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Optimization of conditions for germination of cold-stored *Arabidopsis thaliana* pollen

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Abstract One of the rare weak points of the model plant *Arabidopsis* is the technical problem associated with the germination of its male gametophyte and the generation of the pollen tube in vitro. *Arabidopsis* pollen being tricellular has a notoriously low in vitro germination compared to species with bicellular pollen. This drawback strongly affects the reproducibility of experiments based on this cellular system. Together with the fact that pollen collection from this species is tedious, these are obstacles for the standard use of *Arabidopsis* pollen for experiments that require high numbers of pollen tubes and for which the percentage of germination needs to be highly reproducible. The possibility of freeze-storing pollen after bulk collection is a potential way to solve these problems, but necessitates methods that ensure continued viability and reproducible capacity to germinate. Our objective was the optimization of germination conditions for *Arabidopsis* pollen that had been freeze-stored. We optimized the concentrations of various media components conventionally used for in vitro pollen germination. We found that in general 4 mM calcium, 1.62 mM boric acid, 1 mM potassium, 1 mM magnesium, 18% sucrose at pH 7 and a temperature of 22.5°C are required for optimal pollen germination. However, different experimental setups may deviate in their requirements from this general protocol. We suggest how to optimally use these optimized methods for different practical experiments ranging from morphological observations of pollen tubes in optical and electron microscopy to their bulk use for molecular and biochemical analyses or for experimental setups for which a specific medium stiffness is critical.

Keywords *Arabidopsis thaliana* · In vitro cell culture · Pollen germination · Pollen tube

Abbreviations

ATP	Adenosine tris-phosphate
CCD	Charged coupled device
EGTA	Ethylene glycol tetraacetic acid
FDA	Fluorescein diacetate

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Introduction

In the last two decades, *Arabidopsis thaliana* has evolved as an extremely useful organism for studying a wide range of issues in plant biology. This species gained even more importance and became the main tool for plant cellular and molecular biology studies after the publication of "The Arabidopsis Genome Initiative (2000)." It is therefore astonishing that since that date, less than 5% of the PubMed-listed publications on research performed on the male gametophyte of flowering plants have used *Arabidopsis* pollen. Pollen is a widely used cellular system that on one hand is studied to better understand the reproduction process in flowering plants, and that on the other hand exhibits several features that make it an excellent model system to investigate the principles governing plant cell growth in general. The latter is due to the fact that the pollen tube, a

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cellular protrusion formed from the pollen grain, elongates extremely rapidly with the purpose of delivering the sperm cells to the ovule. Very conveniently for the researcher, pollen germination and the generation of the pollen tube can be achieved in vitro, thus offering an excellent opportunity to observe the processes associated with plant cell growth, cell wall synthesis and intracellular transport in a single cell system.

While microscopic observations are often carried out on few individual cells, other experimental approaches require large amounts of material. Myosin extraction from lily pollen tubes (Yokota and Shimmen 1994), RNA isolation for transcriptional profiling and gene expression in pollen grains and pollen tubes (Becker et al. 2003; Guyon et al. 2000), and vesicle isolation for biochemical and ultrastructural characterization from germinated lily pollen (Van Der Woude et al. 1971) required large amounts of pollen as a starting material. From the need for substantial amounts of *Arabidopsis* pollen material implicated in this type of experiment ensued the necessity to optimize a method for long-term storage that would allow pooling pollen from several harvests.

The principal problem associated with large quantity pollen harvest in *Arabidopsis* is the minute size of its flower (1 mm), the resulting low number of pollen grains per flower, as well as the small size of the individual pollen grain. The comparison to other plant species illustrates this point. To obtain the same amount of pollen in weight collected from a single flower of *Camellia*, pollen from approximately 115,000 plants of *Arabidopsis* needs to be harvested, or in other words, that of more than one million flowers. To automate bulk pollen collection of *Arabidopsis*, Johnson-Brousseau and McCormick (2004) developed a method using a modified vacuum cleaner equipped with three different meshes that has proven to be very useful. Nevertheless, harvesting the amounts of material necessary for biochemical experiments remains time-consuming.

Not only is pollen harvest from *Arabidopsis* comparably tedious, experimentation on germinating pollen is rendered challenging by the fact that in vitro germination is notoriously irreproducible in this species. It is generally agreed upon that existing protocols are highly unsatisfactory as they do not allow the generation of reproducible percentages of germination, even within a single batch of pollen. One reason for this may be that *Arabidopsis* pollen is tricellular, which is known to be associated with reduced in vitro percentages of germination compared to bicellular species (Brewbaker and Kwack 1963; Taylor and Hepler 1997). Moreover, the six stamens of the *Arabidopsis* flower form two distinct groups, which mature at different times (Smyth et al. 1990), causing a difference in the degree of maturity between the two types of pollen harvested from a single flower (Johnson-Brousseau and McCormick 2004). It was also reported that mature pollen grains undergo autolysis in *Arabidopsis thaliana* after anthesis favoring autopollination (Yamamoto et al. 2003). This may be an additional factor responsible for the low germination rate in vitro.

The need for significant amounts of material associated with certain experimental strategies could be met easier, if pollen could be stored without the significant loss of viability or germination capacity. The pollen of many plant species can be dried on silica gel and stored at −20 or −80°C to keep its ability to germinate after several years. Hitherto, this method did not seem to be very successful for *Arabidopsis* pollen. Here, we investigated the effect of cold storage on pollen germination and we optimized several parameters of the growth conditions to allow for optimal percentage germination of frozen-stored pollen.

Various *Arabidopsis* pollen germination media have been proposed in the literature (Boavida and McCormick 2007; Fan et al. 2001; Li et al. 1999); their principal ingredients comprise calcium, boric acid, magnesium, potassium and sucrose, components that are generally found in pollen germination media at varying concentrations. In addition to these elements, the pH of the medium and the growth temperature are two major factors affecting percentage germination and growth (Boavida and McCormick 2007; Chebli and Geitmann 2007).

Here, we describe protocols for germination of frozen-stored *Arabidopsis thaliana* pollen grains that contain optimized concentrations of these ingredients. In addition, we provide standardized methods for pollen germination and growth in different experimental setups including liquid and solid medium, low and high quantity approaches. These methods are useful for different kinds of studies ranging from morphological observations in electron or optical microscopy (cell wall and cytoskeleton label, live observation of vesicle trafficking, monitoring of ion gradients) to the bulk use for molecular and biochemical analyses.

