresh or artificially damaged grains was tested using aniline blue in lactophenol, fluorescein diacetate (FDA) and 2,5-iphenyl tetrazolium bromide (MTT). For strong contrast, simple manipulation and non-fluorescence, MTT was found to be the most suitable. Pollen was efficiently isolated by shaking chopped anthers in germination media. Germination of isolated pollen was achieved for the first time using this procedure. The highest germination rate was obtained using 90.4% maltose in germination media at pH 7.0. Using a similar isolation method, microspores cultured on four selected maturation media showed relatively high viability (up to 29.1%) which was not affected by the sterilization procedure, although mature pollen grains did not form.

Key words: *Cucumis sativus* L., aniline blue in lactophenol, fluorescein diacetate (FDA), MTT, pollen viability, pollen isolation, in vitro germination.

INTRODUCTION

In vitro pollen development has drawn considerable research interest, since novel applications such as in vitro pollination (for review: Zenkteler, 1999) and, in particular, genetic transformation of microspores were recently achieved. Touraev et al. (1997) found that stable DNA integration following biolistic transformation of mature tobacco pollen grains could be achieved by targeting microspores, followed by in vitro maturation of pollen. In vitro maturation of microspores is a complex process which has so far been studied in tobacco (Benito et al., 1988; Tupy et al., 1991; Touraev et al., 1995), wheat (Stauffer et al., 1991), maize (Takacs et al., 1998) and *Antirrhinum majus* (Barinova et al., 2002). Successful genetic transformation was reported for tobacco and *Antirrhinum*.

Recently, cucumber was proposed as an unique model plant for mitochondrial genetic transformation (Havey et al., 2002), due to three attributes of cucumber that could be considered prerequisites for successful biolistic transformation of the mitochondrial genome in microspores: the mitochondria are paternally transmitted (Havey et al., 1998); the microspores possess relatively few, huge mitochondria with a very large genome (Lilly and Havey, 2001); and a mosaic (MSC) phenotype is available (Malepszy et al., 1996) which probably is conditioned by a deletion in the mitochondrial genome (Lilly et al., 2001). Regrettably, our knowledge of in vitro manipulation of cucumber pollen is very limited

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(Ondřej et al., 2002), hampering utilization of these positive cucumber attributes for biotechnological treatments.

To study different aspects of pollen development and fertility or to determine the optimal time for pollination, in vitro techniques need to be improved so that pollen quality can be determined reliably. The first step for successful in vitro manipulation of isolated microspores or pollen grains is an efficient monitoring system to determine the viability of treated grains.

Two basically different approaches can be taken to estimate pollen viability: staining pollen with vital dyes, and in vitro germination assay. Staining techniques aim to determine pollen enzymatic activity, membrane integrity, and the stainability of the nucleus. In vitro germination assays determine the actual germination ability of pollen under suitable conditions.

The appropriateness of the viability test depends on the species, since differences have been reported for optimal staining techniques (Heslop-Harrison et al., 1984; Grigg et al., 1988; Khatun and Flowers, 1995; Rodriguez-Riano and Dafni, 2000). Staining techniques can be used to assess the physiological condition of mature pollen and also microspores.

The most frequently used vital stains to assess pollen viability are tetrazolium salt (MTT) to detect dehydrogenase activity (Norton, 1966), aniline blue to detect callose in pollen walls (Hauser and Morrison, 1964), acetocarmine to detect cytoplasmic content (Nassar et al., 2000), and fluorescein diacetate for esterase activity (FDA) (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984).

In contrast, in vitro germination assay can be used only for mature pollen. The pollen germination rate is greatly influenced by different factors such as the genotype of the accession, the composition of the germination medium, temperature and humidity (Marcellan and Camadro, 1996; Adhikari and Campbell, 1998; Rossel et al., 1999; Adaniya, 2001).

The aim of our study was to find a reliable staining procedure for assessment of the viability of cucumber pollen grains and microspores, to optimize the isolation technique, and to achieve germination of isolated pollen. Using an optimized protocol, we attempted to estimate viability and tested in vitro maturation of isolated cucumber microspores.

MATERIALS AND METHODS

DONOR PLANTS AND GROWTH CONDITIONS

The highly inbred line 'b' derived from the Polish cultivar 'Borszczagowski' developed by Malepszy et al. (1996), was chosen for these experiments because of its abundance of male flowers. Plants were grown under natural light in a greenhouse from May to September at the Biotechnical Faculty, University of Ljubljana. Plants grown earlier or later in non-optimal conditions tended to produce pollen grains of poor quality and were not used for the experiments.

