

SIMPLE METHODS FOR *in vitro* POLLEN GERMINATION AND POLLEN PRESERVATION OF SELECTED SPECIES OF THE GENUS *Agave*

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ABSTRACT. Simple methods to evaluate the viability and to preserve *Agave tequilana* Weber var. azul and *A. angustifolia* Haw pollen were established. Pollen viability was assessed by improving a growing media and evaluating three common pollen germination media components: sucrose, boric acid, and calcium ion. Optimal germination of pollen was obtained at 0.300 M sucrose, 0.324 mM boric acid, and 1.219 mM calcium nitrate and incubated at a temperature of 25°C. Agave pollen was preserved in olive oil and organic solvents. Olive oil, which is immiscible with water, provides an anhydrous environment and limits available oxygen, conditions similar to those provided by some organic solvents. Fresh pollen was put into eppendorf tubes containing n-butanol, n-propanol, isopropanol, extra virgin olive oil and preserved at -20, 4, and 25°C. The germination of the preserved pollen was scored at different time periods during preservation employing the optimal germination medium. The viability of pollen grains preserved at -20°C in olive oil, proved olive oil as an efficient medium for agave pollen preservation for at least 6 months.

KEYWORDS: growing media, pollen tube, pollen viability, olive oil.

1. Introduction

Agave angustifolia Haw and *A. tequilana* Weber var. azul are semelparous and perennial plants that produce flowers only once towards the end of their life cycle (8-12 years) and are cultivated because of their commercial importance for alcoholic beverage production such as tequila and mezcal, therefore becoming important crops for Mexico [1].

Previous studies on gametes of these species have been directed to cytogenetic work [2, 3, 4]; however, aspects such as viability, germination and pollen preservation have not been approached. Pollen viability is generally considered to indicate the ability of the pollen grain to perform its function of delivering the sperm cells into the embryo sac following compatible pollination [5]. Estimation of pollen viability on the basis of its function is cumbersome, time consuming, and not always feasible [6].

Pollen viability has been evaluated in a number of ways. These include recording final seed set after pollination, observation of pollen germination and pollen tube growth *in vivo* [7], the use of vital stains e.g. tetrazolium salts for dehydrogenase activity [8]; aniline blue to detect callose in pollen walls and pollen tubes [9]; acetocarmine or Alexander stain to detect cytoplasmic contents [10], the determination of the intactness of the plasma membrane with fluorescein diacetate following *in vivo* esterase activity [11] and by *in vitro* and *in vivo* germination tests [12, 13]. There is, however, little information about the applicability of most of these methods to species of the genus *Agave*. To assess the viability of agave pollen a germination medium was improved based in the growing medium developed by Tuinstra and Wedel [14], since there, three of the most common nutrient components have been used when formulating an artificial medium for *in*

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vitro pollen germination: sucrose [15, 16 17], boric acid [16, 17], and calcium ions [18], where the amounts of the chemical components can vary depending on the plant species [19, 20, 21].

On the other hand, duration of pollen viability varies greatly among species, and it is related to the type of pollination, varying from minutes to years depending primarily on the taxonomic status of the plant and on environmental conditions such as humidity and temperature. It is well known that the genus *Agave* is pollinated during the day by hymenoptera, butterflies and hummingbirds, and during the night by bats and moths [22, 23]. Although there are several exceptions e.g. *Zea mays* L. [24], pollen of many flowering plant species survive to considerable desiccation conditions and can be stored in air-dry environments [25, 26, 27, 28, 29, 30]. In general, plants with entomophilous pollination have pollen with longer viability than those of anemophilous pollination [31]. There have been several investigations on the effects of time, temperature and moisture content of storage conditions on pollen survival [32, 33, 34]. Pollen preservation makes it easy to hybridize individuals with different flowering times and different geographical locations for the purpose of genetic improvement. Works on pollen storage also include basic studies such as viability, anhydrobiosis and germination. Pollen studies in which its conservation has been obtained using organic solvents or by cryogenic storage using liquid nitrogen have led to its conservation for long periods of time in several species. The use of organic solvents provides anhydrous storage conditions by limiting available oxygen. On the other hand, oils are immiscible with water so they are also likely to provide ambient conditions for pollen similar to those provided by organic solvents [35, 36, 37].

The objectives of the current work were to determine the efficacy of organic solvents and olive oil for *Agave angustifolia* Haw and *A. tequilana* Weber var. azul pollen storage, as well as the establishment of an optimum culture medium for pollen germination and pollen tube growth.