Materials and methods

Arabidopsis thaliana growth and pollen harvest

Arabidopsis thaliana ecotype Columbia 0 plants were grown in trays in a glasshouse at 22°C day temperature and 20°C night temperature, 50% humidity under 16 h daylight and 300 μE m^{-2} s^{-1} light intensity. Approximately 150 seeds (prepared by mixing 5 mg of seeds in 40 mL of 0.1% agar in water to avoid seed clumps) were sown per plate. The mixture was then uniformly dribbled on the soil surface. Plants were irrigated each day and fertilized every second day with Plant-Prod® 20–20–20 fertilizer at 200 ppm.

Pollen was collected every day from the time flowers bloomed using a modified vacuum cleaner as described by Johnson-Brousseau and McCormick (2004). Briefly, using plumbing fittings, three different sized Lab Pak® nylon meshes (80, 35 and 5 μm) were fixed in sequence on a plastic pipe, which was then connected to a 700 W Shark® vacuum cleaner. Pollen was collected by passing the modified plastic pipe over the *Arabidopsis* flowers with gentle shaking.

Storage of pollen grains

Pollen was removed from the 35- and 5-μm nylon meshes and used directly or stored in 1.5-mL microfuge tubes. Unless noted otherwise, pollen was dried on silica gel for 2 h at room temperature prior to cold storage either at −20°C or at −80°C.

Pollen grain rehydration

Pollen was rehydrated before each experiment. For this purpose, after removal from freezer, pollen was placed in a humid chamber for 30 min at room temperature. Care was taken not to let the grains get in direct contact with liquid water.

Germination media

For all experiments, unless specified elsewhere, two different versions of the growth medium were used for pollen germination; a liquid version and a solid version containing 0.5% agar (SIGMA A1296). Unless specified otherwise, the germination medium contained 18% sucrose, 0.01% (1.62 mM) boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, and 1 mM KCl with a pH adjusted to 7. For solid medium preparation, agar was added to the mix and heated to dissolve.

For comparison, the following media were used with a modified concentration of sucrose (18% instead of the concentration originally published): Brewbaker and Kwack (BK) medium (Brewbaker and Kwack 1963), Lily pollen germination medium (Parre and Geitmann 2005). The media that were used exactly as published had originally been developed by the following groups: Wu and coworkers (Fan et al. 2001), Yang and coworkers (Li et al. 1999), and McCormick and coworkers (Boavida and McCormick 2007).

Experimental setups

Pollen germination on a drop of liquid medium

Two-hundred-microliter liquid growth medium was placed on a microscope slide forming a dome-shaped drop. Hydrated pollen grains were sprinkled on top of the drop using a fine brush. The slides were placed in a humid chamber to avoid dehydration of the medium.

Pollen germination in liquid medium in Erlenmeyer flasks

Three-milliliter liquid growth medium were put in a 25-mL Erlenmeyer flask. Hydrated pollen grains were mixed with the medium by vigorous shaking to avoid clump formation. Two or three whole *Arabidopsis* flowers were added to the medium unless specified otherwise. The Erlenmeyer flasks were covered with a Parafilm® layer containing small holes and placed on a shaker at 70 rpm.

Pollen germination on solid surface

Hot agar-containing medium was poured onto a microscope slide to form a layer with a thickness of about 0.5 mm and left to cool. Hydrated pollen was then sprinkled on the surface using a fine brush. The slides were placed in a humid chamber.

Pollen germination in solid medium

Solid medium was prepared as described before and left to cool to 42°C. Hydrated pollen grains were rapidly mixed with the medium by vigorous stirring. The medium was then poured onto a microscope slide to form a layer with a thickness of about 0.5 mm and placed in a humid chamber.

Unless specified otherwise, subsequent incubations were carried out at 22.5°C. Temperature control was ensured by placing the samples in a Sanyo® MIR-153 incubator. Images were taken at 2, 4 and 6 h after the beginning of incubation.

Viability test

Pollen grain viability was assessed using fluorescein diacetate (FDA), which was dissolved in acetone at 10 mg mL^{-1} and stored at −20°C. Prior to each experiment, FDA was diluted in a 10% sucrose solution to a final concentration of 0.2 mg mL^{-1}. Hydrated pollen was dipped in 250 μL of the FDA solution on a glass slide and kept in the dark for 5 min. Observations were made with a Zeiss Imager-Z1® microscope with excitation light at 470 nm and a 515–565 nm band pass emission filter. Only viable pollen grains emit a fluorescence signal under these conditions.

Microscopy

Observations of samples were done either with a Zeiss Imager-Z1 microscope equipped with a Zeiss AxioCam MRm Rev.2 camera and AxioVision Release 4.5 software or with a Nikon Eclipse TE2000-U inverted microscope

equipped with a Roper fx cooled CCD (charged coupled device) camera and ImagePro (Media Cybernetics, Carlsbad, CA) software.

Determination of the germination, growth rate and pollen tube length

For each experiment, at least ten images per sample were taken at random positions and the percentage of germination was quantified. Pollen grains were considered germinated when the pollen tube length was greater than the diameter of the pollen grain (Tuinstra and Wedel 2000). To determine pollen tube length, at least fifty tubes were measured for each experiment.

Results and discussion

Influence of storage conditions on pollen grain viability

Tests using FDA that fluoresces under UV in living cells (Schnurer and Rosswall 1982) revealed a decrease in pollen viability with duration of cold storage. While 80% viability was observed for fresh pollen or pollen stored at −20°C for 24 h only, this percentage decreased to 12% for pollen stored for 10 months with the most significant drop occurring at approximately 6 months (Fig. 1). Storage temperature (−20°C vs. −80°C) did not affect pollen viability differently. At both temperatures, viability was around 60% for pollen grains stored for 2–5 months (Fig. 2). To ensure satisfactory germination of cold-stored pollen, we therefore suggest using up frozen-stored *Arabidopsis* pollen within a 5-month period.

