Sugarbeet Pollen Germination in Vitro[1]

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ABSTRACT

Factors affecting in vitro germination of sugarbeet (*Beta vulgaris* L.) pollen were investigated to provide improved methods of testing pollen viability. Pollen germinated most frequently and consistently in a liquid medium (pH 5.5, 100 ppm H_3BO_3, 100 ppm $Ca(NO_3)_2 \cdot 4H_2O$, and 3.2 x 10^5 ppm sucrose in distilled water) at 23 C for 24 hours. Pollen collected less than 24 hours after dehiscence, humidified 15 minutes, and stirred into liquid medium at 0.3 mg/ml, germinated up to 82%, but mass-collected pollen commonly germinated 25 to 50%. Pollen tube length averaged about 570 μm 24 hours after germination. Individual tubes over 1300 μm were observed. Germination inconsistencies may result more from germination conditions and techniques than from the environment of pollen production. The methods described should provide relatively consistent and accurate in vitro measures of pollen viability.

Additional Key Words: *Beta vulgaris* L., pollen viability, culture medium

Pollen is a unique plant tissue that potentially can be used and manipulated to the advantage of the geneticist, breeder, physiologist, or germplasm curator. Since pollen of most species is haploid and each cell is independent, pollen has the potential to provide a microbial-like system for evaluation, assay, and selection. Interest in pollen has been enhanced recently by research demonstrating that many genes active in the sporophyte also are active in the gametophyte (17, 19, 21, 22, 23).

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Whatever the interest and use, the viability of the pollen is necessary information. Commonly used pollen viability assays fall into three categories based upon (i) pollen staining or fluorescence, (ii) fertilization or seed set, and (iii) pollen germination.

Vital stains of pollen have not been found to be precise viability indicators, and are generally considered useful only where an imprecise estimate of viability is adequate (18). In sugarbeet, Hecker (7) reported a positive relationship between MTT (tetrazolium bromide) stain and in vitro germination of sugarbeet (*Beta vulgaris* L.) pollen.

Pollen viability testing by seed set, or germination on the stigma, does not provide exact measures of viability and requires too much time for most purposes. Hecker et al. (8) have reported a general relationship between sugarbeet seed set and in vitro pollen germination.

The ability of pollen to germinate on artificial media is widely used as a test of viability, especially for bicellular pollen. This requires a near-optimum germination medium and environment. Adequate media now exist for bicellular pollen from many species (20). However, tricellular pollen generally is short-lived and difficult to germinate (3), and adequate media or germination conditions have not been developed for many species (20), including sugarbeet.

In vitro germination of sugarbeet pollen, which is tricellular, was reported by Artschwager and Starrett (2) in 1933 as abundant on media containing 40% sucrose and 1.5% agar at 32 C; their photograph, presumably one with abundant germination, shows about 30% of the pollen germinated. Succeeding authors (1, 9, 10, 11, 12, 13, 15) reported in vitro germination of 0 (zero) to 40% for various media and conditions, except that Kato and Hosokawa (11) had germination of up to 50% on media supplemented with peptone. However, Nakashima et al. (16) found a negative peptone effect and concluded that the medium proposed by Brewbaker and Kwack (4), containing 25% sucrose and 1% agar, was the best among all the media they tested. The most extensive research has been reported by Glenk et al. (6). Using semisolid media, 5% gelatin, 30% sucrose, and 0.03% boric acid, they reported germinations of 20 to 50%, dependent on genotype and time after dehiscence, but as high as 85% for pollen collected immediately after anther dehiscence. However, they experienced considerable variation, primarily due to season and location effect and elapsed time between dehiscence and pollen collection.

A consistent feature of all these sugarbeet pollen studies is low average germination, commonly 10 to 20%, and high variability. If pollen germination and tube growth in vitro are to be tools for sugarbeet scientists, consistent reliable methods and techniques for germination and growth need to be developed. The purpose of this paper is to report the effects of major cultural factors on in vitro sugarbeet pollen germination and tube growth.