2. Materials y methods

Inflorescences of *Agave tequilana* Weber var. azul and *A. angustifolia* Haw were collected during the 2006 flowering season in the region of Jalisco, Mexico, and taken to the laboratory for further processing.

2.1 Pollen germination assays

Anthers were selected just before dehiscence. Undehisced anthers were dried at room temperature for 16–24 hours to induce dehiscence. Pollen grains were collected from these anthers and treated as follows: Experiments under a factorial design were conducted in order to evaluate the effects of sucrose, boric acid and calcium nitrate on *Agave tequilana* Weber var. azul and *A. angustifolia* Haw on pollen germination. Experiments were conducted in Petri dishes (60X15 mm) containing 5 mL of 0.5% (w/v) agar medium supplemented with different concentrations of three nutritional components: sucrose (0.060, 0.300, and 0.500 M), boric acid (0.324, 0.971, and 1.618 mM,) and calcium nitrate (0.610, 1.219, and 1.829 mM). A total of 27 combinations of germination media were tested. The pH of all treatments was adjusted to 5.8 before adding the agar and without any sterilization process. The pollen grains were dispensed onto the germination media directly from a bulk of anthers. Petri dishes were incubated under dark conditions for 30 minutes at 25°C. Pollen grains were evaluated for germination under an Olympus BH-2 light microscope at a 20X magnification. Germination was quantified as the percentage of germinated pollen grains (100 grains quantified per replication) when the pollen tube emerged through the ectoaperture ([Figure 1a](#)) and when the tube length was greater than the diameter of the pollen grain ([Figure 1b](#)). No staining of the pollen grains



was necessary. The factorial experiment was blocked in time with 3 replications (one Petri dish per replication) following a previous report [14].

In order to find the best incubation temperature, three temperatures were selected from those that are commonly found in ranges under field conditions where these species grow: 4, 25 and 35°C [38]. Fresh pollen was sown in Petri dishes which contained the optimal medium (0.5% agar, 0.300 M sucrose, 0.324 mM boric acid, and 1.219 mM calcium nitrate) found in the previously mentioned experiment and incubated at the designated temperature treatment for 30 minutes. Then, pollen germination was quantified as previously described. The experiment was blocked in time with three replications following a randomized complete block design.

2.2 The effects of solvents, olive oil and temperature on pollen preservation

Pollen grains were preserved in Eppendorf tubes containing n-butanol, isopropanol, n-propanol (without dilution) and extra virgin olive oil (25 mg/100 µL), at three different temperatures -20, 4 and 25°C. The viability of the pollen grains stored in solvents and in olive oil was tested by separating the grains from the solvent or the oil. In the case of the pollen grains stored in oil, these were soaked in a drop of water and then dispersed onto Petri dishes containing the optimum germination medium. The germination was quantified after 15, 30, 50, 90, and 180 days of storage.

In all the experiments, the resulting data were normalized to mean = 0 and variance = 1 [$Z \sim N(0, 1)$] prior to the ANOVAs and the Multiple Range Tests (LSD), however, data are expressed in original percentage values in order for the results to be meaningful.

3. Results and discussion

3.1 Pollen germination assays

3.1.1 The effect of culture medium components

The analysis of variance of experimental data showed the significative effects on pollen germination of sucrose ($p=0.0000$), boric acid ($p=0.0000$), calcium nitrate ($p=0.0000$) and their interactions, boric acid:calcium nitrate ($p=0.0081$), boric acid:sucrose ($p=0.0039$) and calcium nitrate:sucrose ($p=0.0007$).

The LSD test showed that for *A. angustifolia* Haw, sucrose concentrations were significantly different among the three levels, while for *A. tequilana* Weber var. azul the two highest concentrations had the same effect. It is important to note that for both species, the highest sucrose concentration (0.500 M) caused the bursting of a high number of pollen tube tips (Figure 1c). The same test showed that calcium nitrate at 1.219 mM had an effect statistically significant different at $p=0.05$ respect to the 0.610 and 1.829 mM concentrations. Boric acid had a significant impact on pollen germination at 0.324 mM with the highest germination percentage, although there were significant differences among the three levels (Table 1).

Table 1. Means of pollen germination under the effects of sucrose, boric acid and calcium nitrate in agar medium for *A. angustifolia* Haw and *A. tequilana* Weber var. azul.