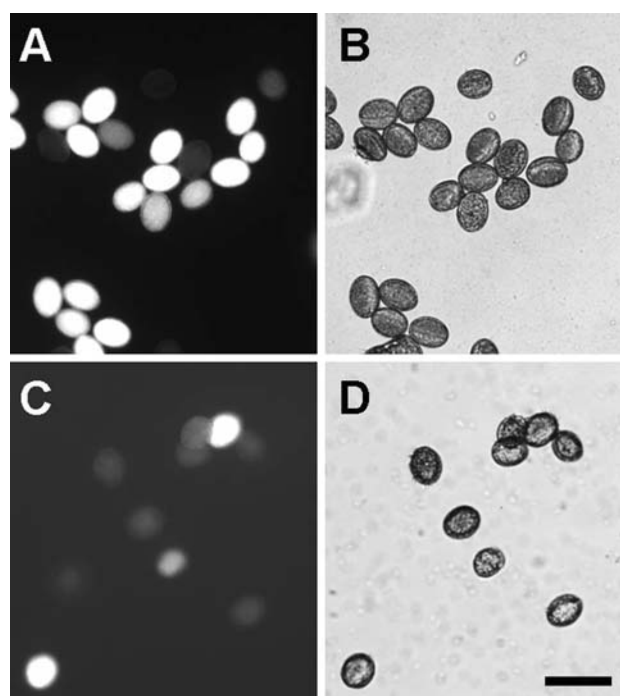

Fig. 1 *Arabidopsis thaliana* pollen viability test using fluorescein diacetate (FDA). Viable pollen fluoresces bright white under UV light. Fresh pollen (**a**, **b**) and pollen stored for 12 months at −20°C (**c**, **d**). *Bar* 20 μm

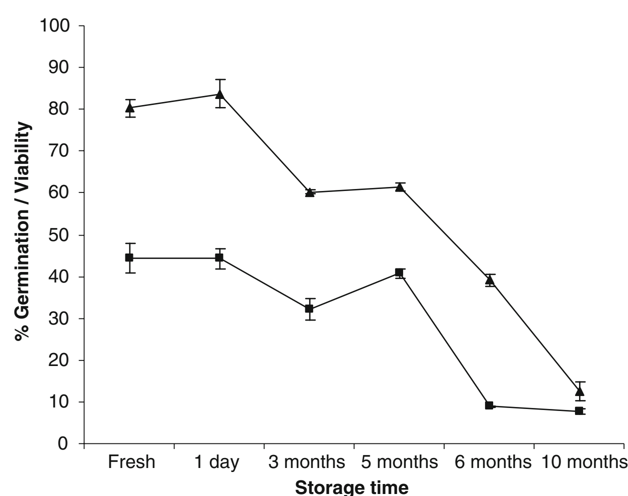

Fig. 2 Change of *Arabidopsis* pollen viability (*filled triangle*) and percentage germination (*filled square*) with duration of cold storage. Stored pollen was kept at −20°C after 2 h of dehydration following harvest. *Vertical bars* represent the standard deviation ($n = 5$)

Effect of storage conditions on germination

To be able to pool pollen from different harvests for analyses requiring significant amounts of material, the optimization of storage conditions is pivotal. Generally, drying pollen before freeze-storing is advantageous, and therefore, we tested different times for drying of *Arabidopsis* over silica gel. Drying for 24 h reduced the percentage of germination of the pollen before freezing by approximately 20%, whereas pollen dried for 2 h had the same percentage of germination as fresh, nondried pollen. We therefore used the 2-h drying period for all subsequent pollen batches.

We then examined the effect of prolonged freeze storage at −20°C on germination. Our data reveal that during the first 5 months of freeze storage, the percentage of germination does not decrease significantly compared to fresh pollen. However, after this time, the percentage of germination decreased to be below 10% by the time pollen had been in storage for 10 months or longer. These data are consistent with the decrease in pollen viability observed for the same period of time (Fig. 2).

Contrary to pollen viability, storage temperature strongly affected pollen germination. The percentage of germination of pollen stored at −80°C for 4 months was 11%, while that of pollen of the same age stored at −20°C

was 40%. The latter is therefore clearly a preferable temperature for storing *Arabidopsis* pollen—contrary to other species such as lily whose germination activity is conserved very well at the lower temperature. Even though the percentage of germination was lower for pollen stored at −80°C, we observed that this percentage was maintained for periods exceeding 1 year. This loss of ability to germinate may in part be due to lysosomal degradation of the cytoplasmic components that is characteristic for *Arabidopsis thaliana* pollen grains (Yamamoto et al. 2003).

Optimization of medium composition for the germination of frozen-stored pollen in various experimental setups

In different experimental setups, pollen is exposed to different conditions, such as availability of oxygen, that might influence its requirement for the individual elements present in the germination medium. We therefore optimized the concentrations for four different experimental setups:

1. Liquid drop: pollen is mixed with liquid medium forming a drop of 180 µL placed on a microscope slide.
2. Bulk germination in liquid (Erlenmeyer): pollen is mixed with 3-mL liquid medium in an Erlenmeyer flask.
3. On solid surface: pollen is sprinkled onto the surface of an agar-stiffened layer of medium.
4. Within solid medium: pollen is mixed into an agar-stiffened medium prior to gelation.

We used concentration series for each of the components of the liquid germination medium and assessed the percentage of germination at 2, 4, and 6 h after incubation for each of the experimental setups. While different pollen batches were used for different experiments (thus resulting in different percentages of germination for the control samples), pollen with identical storage durations were used for all the samples of an individual series of experiments. Table 1 summarizes the optimized concentrations. In the following, we discuss some of the results in more detail.

Calcium

The presence of Ca^{2+} in the growth medium is known to be required for in vitro pollen germination and tip growth of most plant species (Brewbaker and Kwack 1963; Chebli and Geitmann 2007; Li et al. 1999; Picton and Steer 1983). It plays a role in cell wall formation and rigidity, directs vesicle trafficking and controls actin dynamics (Chebli and Geitmann 2007) and was also found to affect the period and amplitude of growth rate oscillations (Geitmann and Cresti 1998; Holdaway-Clarke et al. 2003). Normal pollen

Table 1 Optimized conditions for in vitro *Arabidopsis* pollen germination in four different experimental setups

	Setup 1	Setup 2	Setup 3	Setup 4
Temperature (°C)	30.0	22.5	22.5	22.5
pH	7.0	7.0	7.0	7.0
Calcium (mM)	4.0	4.0	2.0	10.0
Boron (mM)	0.49	0.49	1.62	1.62
Magnesium (mM)	1.0	1.0	1.0	1.0
Potassium (mM)	1.0	1.0	1.0	1.0
Sucrose (%)	18	18	18	18

Setup 1 pollen in liquid drop, *Setup 2* pollen in Erlenmeyer, *Setup 3* pollen on solid surface, *Setup 4* pollen within solid medium

tube growth can only take place in the presence of a calcium concentration that is situated within a certain range (Brewbaker and Kwack 1963; Holdaway-Clarke et al. 2003; Picton and Steer 1983) that varies between species (Steer and Steer 1989). Within this range, pollen tube tip extension rates are relatively insensitive to small changes in the calcium concentration (Picton and Steer 1983), whereas outside of this range, growth is severely hampered.