MATERIALS AND METHODS

The experiments necessary for the development of the in vitro germination techniques described in this report were conducted in 1984-86 at Fort Collins, Colorado. We used diploid sugarbeet plants, unless specified otherwise, of various genetic types, mostly heterogeneous, that were photothermally induced and grown to the flowering stage in a greenhouse maintained at 28 C days and 18 C nights. However, significant deviations from these temperatures sometimes occurred on hot days and cold nights. Pollen usually was collected in the morning by shaking the flowering branches over a glass plate. With a flat blade, the pollen was scraped onto smooth paper that was inclined and tapped to remove anthers and debris, leaving relatively pure pollen. The pollen was weighed and divided to facilitate a constant density of 0.3 mg of pollen per ml of medium. The pollen samples were then transferred on watch glasses into a simple humidification chamber for 15 minutes. The chamber was a covered plastic box lined with wet blotting paper. The preweighed pollen was then transferred into 2 or 4.5 ml of liquid medium, stirred, and dispensed into 4- or 6-cm disposable petri plates, respectively, or the pollen was spread on an agar medium surface. Even distribution of the pollen on the agar medium surface was accomplished by suspending the pollen in a small quantity of 32% sucrose solution, then pouring it in a thin film onto the medium. The covered plates were placed at the desired temperature, dependent on the experiment, for 24 hours, unless a special time schedule was required. Addition of two drops of a 1:1 solution of polyethylene glycol (PEG 400) and formaldehyde into or onto the medium and refrigeration stopped pollen tube growth and kept the cultures free of microbial contamination until pollen germination counts could be made directly in the dishes at 100x. Except for special instances, pollen was considered germinated if the tube was longer than one pollen diameter. In all germination tests, 300 or more pollen were counted in each of two to four replications (plates) of completely random design experiments. Pollen tube lengths were determined by transferring a random sample to a microscope slide, then 10 to 30 tubes were measured on projected microscope images with a map-measuring instrument.

Our exploratory and initial experiments were done using the solid medium of Brewbaker and Kwack (4) (B-K medium), consisting of 0.1 g H_3BO_3, 0.3 g $Ca(NO_3) \cdot 4H_2O$, 0.1 g KNO_3, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 20 g agar, and 320 g sucrose, made to 1 L with distilled water. Medium modifications were tested at varied concentrations (weight/volume) of sucrose, calcium, potassium, magnesium, and boron. The majority of the experiments in this report were done with liquid B-K medium, which only lacked agar. The medium pH was adjusted to 5.5 with HCl

or NaOH. The solution of salts, without sucrose, was stored refrigerated for several weeks as a stock.

We also tested the effects of agar, medium pH, medium additives, germination temperature, pollen density in the medium, light, pollen moisture content, flowering stage, and greenhouse fumigation.

In vivo pollen germination was done by brushing pollen onto compatible stigmas of male sterile plants, excising stigmas after 2 hours, staining in 5:1 (v/v) aniline blue-neutral red (0.05% aniline blue in 0.067 M K_2HPO_4 buffer and 0.1% neutral red in water) on slides, and examining with a combination of epifluorescence and transmitted light.

RESULTS AND DISCUSSION

The first factor tested was sucrose concentration in the medium. Our results with B-K solid medium (Table 1) showed that germination was maximized between 24 and 32% sucrose. Germinations at 16 and 40% sucrose were significantly lower. Considerable variation in germination occurred among pollen sources; these were various heterogeneous sources sampled on separate days. In Experiment 2 of the solid medium experiments (Table 1) there were no germination differences between 29 and 34% sucrose. The variation among pollen sources, in this case, should have been primarily due to genotype, since the pollen was collected on the same day from the four sources.

Table 1. Sugarbeet pollen germination (%) on solid and liquid B-K media with varying sucrose content.