Flowers were collected in the morning, immediately after anthesis, and kept at high humidity in a closed vessel with a wet paper towel (Lee et al., 1985) to avoid pollen dehydration, and pollen was taken within one hour.

STAINING TECHNIQUES TO ESTIMATE POLLEN VIABILITY

Pollen viability was assessed using various staining methods. Using aniline blue in lactophenol staining (synonym: cotton blue) modified from Hauser and Morrison (1964), pollen samples were dispersed in a drop of stain mixture containing 10 ml phenol, 10 ml lactic acid, 10 ml glycerol, 10 ml distilled water and 200 mg/l aniline blue. Viable pollen turned blue, while nonviable pollen remained uncolored. Fluorescein diacetate (FDA, Heslop-Harrison and Heslop-Harrison, 1970) was made up as stock solution in acetone at 2 mg/ml. Immediately before use, dilutions were prepared by adding drops of the stock to 2 ml 15% sucrose solution until saturation was reached, as indicated by the appearance of persistent turbidity. Pollen samples were dispersed in a drop of staining solution on a microscope slide and incubated for 10 min in a humidity chamber. The fluorochromatic reaction was excited using epifluorescence under blue-light excitation (510 nm dichroic mirror, 525 nm barrier filter, Carl Zeiss, Jenalumar). Observation and grain counts were completed within 10 min after placing a coverslip over the specimen. Viable pollen had yellow-green fluorescence, while nonviable pollen gave ghost fluorescence. Tetrazolium salts were prepared following the method described by Rodriguez-Riano and Dafni (2000). The substrate 2,5-diphenyl tetrazolium bromide (MTT) was diluted to a 1% concentration in a 5% sucrose solution. The pollen sample was put in a drop of reagent and allowed to dry. The reaction was enhanced by gently heating the

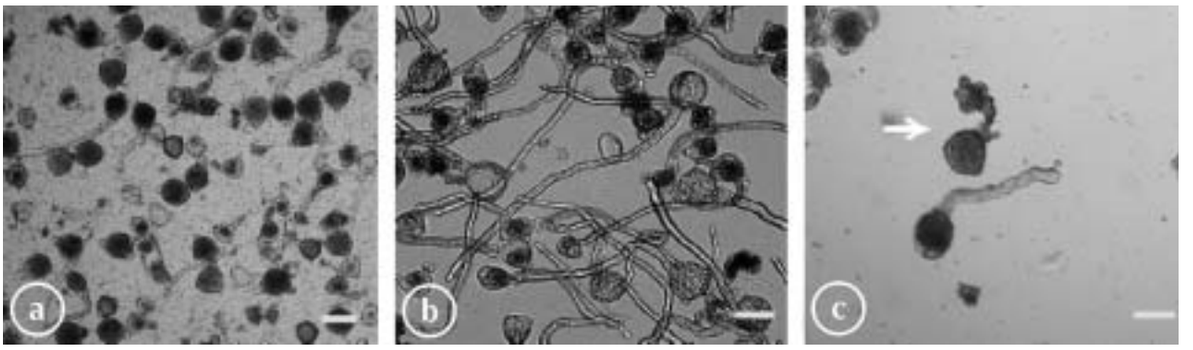

Fig. 1. Light microphotographs of cucumber pollen germination. (**a**) Germination achieved using squeezing method, (**b**) Germination achieved using stirring method, (**c**) Typical bursting of pollen grain (arrow). Bar = 30 μm.

sample, followed by the addition of a drop of glycerol to the dried sample. Pollen was considered viable if it turned deep pink. To test the selectivity of the stain reagent, assays with artificially damaged pollen were performed. Artificially damaged pollen was obtained from flowers treated with 95% ethanol for 10 min, or with dichloroisocyanuric acid 125 g/l for 2 h and rinsed three times in distilled water.

ISOLATION AND IN VITRO GERMINATION OF CUCUMBER POLLEN

Fresh and isolated mature cucumber pollen were tested in various experiments. Fresh pollen was obtained by gently chopping anthers directly on a microscope slide in staining solution. Various isolation techniques were tested for isolating pollen or microspores, of which only two are described in detail. Isolated pollen was obtained either by gently squeezing isolated anthers with a glass rod in germination media (hereafter called the squeezing method); alternatively, anthers were chopped with a razor blade and stirred at 400 rpm for 6 min on an orbital shaker at 24˚C (hereafter called the stirring method). Pollen was purified by filtering the suspension through 100 μm stainless-steel mesh; the filtrate was sedimented by centrifugation at 1000 rpm (35 g) for 3 min. Then the pollen was dispersed in 1 ml medium and used for germination assays or viability testing.