We used different calcium concentrations: 0, 2, 4 and 10 mM. These are the total concentrations of calcium in the medium resulting from the equimolar addition of two different sources of calcium: calcium chloride and calcium nitrate. We found that growth in liquid medium (Setups 1 and 2) was optimal at 4 mM Ca^{2+} and could not be enhanced further by higher concentrations, whereas growth in solid medium was augmented by 10 mM calcium compared to 4 mM (Fig. 3). Pollen tubes growing on the surface of solid

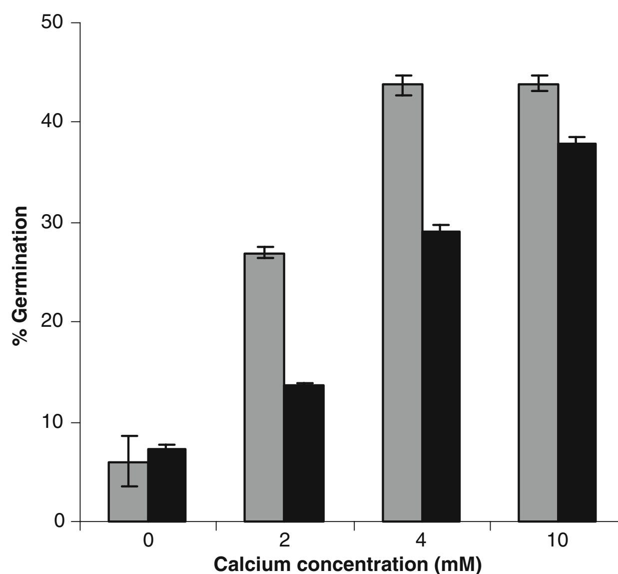

Fig. 3 Effect of calcium concentration on the percentage of germination of *Arabidopsis thaliana* pollen grown in liquid drop (*gray*) and in solid medium (*black*) after 6 h of growth. *Vertical bars* represent the standard deviation ($n = 5$)

medium (Setup 3) required only 2 mM of calcium for optimal germination that was not enhanced or inhibited by higher concentrations up to 10 mM (Fig. 4). In all three cases, the optimal percentage of germination was approximately 40%. The difference in calcium requirement between the experimental setups might be due to oxygen availability affecting the metabolism of the pollen tube, since oxygen is more readily available for pollen growing on the surface of a solid medium than in liquid or in solid medium.

In all three experimental setups, we observed the presence of germinated pollen in the control samples devoid of added calcium. Percentages of germination in these "calcium-free" samples were up to 22% on the surface of solid medium. The addition of EGTA (ethylene glycol tetraacetic acid) at a concentration of 0.1 and 0.2 mM to quench any contaminations with calcium did not reduce the percentage of germination (data not shown). Similar observations were made for *Tradescantia virginia* pollen upon Ca^{2+} quenching with EGTA (Picton and Steer 1983). This may be explained with the presence of a stock of calcium already present in or on the surface of the pollen grains.

Since the two sources of calcium in the medium contained chloride and nitrate [$CaCl_2$ and $Ca(NO_3)_2$], changing the calcium concentration also changed Cl^- and NO_3^- concentrations. To ensure that the observed effects were only related to variations in the calcium concentration and not to the alterations in chloride and/or nitrate contents, we increased the concentrations of these two ions to match those in the 10 mM Ca^{2+} sample using hydrochloric acid and nitric acid. Results showed that there were no significant differences between the controls (pollen tubes germinating without addition of Cl^- and NO_3^-) and the respective media containing the increased amounts of chloride and nitrate ions (Fig. 4). From this we conclude that the effect observed under different calcium concentrations was only due to the variations in the concentration of calcium ions.

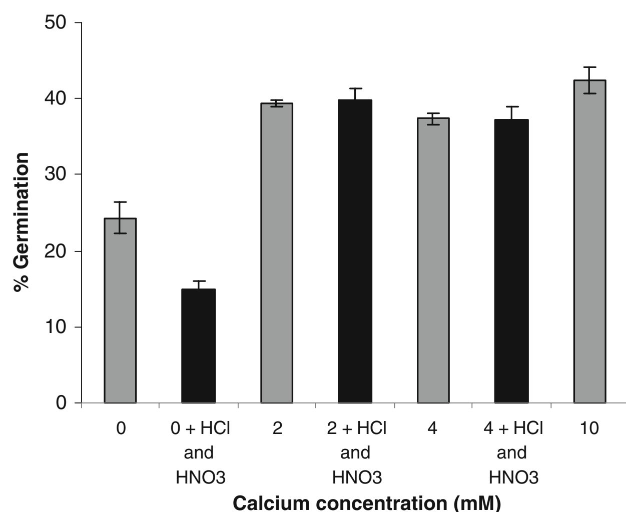

Fig. 4 Effect of calcium concentration on the percentage germination of *Arabidopsis thaliana* pollen grown on solid medium after 6 h of growth. In a parallel series (*black bars*), chloride and nitrate levels were adjusted to that of the 10 mM Ca^{2+} sample. *Vertical bars* represent the standard deviation ($n = 5$)

Boron

In the pollen tube, boron is involved in cell wall formation, protein assembly into membranes and cell wall (Blevins and Lukaszewski 1998). Through its effect on H^+-ATPase activity, boron affects pollen germination, tube growth