Experiment and Pollen source	Sucrose					
Exp. 1 (solid)	**16%**	**24%**	**32%**	**40%**		
4-18	5	4	2	0		
4-20	16	15	12	0		
5-9	8	31	29	8		
5-24	3	8	12	3		
$\bar{X}$	8±0.6	15±1.1	14±2.4	3±0.9		
Exp. 2 (solid)	**29%**	**30%**	**31%**	**32%**	**33%**	**34%**
6-19-1	11	11	10	8	11	10
6-19-2	11	14	12	12	12	14
6-19-3	1	1	2	1	2	1
6-19-4	7	10	9	10	9	8
$\bar{X}$	8±0.6	9±0.6	8±0.5	8±0.5	8±0.4	8±0.6
Exp. 3 (liquid)	**16%**	**24%**	**32%**	**40%**		
4-1	31±4.8	35±0.6	40±0.8	27±0.1		
Exp. 4 (liquid)	**20%**	**26%**	**32%**	**38%**	**44%**	
5-1	21±1.3	28±1.7	29±2.0	24±1.3	7±1.2	

Varying the agar content (0.5 to 2%) had no effect on solid medium germination. Also, sterile filtration of the medium had

no effect. However, autoclaving the medium reduced pollen germination. Separate experiments with no sucrose in the B-K solid medium resulted in essentially no germination, and little pollen lysis. Some of the initial germination variability on solid medium resulted from density differences of pollen on the medium surface. This density effect was first reported by Brewbaker and Majumder (5) for all angiosperm pollens. Dusting pollen onto solidified medium resulted in uneven pollen distribution and variation in germination. Pollen densities and resultant germinations were made more uniform by suspending pollen in a 32% sucrose solution before applying it to the medium surface.

Because of relatively low germination on B-K solid medium and inconsistency problems, pollen germination was tested in B-K liquid medium. Germinations were higher in liquid than on solid media (Table 1 and 2). B-K liquid medium with 32% sucrose was used to make the paired comparisons in Table 2. This liquid medium was adopted as the standard for testing other germination factors. When sucrose was deleted from the liquid medium, the pollen lysed immediately and no germination occurred.

Table 2. Sugarbeet pollen germination (%) on solid and liquid B-K media.

Pollen source	Medium	
	Solid	Liquid
11-7-1	15	14
11-7-2	11	19
11-16-1	6	21
11-16-2	12	30
12-4	11	31
12-11-1	3	17
12-11-2	1	8
12-19-1	2	53
12-19-2	4	35
12-19-3	5	43
12-19-4	5	28
12-28-1	7	17
12-28-2	15	35
$\bar{x}$	8±0.3	27±1.0

Sucrose is generally considered to serve two functions in the medium, that of an energy source and an osmoticum. Evidence is abundant that sucrose in the medium is metabolized by germinating pollen (18). Mulcahy and Mulcahy (14) proposed that tricellular pollen may be heterotrophic from the start of germination. However, when we substituted polyethylene

glycol (PEG 400 to 20,000) for sucrose in the medium, pollen germination was unaffected, although tube growth tended to be abnormal and shortened.

Glucose and lactose also were tested as medium sugars. Pollen germination in liquid glucose media of 15, 25, and 35% was 11, 1, and 0%, respectively, compared with 14% in a B-K liquid medium (32% sucrose). A 15% lactose medium had 15% germination. Lactose likely served solely as an osmoticum, like PEG, because lactose is not known to be utilized or even to occur in plants, except in rare cases.

In tests with B-K liquid medium (32% sucrose), varied concentrations of salts and boric acid were tested for effect on germination. Calcium nitrate was tested from 0 to 1000 ppm. The other salts were tested from 0 to 500 ppm. Both calcium and boron enhanced in vitro germination of sugarbeet pollen (Table 3). Magnesium and potassium had no consistent effect. Subsequent experiments showed the essentiality of boron and the necessity of calcium for maximum germination and tube growth (Table 4).