Treatment	Pollen germination (%)			
	<i>A. angustifolia</i>	Range of values	<i>A. tequilana</i>	Range of values
Sucrose (M)				
0.060	40 a	37.7 - 43.0	23 a	17.6 - 28.4
0.300	79 c	76.1 - 81.5	70 b	64.8 - 75.2
0.500	70 b	65.4 - 71.0	68 b	62.3 - 73.7
Boric acid (mM)				
0.324	78 c	74.5 - 79.9	61 c	55.6 - 66.4
0.971	59 b	55.2 - 60.6	46 b	40.5 - 51.5
1.618	53 a	49.4 - 54.8	32 a	26.8 - 37.2

Calcium nitrate (mM)				
0.610	58 a	55.1 - 60.5	52 a	46.7 - 57.3
1.219	68 b	65.5 - 70.9	64 b	58.5 - 69.5
1.829	64 a	58.5 - 63.9	56 a	50.4 - 61.6

Values with the same letter are not significantly different at $P \leq 0.05$ using LSD test.

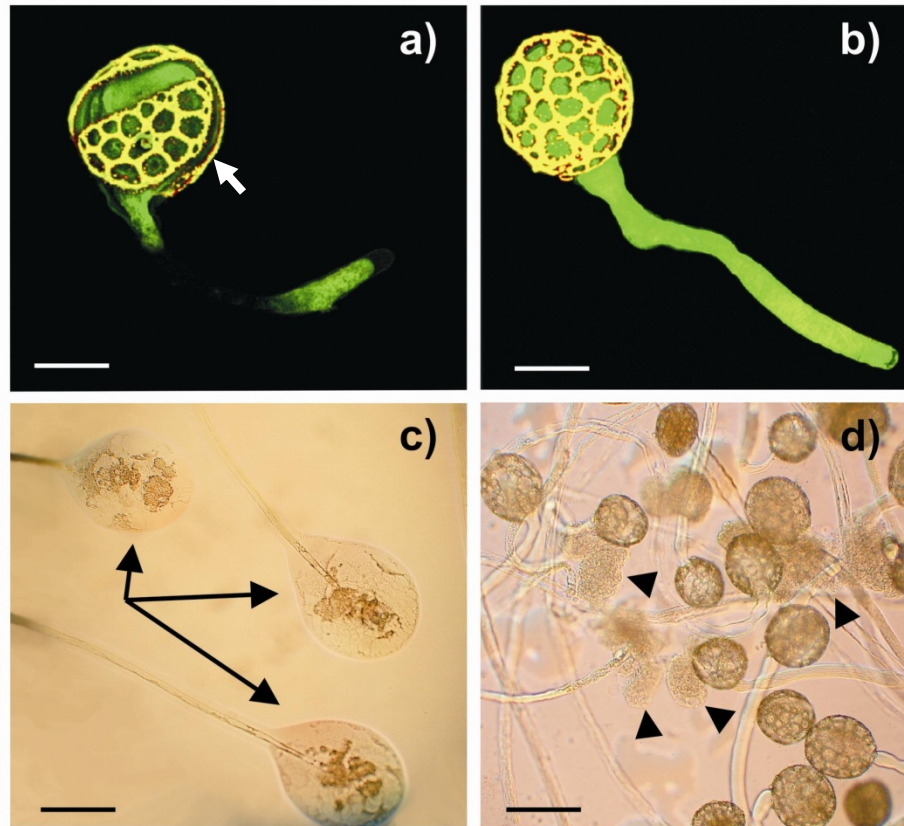


Figure 1. Photomicrographs of *Agave* pollen germination. **a)** Pollen tube emerging through an ectoaperture (white arrow) (bar = 40 μm). **b)** Pollen tube length greater than the diameter of the pollen grain (bar = 40 μm). **c)** Bursting pollen tube tips (black arrows) (bar = 75 μm). **d)** Pollen grain contents leaching onto the medium (arrowheads) (bar = 85 μm). **a)** and **b)** taken with a Leica TCS-NT confocal microscope. **b)** and **c)** taken with an Olympus BH-2 light microscope.

3.1.2 The effect of incubation temperature on pollen germination

When pollen was distributed onto the germination medium, high and low temperatures affected pollen viability under the three incubation temperatures 4, 25 and 35°C, being 25°C significantly higher ($p=0.05$) (Table 2).

Table 2. Means of pollen germination in agar medium under the effects three different temperatures for *A. angustifolia* Haw and *A. tequilana* Weber var. azul.

Incubation temperature °C	Pollen germination (%)			
	<i>A. angustifolia</i>	Range of values	<i>A. tequilana</i>	Range of values
4	50a	44 – 56	42a	36 – 48
25	77b	71 – 83	62b	56 – 68
35	45a	39 – 51	37a	31 – 43

Values with the same letter are not significantly different at $P \leq 0.05$ using LSD test.