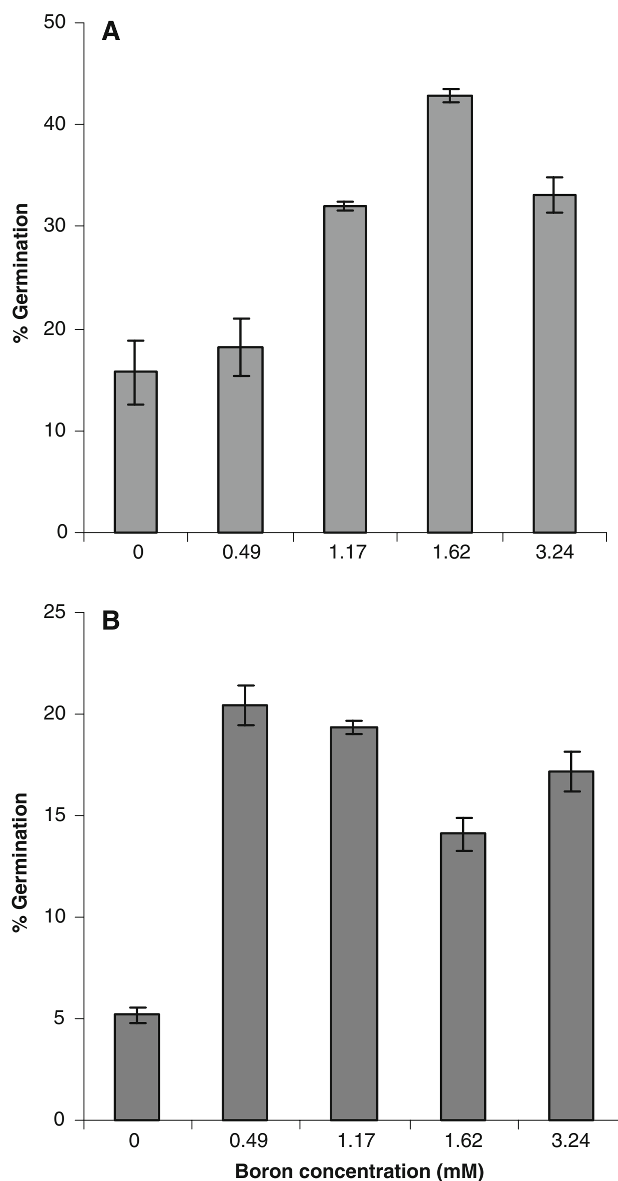

Fig. 5 Effect of boron concentration on the percentage germination of *Arabidopsis thaliana* pollen grown on solid medium (**a**) and in liquid medium (**b**) after 6 h of growth. *Vertical bars* represent the standard deviation ($n = 5$)

(Feijó et al. 1995; Wang et al. 2003) and oscillation behavior (Holdaway-Clarke et al. 2003). Boric acid (100 ppm) was found to be essential for pollen germination (Brewbaker and Kwack 1963). In *Picea meyeri*, boron deficiency decreases pollen germination and affects callose and nonesterified pectin accumulation on the cell wall (Wang et al. 2003).

Five different concentrations of boric acid (0, 0.49, 1.17, 1.62 and 3.24 mM) were used. Germination of pollen growing on the surface of solid medium (Setup 3) was the highest for 1.62 mM of boron, whereas higher concentrations were inhibitory (Fig. 5a). In liquid media (Setups 1 and 2), the highest percentage of germination was obtained for concentrations as low as 0.49 mM (Fig. 5b). Similar results were observed in *Picea meyeri* where 0.01% boric acid (1.62 mM) yielded optimal germination, and higher concentrations were detrimental for pollen tube germination (Wang et al. 2003). One possible reason for higher boron concentrations required for optimal germination when using a solidified medium is that the boron may be sequestered by the agar molecules (residues of algal cell walls).

Potassium

Many pollen species such as lily and *Solanum* require potassium for optimal in vitro germination through its possible involvement in the initiation of the osmotic water influx required for pollen germination (Fan et al. 2001). The effect of potassium on growth was proposed to be in the maximization of the association of the calcium ions to the cell wall (Brewbaker and Kwack 1963). In *Arabidopsis*, a potassium channel has been shown to be present in pollen protoplast membrane (Fan et al. 2001) and its mutation reduces the growth of *Arabidopsis* pollen tubes (Mouline et al. 2002). Therefore, we investigated whether or not varying the concentration of potassium in the medium influences pollen germination in *Arabidopsis*.

Different concentrations ranging between 0 and 10 mM of potassium chloride were tested. Results showed that in liquid growth medium (Setups 1 and 2), potassium concentrations of 1 mM or higher increased the percentage of germination by more than 30%, whereas this increase was more than 60% in solid medium (Setups 3 and 4). No inhibitory effect was observed for higher concentrations of potassium (Fig. 6).

Sucrose

Since the pollen tube does not perform photosynthesis, a carbon source is required for energy supply and carbohydrate skeleton formation. Therefore, sucrose is generally added to pollen germination media, but the optimal concentration varies greatly between species, for instance

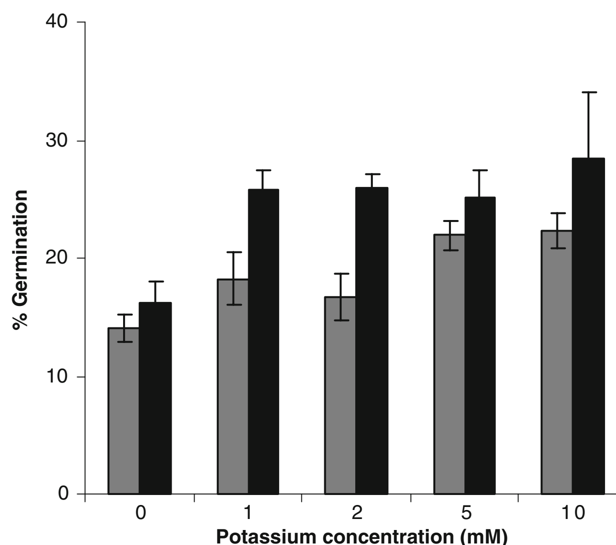

Fig. 6 Effect of potassium concentration on the percentage germination of *Arabidopsis thaliana* pollen grown in liquid drop (*gray*) and in solid medium (*black*) after 6 h of growth. *Vertical bars* represent the standard deviation ($n = 5$)

optimal *Papaver* pollen growth in vitro occurs at 5% sucrose, *Camellia* at 8%, *Lilium* and *Solanum* at 10% (unpublished data). While it was previously observed that sucrose concentrations higher than 15% reduced or prevented *Arabidopsis* pollen germination (Boavida and McCormick 2007), we tested different concentrations of sucrose ranging from 0 to 25%. For our optimized medium, 18% sucrose yielded the highest percentage germination (50%) regardless of the stiffness of the medium (Fig. 7). This percentage was significantly reduced when sucrose concentration was outside the optimal range of 15–20%. In addition to lowering the percentage germination, higher