Table 3. Sugarbeet pollen germination (%) in liquid B-K medium (32% sucrose) with different concentrations of salts and boric acid. As the concentration of each additive was varied, the other three additives were held constant at the standard quantity.

Medium additive	Standard quant. (ppm)	Concentration (ppm)					
		0	100	200	400[†] 300	700[†] 400	1000[†] 500
$Ca(NO_3)_2 \cdot 4H_2O$	300	6±0.3	16±0.2	19±1.3	13±0.5	15±1.2	7±0.7
$MgSO_4 \cdot 7H_2O$	200	26±1.7	20±1.5	27±3.0	32±1.3	23±1.1	21±2.6
KNO_3	100	28±1.0	27±0.9	28±2.2	27±0.5	25±1.1	27±1.0
H_3BO_3	100	15±1.3	32±0.9	35±2.6	30±1.0	26±0.9	13±1.0

[†] Concentration for calcium nitrate only.

The optimum pH of the medium for pollen germination is quite different for different plant species, and critical in some cases. The data in Table 5 indicate that a liquid medium pH of 5.0 to 6.0 was optimum for sugarbeet pollen. Most of the liquid medium experiments in this study were done at pH 5.0; however, more extensive recent experiments indicate that 5.5 may be better for most beet pollen sources. Stock solutions of salts and boric acid were stored (refrigerated) for several weeks; however, the pH gradually declined. Upward pH adjustment with NaOH, when necessary, may have reduced pollen germination and introduced experiment to experiment variability, as noted later. Storage of complete medium, including sucrose, was not satisfactory.

Pollen from plants in the mid and late flowering stages had the best germination. For greenhouse grown plants the most viable mass-collected pollen resulted from blowing pollen off the plants

the evening prior to the day of collection. This reduced the amount of pollen in the collection that was more than 1 day old. We have found that pollen viability deteriorates rapidly following anthesis. Freshly collected nonhydrated pollen, 15 minutes from collection to placement in the germination medium, germinated 1 to 21%, depending on pollen source (Table 6). In every case, hydration in a saturated atmosphere at 23 C restored germinability (Table 6 and 7), but restoration declined as time of exposure in a low humidity lab environment increased (Table 7). Desiccation of the pollen over anhydrous calcium chloride for 24 hours at 5 C, a necessary step in cryopreservation of sugarbeet pollen (8), had no detrimental effect on germination in five of six pollen sources, provided the pollen was hydrated prior to germination (Table 6). Adequate hydration in all these treatments occurred in 15 minutes. Hydration of up to 4 hours resulted in no better germination. Based on our experiments, we conclude that all pollen should be humidified 15 minutes prior to germination. Variability among samples and sources was reduced by this standard treatment. Pollen collected immediately after dehiscence and placed into medium may not benefit from humidification. However, the quantity of pollen required and the extent of the experiments usually obviate this technique. Pollen sources in Table 6 were different dates of collection and genotypes.

Table 4. Sugarbeet pollen germination and tube length in liquid B-K medium (32% sucrose) with varying boron and calcium.

H_3BO_3 ppm	$Ca(NO_3)_2 \cdot 4H_2O$ ppm	Germination %	Tube length µm
Exp. 1			
0	0	0a'	0a'
0	300	1a	13ab
100	0	11b	52b
100	100	20c	133c
100	300	21c	124c
Control		14b	76b
Exp. 2			
100	0	24b'	225a'
100	50	27b	392b
100	100	29b	444b
100	300	14a	183a
Control		22b	418b

' Means within columns within experiments that are followed by the same letter are not significantly different (P = .05).

Table 5. Pollen germination (%) on media of varying pH.