Unless stated otherwise, the medium used in germination experiments was 'basic germination medium' consisting of 15% sucrose (w/v), 0.25 mg/l H_3BO_3 and 1 mM $Ca(NO_3)_2$ at pH 7.0. Variations of the basic germination medium involved either altered carbohydrate content or altered pH: sucrose replaced by 15% maltose or 15% glucose, and pH lowered to 5.0 or raised to 8.5.

For each experiment, isolated pollen obtained from 30 flowers was distributed on microscope slides, in at least 3 replicates. For germination tests, isolated pollen was incubated for 60 min in a humidity chamber (Lee et al., 1985) at 24˚C. After incubation, photos were taken with a Nikon Coolpix 990 camera and at least 300 pollen grains per treatment were examined under a microscope and rated as germinated, not germinated or burst. Pollen grains were considered germinated when pollen tube length equalled or exceeded the diameter of the pollen grain (Fig. 1a,b). Bursting was characterized by an irregular mass of cytoplasm, with starch grains protruding from the pollen (Fig. 1c).

ISOLATION OF MICROSPORES IN MATURATION MEDIA

The microspore developmental stage was determined by staining with 1 μg/ml propidium iodide in 50 mM TRIS-HCl and 0.5% TritonX-100 at pH 7.0. Flowers were sterilized (when applicable) by shaking the flower buds in 16.6 g/l dichloroisocyanuric acid for 10 min with the addition of a few drops of Tween 20 surfactant, followed by three rinses in sterile water. Higher concentrations of sterilizing agent and/or longer duration of treatment were rejected as harmful (data not shown). Predominantly uninucleate microspores were isolated in each maturation medium by the stirring method at 200 rpm for 10 min, as described previously for isolation of pollen grains. The filtrate was sedimented by centrifugation at 700 rpm for 5 min and the pellet was resuspended in maturation media to achieve a final density of $1–2 \times 10^4$ microspores per ml. Isolated microspores were cultured in darkness at 25˚C on a rotary shaker at 25 rpm.

Isolated microspores were cultured on four different maturation media reported as suitable for

TABLE 1. Viability tests using fresh or damaged cucumber microspores and mature pollen as assessed by aniline blue in lactophenol, FDA and MTT test (average of 3 repetitions)

Viability test	Viability of microspores (%)			Viability of mature pollen grains (%)		
	Fresh	Damaged		Fresh	Damaged	
		Ethanol	Dichloroiso-cyanuric acid		Ethanol	Dichloroiso-cyanuric acid
Aniline blue in lactophenol	93.4	85.9	100.0	96.1	99.0	19.3
FDA	87.3	0.0	0.0	93.3	95.6	0.0
MTT	93.1	0.0	0.0	94.6	97.7	2.7

Nicotiana or *Antirrhinum*: medium A according to Kyo and Harada (1986), medium M1 according to Tupy at al. (1991), medium MR 26 according to Benito Moreno et al. (1988) and medium AT3 according to Barinova et al. (2002).

RESULTS AND DISCUSSION

VIABILITY TESTING

Five common methods for estimating the viability of fresh or damaged microspores or pollen grains of cucumber were tested. Two methods (acetocarmine according to Jahier, 1996; p-phenylenediamine according to Rodriguez-Riano and Dafni, 2000) were excluded as not suitable for cucumbers by preliminary testing (data not shown). The methods examined in detail were aniline blue in lactophenol, FCR and MTT. Artificial damaging of pollen was attempted using ethanol or the sterilizing agent dichloroisocyanuric acid at high concentration. The results, presented in Table 1, showed that according to viability estimates the pollen was damaged more efficiently by dichloroisocyanuric acid than by 95% ethanol, which damaged microspores but not pollen grains. Aniline blue in lactophenol completely stained both fresh (Fig. 2a) and damaged microspores, but clearly distinguished between undamaged and aborted mature pollen (Fig. 2d). FDA sufficiently discriminated putatively viable and non-