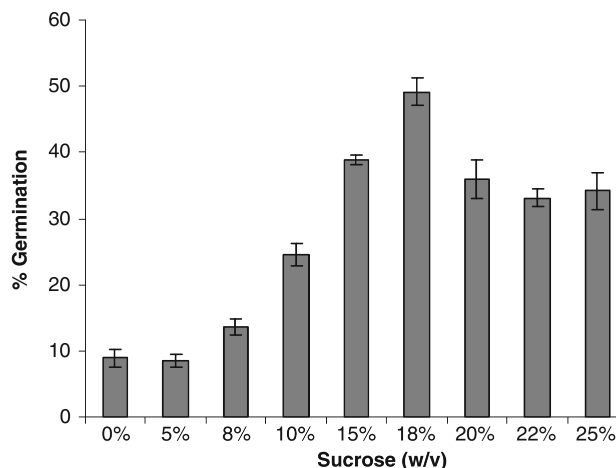

Fig. 7 Effect of the sucrose concentration on the percentage germination of *Arabidopsis thaliana* pollen after 6 h of growth on solid medium. *Vertical bars* represent the standard deviation ($n = 5$)

sucrose concentrations reduced pollen tube elongation (Fig. 8). The requirement for relatively high sucrose concentration for *Arabidopsis* pollen when comparing to other species might be related to the fact that this species has a dry stigma (Elleman et al. 1992; Zinkl and Preuss 2000) thus providing an environment with high osmolarity. *Arabidopsis* culture medium with 18% sucrose might provide the environment that is closest to the in planta situation.

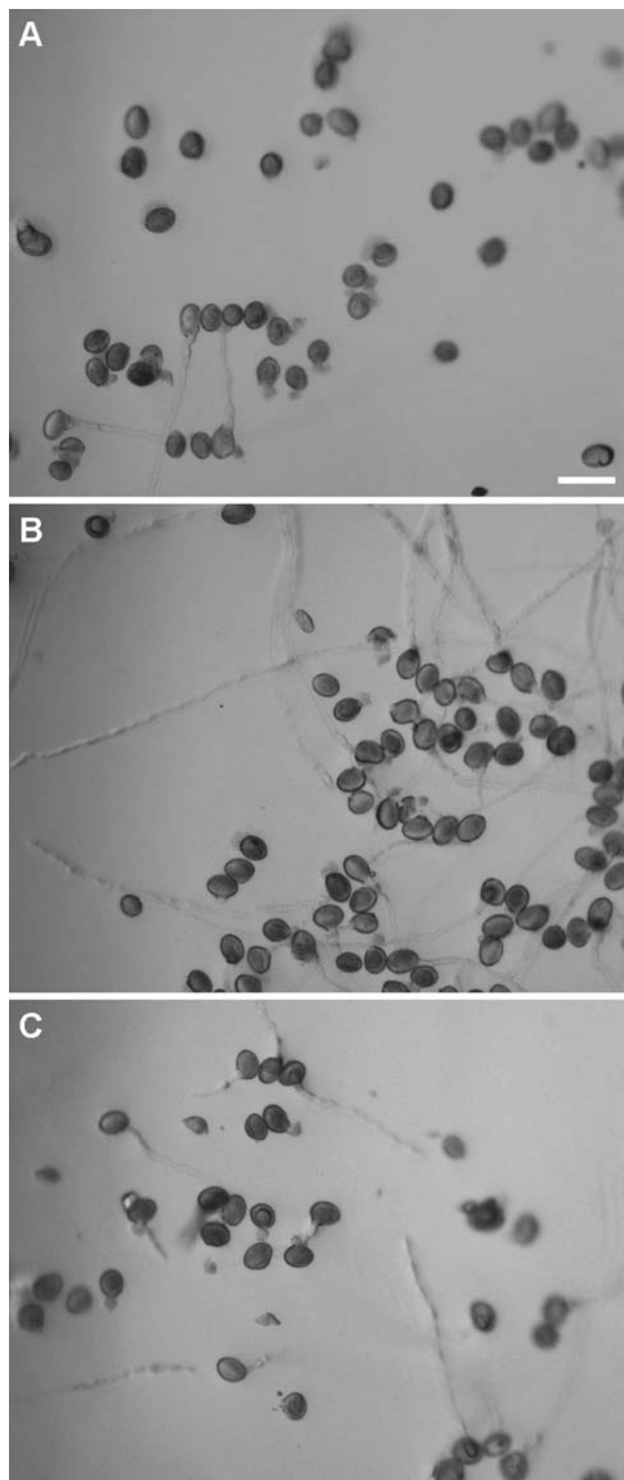

Fig. 8 *Arabidopsis* pollen germination on agarose medium containing 5% (**a**), 18% (**b**), and 25% sucrose (**c**). *Bar* 50 µm

pH

Medium pH is a critical condition for in vitro pollen tube growth. For *Lilium*, *Solanum* and *Camellia* pollen tubes, the optimum pH is between 5 and 6. Lower or higher pH values drastically reduce the percentage of germination and are unable to sustain pollen tube growth (Chebli and Geitmann 2007). To optimize the pH for *Arabidopsis*, we tested different values: 5, 6, 6.8, 7 and 8. Adjustment of the pH was made immediately prior to each experiment. For solid media, pH was adjusted prior to the addition of agar. In solid medium, the optimum pH for pollen germination was 7. When pollen was grown at a slightly different pH