Medium and exp.	Initial pH						
	4.48	**5.0**	**5.17**	**5.48**	**6.0**	**6.17**	**S_x**
Solid	4	11	10	8	9	7	1.2
	4.0	**4.5**	**5.0**	**5.5**	**6.0**		
Liquid (4-18)	2	13	36	31	26		1.1
Liquid (5-3)	—	13	38	45	39		1.5

Table 6. Pollen germination (%) in liquid medium following desiccation for 24 hours over anhydrous calcium chloride at 5 C,and 15 minutes hydration at 23 C.

Pollen treatment	Pollen source					
	11-14-1	11-14-2	11-23-1	11-23-2	12-6-1	12-6-2
Fresh collection	21	8	1	3	2	9
Fresh, hyd.	28	26	26	42	20	14
45 min. lab exposure	3	3	0	0	0	0
45 min. lab exposure, hyd.	19	36	14	28	26	26
24 hour desiccation	1	0	0	1	0	0
24 hour desiccaton, hyd.	4	29	35	41	25	18
$LSD_{(0.05)}$	5.7	10.0	4.7	6.3	9.9	7.5

Table 7. Germination in liquid medium of a heterogeneous pollen source exposed in the lab at about 23 C, and hydrated (15 min.) at 23 C.

Pollen treatment	% Germination
Fresh collection	2.1
Fresh, hyd.	37.5
45 min. lab exposure	0.1
45 min. lab exposure, hyd.	31.9
3 hr. lab exposure	0.0
3 hr. lab exposure, hyd.	24.1
24 hr. lab exposure	0.0
24 hr. lab exposure, hyd.	28.6
72 hr. lab exposure	4.1
72 hr. lab exposure, hyd.	17.0
$LSD_{(.05)} = 4.5$	

Even though post-collection humidification enhanced pollen germination, pollen collected from plants growing in a humid environment appeared to have reduced in vitro germinability. Germination also was reduced in pollen from plants suffering any type of stress. Day-to-day variation in germination of pollen from the same plants was frequently quite large.

The number of pollen grains per unit of medium had a significant effect on germination. We found that 0.07, 0.28, 0.60, 0.89, 1.18, and 1.49 mg of pollen per ml of liquid medium germinated 6, 18, 20, 15, 13, and 10%, respectively. We adopted a standard procedure of 0.3 mg of pollen per ml of medium. This density facilitates counts after germination, and requires rela-

tively small quantities of pollen. Pollen from diploid plants weighed about 310 mg/cc at 18% moisture.

In our experiments, the inclusion of a wetting agent in the liquid medium had no effect on pollen germination. Likewise, light and darkness had no differential effect on pollen germination. Temperature, however, affected germination. Constant temperatures of 7, 10, 23, and 32 C for 24 hours resulted in germinations of 6, 16, 24, and 18%, respectively. Germination on the lab bench, where the temperature fluctuated from 21 to 24 C, was 24%. Since rigid temperature control was not critical, all germinations were done in the 21 to 24 C lab. Pollen germination was the same in liquid cultures that were stationary or shaken, and germinations were not significantly different when stirred into the medium or floated on the surface, 32 and 37%, respectively.

In vitro and in vivo germinations of mass-collected pollen were compared in three experiments; the mean germinations were 41 and 43%, respectively. These differences were not significant. Germination counts in vivo were difficult and laborious. We did not observe the high frequency of in vivo germination (over 90%) and sharp contrast compared with in vitro germination (6 to 9%) reported by Jassem (9). Our experiments indicate that in vitro pollen germination is a good measure of in vivo germination, and is an adequate test for viability.

To determine the rate of germination and tube growth over time, several experiments are summarized in Table 8. Germination and pollen tube growth essentially were complete after 24 hours. The average length was 569 μm. Tube lengths at 24 hours ranged from 65 to 870 μm in these experiments. In other experiments, tubes longer than 1300 μm were observed, which should be sufficiently long to reach from stigma to ovule.

Table 8. In vitro pollen germination and tube growth over time; 24 hours was the control treatment.