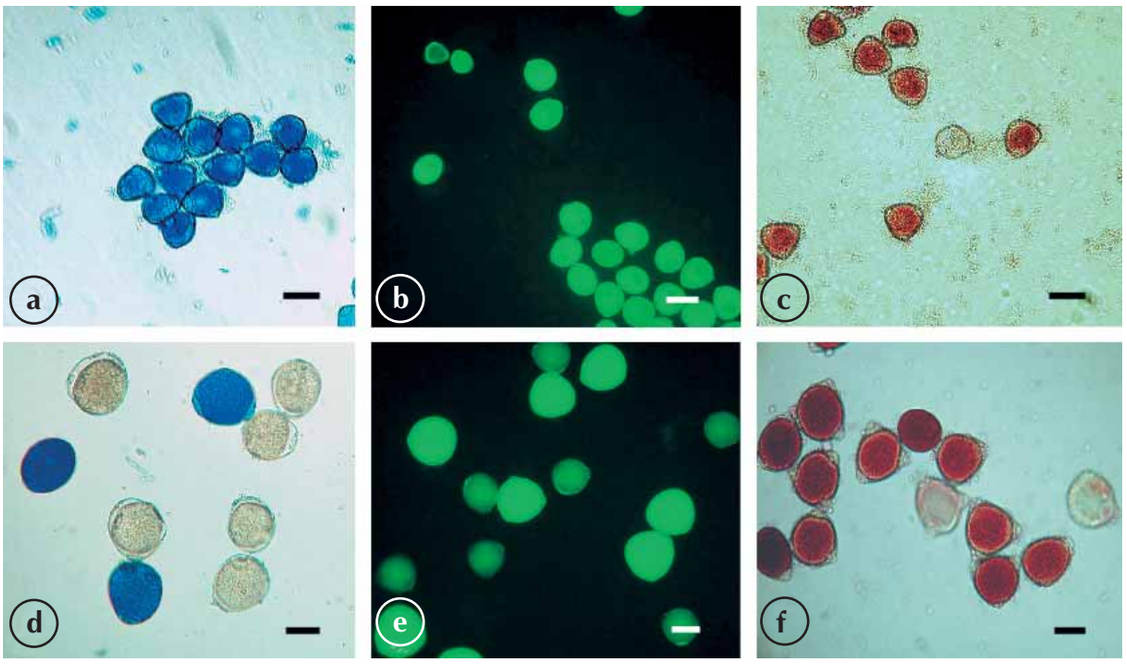

Fig. 2. Staining of cucumber microspores (**a–c**) and pollen grains (**d–f**) with vital dyes: (**a,d**) Aniline blue in lactophenol method, (**b,e**) FDA method, (**c,f**) MTT method. Bar = 30 μm.

TABLE 2. Influence of isolation method and variation of media composition and pH on pollen germination and bursting

Method	Pollen germination	Pollen burstnig
Isolation method		
Isolation by squeezing	21.1 ± 5.2 (9*) a**	20.7 ± 4.4 (9) b
Isolation by stirring	80.1 ± 5.9 (9) b	15.7 ± 3.4 (9) a
Carbohydrate content-stirring method		
Sucrose	80.1 ± 9.2 (18) b	12.2 ± 2.6 (6) a
Glucose	73.3 ± 11.8 (18) a	23.8 ± 3.6 (6) c
Maltose	90.4 ± 7.0 (18) c	16.2 ± 2.7 (6) b
pH value-stirring method		
pH 5.0	82.4 ± 6.0 (6) b	17.2 ± 4.0 (6) b
pH 7.0	85.7 ± 3.7 (6) b	12.8 ± 2.9 (6) a
pH 8.5	75.9 ± 5.4 (6) a	19.6 ± 3.4 (6) b

*No. of replicates; **Identical letters on columns indicate no significant difference by Duncan's multiple-range test ($p < 0.05$).

viable microspores and pollen grains (Fig. 2 b,e) and MTT similarly showed very good contrast between putatively viable and non-viable microspores and pollen grains (Fig. 2 c,f). Viability estimates for fresh microspores and pollen grains were high with all three methods tested and ranged from 87.3–96.1%, while data obtained for damaged ones differed considerably depending on the staining method or damaging procedure.

The optimal method for reliable pollen or microspore viability testing has often been shown to be specific to the species. For instance, Rodriguez-Riano and Dafni (2000) found substantial variability of germination rates according to the viability test used for fresh or damaged pollen grains of eight species. The authors suggested MTT and the peroxidase test as the most reliable. Our results showed false viability of both damaged microspores and pollen grains when aniline blue in the lactophenol test was used. For this staining procedure, the results are in agreement with data obtained by Grigg et al. (1988) in *Solanum muricatum* Ait, where viability as determined by aniline blue was more overestimated than in the FDA test. The FDA test has been effectively used for viability estimation in several plant species including *Cucurbita* (Nepi and Pacini, 1993; Pacini et al., 1997; Nepi et al., 2001), as phylogenetically closest to *Cucumis*. Our results indicate that the FDA and MTT tests are both adequate for viability testing of damaged and undamaged pollen grains or microspores. Advantage of MTT over FDA were higher contrast between viable and non-viable grains, a faster protocol offering a longer time interval for sample examination, and the lack of need for fluorescence.