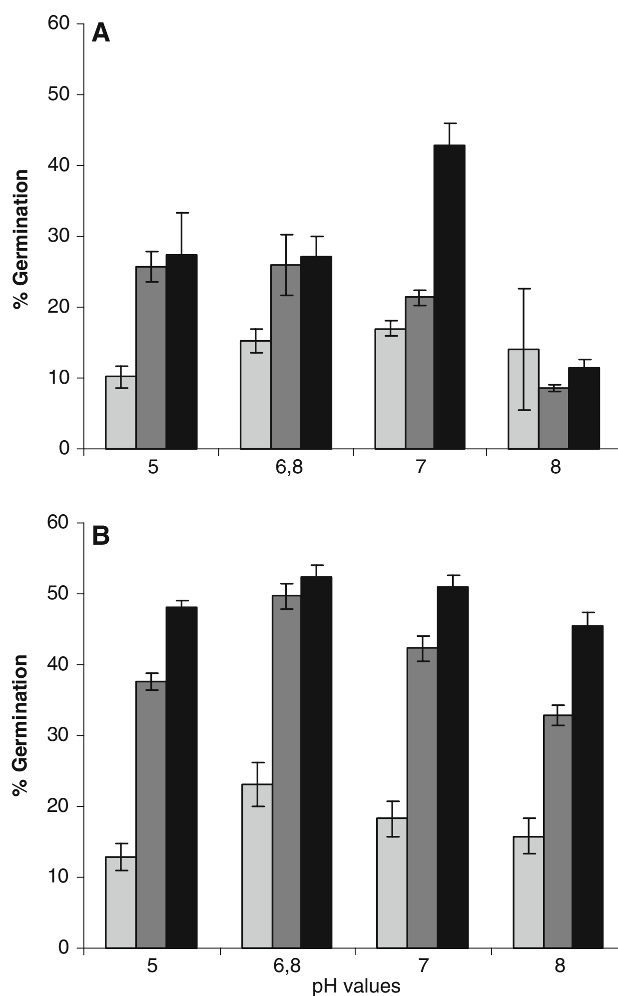

Fig. 9 Effect of the pH on the percentage germination of *Arabidopsis thaliana* pollen grown on solid surface (**a**) or in liquid medium (**b**) after 2 h (*light gray*), 4 h (*dark gray*), and 6 h (*black*) of growth. *Vertical bars* represent the standard deviation ($n = 5$)

(6.8), the percentage of germination was reduced by 40% when quantified after 6 h of growth (Fig. 9a).

However, interestingly, we observed that in a solid medium (Setup 4) with a slightly acidic pH, pollen grains were able to germinate faster than in a medium with pH 7. After 4 h of germination, the percentage of germination reached a high value that at this point of time was higher than that in medium with pH 7 (Fig. 9a).

In liquid medium (Setup 1), we noticed that the pH variation did not have a dramatic effect on the percentage of germination achieved after 6 h. However, the more acidic the medium (pH between 5 and 6.8) the faster the pollen germinated, since percentages of germination were bigger at 2 h in these samples (Fig. 9b). Therefore, in experiments focusing on shorter time periods after germination, a slight acidification of the liquid medium would be advantageous to obtain optimal percentage of germination. On the other hand, total germination as quantified after 6 h is higher at pH 7.

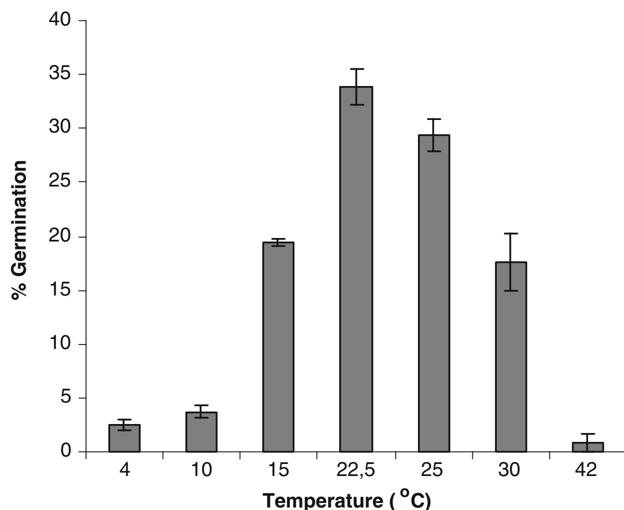

Fig. 10 Effect of the temperature on the percentage germination of *Arabidopsis thaliana* pollen grown on solid medium. *Vertical bars* represent the standard deviation ($n = 5$)

In our experiments, we noticed a decrease by 0.4 pH units after 6 h of growth in liquid medium. This acidification of the medium may affect pollen germination with time. This is consistent with earlier reports that with time, growing pollen tubes tend to acidify the medium eventually resulting in growth to stop (Tupý and Říhová 1984). An acidification of 0.1 units was also observed in a control liquid sample without any pollen. This decrease in the pH might be due to the metabolism of microorganisms, present in the medium, on the glassware and in higher amounts on the pollen itself. It may also be due to the metabolism of the pollen and pollen tubes where a high degree of ions exchange happens between intra- and extracellular compartments. The use of Tris buffer (5 and 10 mM) reduced the percentage of germination (not shown), which is why we did not pursue further experimentation with buffers.

Temperature

Pollen germination shows a temperature-dependent behavior. A controlled temperature was proven to be important for optimal germination and pollen growth in *Arabidopsis thaliana* (Boavida and McCormick 2007). Since various experimental strategies may require incubation temperatures other than room temperature, we tested a temperature range from 4 to 42°C. Best germination for solid medium growing pollen tubes was obtained for temperatures ranging from 22 to 25°C (Fig. 10). The same was observed for pollen grown in liquid medium in Erlenmeyer flasks, whereas the optimal temperature for pollen in a liquid drop was 30°C (Table 1). A similarly surprising optimal germination temperature was observed by Boavida and McCormick (2007) who describe that 28°C increases germination in the Colombia ecotype.

Approximately 5% of the pollen grains were able to germinate at 4°C in liquid medium and these tubes displayed normal morphology (Fig. 11) with a mean length of 110 μm after 24 h of growth. We tested this condition,

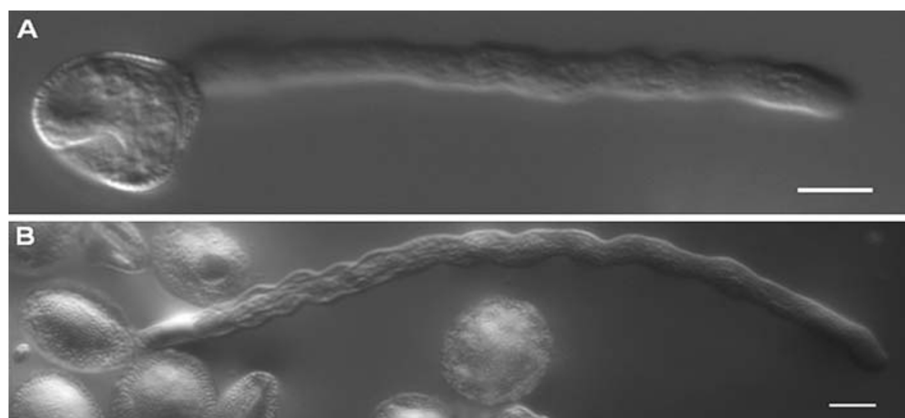

Fig. 11 Micrographs of *Arabidopsis* pollen tubes grown at 4°C for 24 h (**a**) in liquid medium and (**b**) on solid medium. *Bars* 10 μm