Time	Germination %	Tube length μm
10 min.	3.2	18
30 min.	11.3	43
60 min.	13.3	94
90 min.	13.1	141
2 hours	18.3	187
3 hours	24.4	386
6 hours	26.4	400
24 hours	32.2	569
72 hours	31.3	578
$LSD_{(.05)}$	4.3	83

From three experiments comparing the germination and tube length of pollen from diploid, triploid, and tetraploid plants, the average germination was 43, 3, and 39%, respectively. The tube length after 24 hours was 296, 329, and 349 μm, respectively. Pollen from normal-cytoplasm triploid plants, although low in germination (3%), produced normal tubes about the same length as those of pollen from diploid and tetraploid plants.

Addition of Tris (10^{-3} to 10^{-5} M) to the B-K liquid medium had no effect on pollen germination. Likewise, the addition of 0.005 to 2 ppm indoleacetic acid to the B-K liquid medium had no effect on germination, nor did addition of EDTA at 100 ppm.

Tests of greenhouse fumigant effects on pollen viability showed no significant deleterious effect of five different materials (Carmel Chemical Corporation materials GH-16, GH-19, GH-21, GH-27, and GH-33)[2] routinely used in our greenhouses to control aphids and spider mites. These materials were dispensed with a fogging applicator.

Figure 1 shows pollen germinated in vitro for 24 hours, including one pollen with two supernumerary germ tubes, a relatively frequent event. Two or more germ tubes occur in about 3% of germinated pollen from both diploid and tetraploid plants.

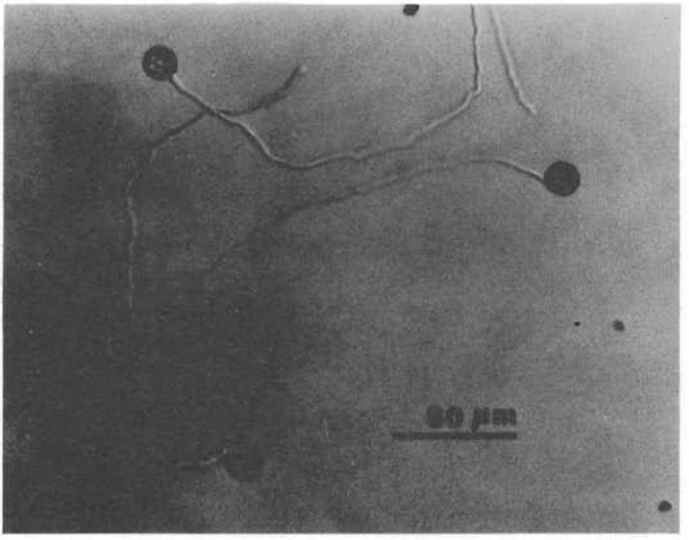

Figure 1. Sugarbeet pollen germinated in a liquid medium.

[2] Company and commercial names are shown for the benefit of the reader and do not imply endorsement or preferential treatment of the product listed.

Although the experiments reported in this paper used B-K medium, subsequent experiments have indicated deleterious interactions of magnesium with calcium. Hence, a simplified medium, 0.1 g H_3BO_3, 0.1 g $Ca(NO_3)_2 \cdot 4H_2O$, and 320 g sucrose made to 1 L, now is recommended. This medium has been satisfactory in all subsequent experiments.

Subsequent experiments also have shown a deleterious effect of sodium on pollen germination and tube growth. Hence, it is recommended that pH adjustments be made with KOH and HCl. Separate tests indicated that chloride and nitrate have no effect on germination.

Differences of in vitro pollen germination among our numerous experiments indicate that sugarbeet pollen is sensitive to various factors, some of which we have failed to identify. A comparison of pollen from genetically similar plants grown in a controlled-environment chamber and a greenhouse, sampled five times over a period of 16 days, produced means and variances that were not significantly different for the two environments. These results indicate that relatively variable greenhouse environments probably are not a major contributor to inconsistencies in germination results. Hence, post-collection techniques and conditions, including medium ingredients, may be the most important factors.

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