The results show that although a high germination percentage was obtained at the 0.300 and 0.500 M sucrose concentrations, at 0.500 M, there were high amounts of bursted pollen tubes, resulting in the leach of metabolites into the medium (Figure 1d), probably due to the presence of sucrose, because the composition of the growth medium can affect pollen metabolism. Higher sucrose levels in the growing medium may alter the permeability of the pollen grain plasmalemma and the growing pollen tube, resulting in the leach of grain content and ions into the medium [39], this and other monosaccharides enter the pollen grain and increase the high internal concentrations [39, 40, 41]. Also, sucrose fuels ethanolic fermentation during *in vitro* growth accumulating the levels in the growing medium reaching concentrations near to 100 mM ethanol that may inhibit the growth of pollen tubes [42, 43]. In agreement to Golan-Goldhirsh et al [44] pollen tube bursting could be avoided by using polyethylene glycol (PEG) instead of sucrose.

In this study, agave pollen germination was evaluated over a range of temperatures similar to those that are commonly encountered under field conditions, and then according to these results, we could speculate that pollen germination is not influenced by temperature changes in the field. Thus, evaluation of pollen germination was possible following incubation at room temperature, therefore simplifying the assay procedure.

3.2. The effects of solvents, olive oil and temperature on pollen preservation

Figure 2 shows the time courses of the germination rates of pollen preserved in n-butanol, n-propanol, isopropanol, and olive oil at -20, 4, and 25°C of both *Agave* species. Pollen preserved in n-butanol, n-propanol, isopropanol under the different temperatures completely lost their ability to germinate within the first fifteen days of storage. By contrast, pollen preserved in olive oil at -20°C maintained its germination from 74 to 79% throughout the 180 days of the study. On the other hand, pollen preserved at 4 and 25°C decreased germination to about 7-15% within the 15 and 30 first days of storage, depending on the agave species.

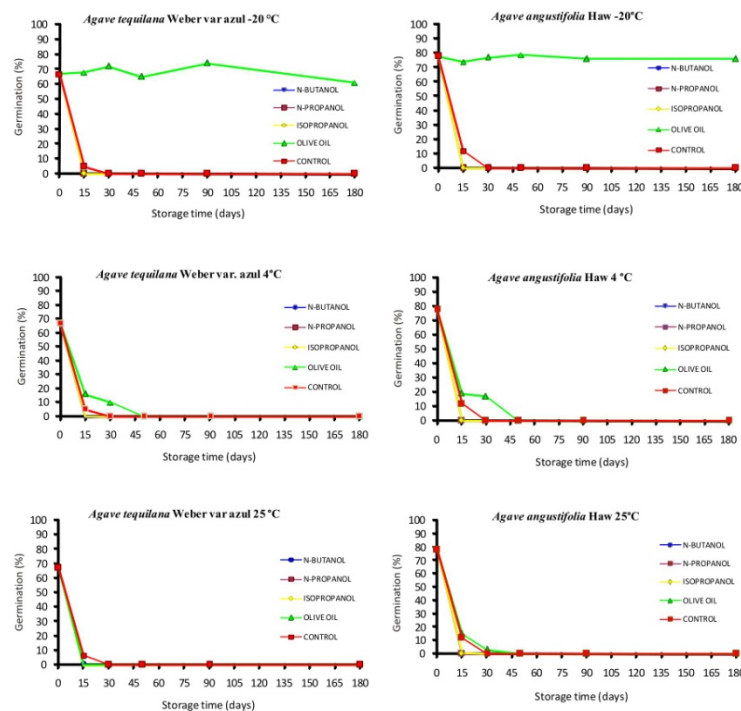


Figure 2. Time courses of the percentage of germination of the two species of Agave pollen preserved in solvents and olive oil at three different temperature levels.

4. Conclusions

Pollen preservation is often desirable, especially for intercrossing species that require long time to mature or flower.

In this study, in the experiments for designing a culture medium for agave pollen germination, it was found that sucrose, boric acid and calcium nitrate combined with an optimum temperature, play an important role in germination rates. Thus, 0.5% (w/v) agar medium supplemented with 0.300 M sucrose, 0.324 mM boric acid and 1.219 mM calcium nitrate constitutes a good culture medium for agave pollen germination assays.

On the other hand, in this work, it was found that all the solvents assessed failed to preserve agave pollen, however, the use of extra virgin olive oil was an optimum medium for pollen preservation for at least 180 days when stored at -20°C. This finding will be an important element for future genetic improvement of agave species when wide hybridizations and germplasm preservation are involved.

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