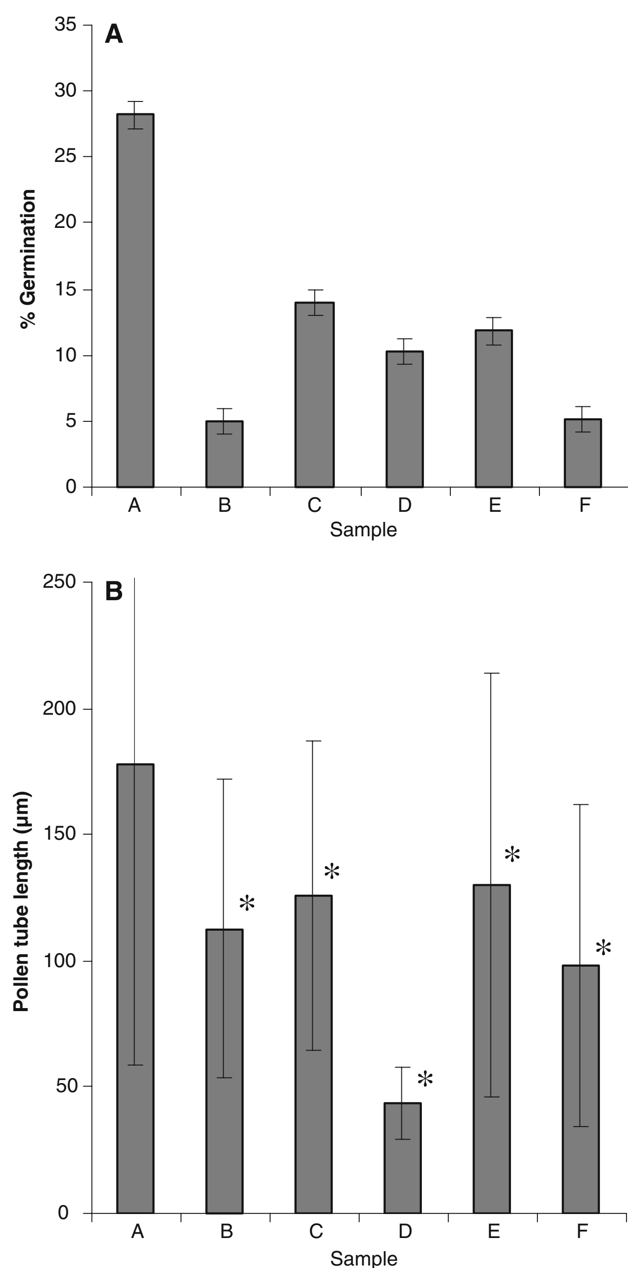

Fig. 12 *Arabidopsis* pollen percentage of germination (**a**) and pollen tube length (**b**) after 5 h of growth on agarose stiffened media based on our optimized protocol (Sample A), Boavida and McCormick (2007) (Sample B), Li et al. (1999) (Sample C), Fan et al. (2001) (Sample D), Brewbaker and Kwack (1963) modified with 18% sucrose (Sample E), and lily medium (Parre and Geitmann 2005) modified with 18% sucrose (Sample F). *Bars* represent the standard deviation ($n = 5$). *Despite large standard deviations for pollen tube length (**b**), mean values of Samples B–F are significantly different from that of Sample A with $P < 0.05$ (Samples B and E) and $P < 0.01$ (Samples C, D, F) (two tailed Student's *t* test)

since it would allow image acquisition of living pollen tubes in the environmental scanning electron microscope as image quality decreases with increasing temperature.

Comparison with other media

To demonstrate the difference between our optimized medium and other media that had been developed for pollen germination of various species, we compared them side by side. Using pollen that had been cold-stored for 3 months, our optimized medium yielded significantly higher percentages of germination than the Lily pollen medium (Parre and Geitmann 2005), the BK medium (Brewbaker and Kwack 1963) and several *Arabidopsis* media (Boavida and McCormick 2007; Fan et al. 2001; Li et al. 1999) (Fig. 12a). The tube length of those grains that had succeeded in germinating was significantly longer in our medium than that in the other media (Fig. 12b). Furthermore, our medium resulted in pollen tubes without apparent morphological disorders, whereas in our hands pollen tubes grown on the Boavida and McCormick (2007) medium were frequently aberrant with apical swellings. One of the reasons why in our hands our medium was superior to the others might be that it had been optimized for bulk-collected, cold-stored pollen, while the others were optimized for freshly collected pollen and/or for pollen from the most recently opened flowers of the *Arabidopsis* plant.

Conclusions

Given the importance of *Arabidopsis thaliana* as a model system, the optimization of the conditions for bulk storage and germination of frozen-stored pollen should contribute to the increased use of this species in pollen research. Furthermore, we provided optimized conditions for different experimental setups that either use large amounts of pollen or require specific conditions such as low temperatures or medium stiffness. Our optimized *Arabidopsis* pollen growth medium is composed of 18% sucrose, 1 mM potassium chloride, 1.62 mM boric acid, 1 mM magnesium sulphate, 2 mM calcium chloride and 2 mM calcium nitrate, at a pH of 7 with an incubation temperature of 22.5°C. For pollen grown in solid medium, twice the amount of calcium is ideal. For pollen grown in a liquid drop, the optimum growth temperature is 30°C (Table 1).

The two main advantages of these methods of *Arabidopsis* pollen germination are the high reproducibility compared to other media described in the literature and the possibility of using large amounts of pollen that has been collected and freeze-stored. Therefore, the availability of flowering *Arabidopsis* plants at the time of the experiment is not a limiting factor. Whenever a batch of flowers is mature, all its pollen can be collected and stored for experiments to be carried out at a later time.

The bulk pollen germination makes our methods useful for a variety of experiments requiring large amount of nucleic acid, proteins or organelles to be extracted from *Arabidopsis* pollen tubes. The wide range of germination conditions makes them appropriate for other experiments where low germination temperatures are required (environmental scanning electron microscopy) or different medium stiffness is a limiting factor to the success of the experiments.

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