GERMINATION AND VIABILITY OF ISOLATED POLLEN GRAINS

Germination tests of mature pollen grains were performed to optimize the isolation procedure and to test the effect of carbohydrate content and media pH (Tab. 2). The germination rate obtained using the stirring method (80.1%) was much higher than with the squeezing method, and it also reduced pollen grain bursting to 15.7%. The carbohydrate content had less influence on the germination rate and bursting of mature pollen grains. Germination was highest on media supplemented with maltose (90.4%). Values of pH lower or higher than the standard value of 7.0 reduced the germination rate and increased pollen grain bursting.

To the best of our knowledge, no report has been published of successful germination of isolated cucumber pollen grains. The high germination frequency (80–90%) obtained by the stirring method is therefore a significant achievement and can be considered an important step for further in vitro manipulations of isolated cucumber pollen. In general, pollen grain bursting was not a major problem. Bursting rates (12.2–23.8%) were influenced by the isolation procedure and the media composition (Tab. 2). Adhikari and Campbell (1998) reported reduced bursting of buckwheat pollen when 15% sucrose was in the germination medium. Barinova et al. (2002) found a positive effect of maltose on in vitro maturation of *Antirrhinum* microspores; maltose also had a positive effect on pollen germination in our study, but the results need further confirmation.

VIABILITY OF MICROSPORES IN MATURATION MEDIA

The viability of microspores from non-sterilized flowers exposed to maturation media was tested using the MTT method. The results (Fig. 3) indicated that viability estimates of microspores obtained from non-sterilized flowers were higher when the microspores were cultured in A (25.1%) or M1 (36.3%) medium than when cultured in MR26 (15.8%) and particularly in AT3 (5.9%) medium.

To analyze the effect of flower sterilization on viability, microspores were isolated from sterilized and non-sterilized flowers and cultured in A and M1 media. The results (Fig. 4) showed no apparent detrimental effect of the sterilization protocol. Viability estimates obtained for sterilized microspores

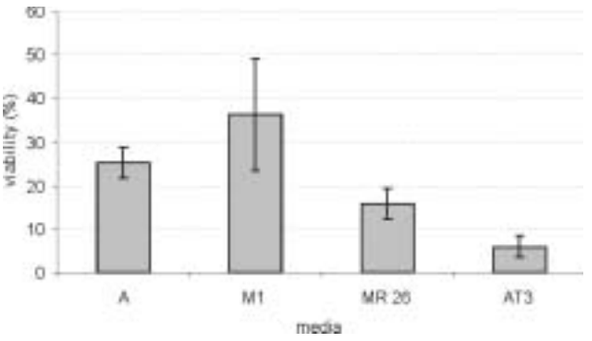

Fig. 3. Viability of microspores after isolation by stirring method in different maturation media tested with MTT method.

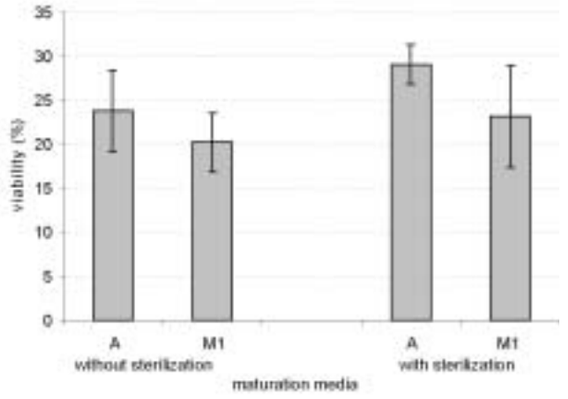

Fig. 4. Viability of cucumber microspores after isolation (with or without sterilization) in two different maturation media assessed by MTT method.

were even slightly higher on both maturation media. The highest viability estimate (29.1%) was for sterile microspores cultured on A medium.

No further development of microspores was observed, the microspores remained more or less in the initial developmental stage, and aggregation of microspores was often noted.

The results indicate that the protocol used produced sterile and presumably sufficiently viable cucumber microspores. The reported isolation and sterilization procedures can be considered first steps in overcoming the recalcitrance of cucumber microspores to maturation attempts. Further steps might focus on other variables for achieving success in maturing cucumber microspores in vitro.

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