Field Tests for Pollen Viability; a Comparative Approach

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Abstract

Many tests for pollen viability have been proposed. The ability to determine this reproductive state in the field can be very important in botanical research as well as for horticultural purposes. After reviewing a large number of tests, we compared four methods of determining pollen viability. Each of these methods can easily be carried out in the field. Viability tests were carried out on 17 species from Israel and were compared to *in vitro* germination trials. No one test worked well on all species. The MTT stain was determined as the most effective for a wide number of species, but tests for individual species should be conducted before final determinations are made.

INTRODUCTION

Information about the ability of pollen grains to germinate when they reach the stigmas of flowers of their own species is valuable both for horticultural purposes and general botanical research. Viability tests provide a means of assessing the potential of pollen to germinate on the stigma. Stone, Thomson and Dent-Acosta (1995) pointed out the need for assessing the viability of pollen used in hand-pollination experiments. In their survey of 283 papers they found that this had been done infrequently.

The literature differs on what a "viability test" actually measures. The term viability has been defined as "having the capacity to live, grow, germinate or develop" (Lincoln, Boxshall, and Clark, 1982). However, the term viability has been used to describe the germination of pollen grains on the stigma (Morse, 1987; Preston, 1991; Vaughton and Ramsey, 1991; Niesenbaum, 1992), *in vitro* germination of pollen grains (Shchori, Goren and Ben-Jaacov, 1992; Beardsell, Knox and Williams, 1993; Lingren et al., 1995), the results of various staining procedures (Bernhardt, Knox, and Calder, 1980; Becker and Ewart, 1990; Mione and Anderson, 1992; Nyman, 1992), and seed set following pollination (Smith-Huerta and Vasek, 1984). Our use of the term viability is based on the definition given by Lincoln, Boxshall, and Clark (1982).

Many different methods have been suggested to determine the viability of pollen (Dafni, 1992; Kearns and Inouye 1993). Vital stains, such as cotton blue in lactophenol and lissamine green, have been used by many workers in the past. Although these stains can distinguish between sterile and partially sterile plants, they do not document the loss of viability in pollen grains very well (Hauser and Morrison, 1964; Ockendon and Gates, 1976; Heslop-Harrison et al., 1984; Knox, 1984).

Artificially pollinating flowers and assessing seed production might seem to be the most accurate means of determining pollen viability, but the amount of pollen used per stigma (Cruzan, 1986; Young and Young, 1992), the complications of fruit abortion (Stephenson, 1981; Primack, 1987), and the effect of environmental conditions such as temperature, humidity and water availability (Chang and Struckmeyer, 1976; Schoper, Lambert and Vasilas, 1986; Burgos, Egea, and Dicenta, 1991; Gudin, Arine, and Pellegrino, 1991; Shivanna et al., 1991) can alter the results of pollination trials. Additionally, waiting for seed maturation is time consuming and may mean the flowering season for that species is over by the time the results are known. Seed counts are also more qualitative than quantitative in many species because a limited number of pollen grains may be all that is necessary for full seed set (Shivanna and Johri, 1985; Stone, Thomson, and Dent-Acosta, 1995). Also, tests for self-incompatibility mechanisms should be done if seed production is to be a measure of viability. Staining techniques also give no information about incompatibility.

In vitro germination, while it does provide a quantitative measure, is time consuming and

can also be influenced by such things as temperature, hydration, density of pollen, % sucrose used, and the presence or absence of a number of ions (King, 1960; Corbet and Plumridge, 1985; Gilbert and Punter, 1991; Heslop-Harrison and Heslop-Harrison, 1992).

Stone, Thomson, and Dent-Costa (1995) pointed out the complications of pollen hydration state and the performance of viability tests. We believe that for many studies fresh pollen should be used and should be tested with a minimum of elapsed time between collection and analysis. Thus, viability tests that can be done in the field can be very useful. Other workers have done comparison testing of techniques for determining viability (Sarvella, 1964; Parfitt and Ganeshan, 1989; Kapyla, 1991; Trognitz, 1991; Sedgley and Harbard, 1993; Khatun and Flowers, 1995), but generally these have each been done on only one or two species of interest to the worker(s) and some of the methods tested are not suitable for field use. Our object was to test a group of techniques that can be used in the field in order to determine which ones might give the most accurate results over a large number of species.

MATERIALS AND METHODS

Plant Material

In all cases we worked with fresh pollen. In some cases where flowers were found within a few hundred meters of the laboratory, inflorescences were brought into the laboratory and the pollen was removed there. Otherwise, anthers were collected from flowers in the field, the pollen removed and stained and counted under field conditions. For the tests reported, pollen was only taken from recently opened anthers. Pollen from enough flowers to provide adequate amounts for the four tests and a germination series was extracted and mixed on a microscope slide. In a few cases additional flowers had to be harvested in order to have enough pollen for the germination series.

Stains for Pollen Viability

The literature was reviewed to determine the types of tests used in the past for pollen viability and to determine what problems had been reported for these tests (see Dafni and Firmage 2000) for a list of tests reviewed and the advantages and disadvantages of each test).

We chose four staining methods for our field tests. We also performed in vitro germination tests to check the accuracy of the tests chosen. We did not use Fluorescein (FCR). It is effective if used within the correct time period (Fritz and Ludaszewski, 1989) and with the proper osmotic solution for the species being tested (Heslop-Harrison et al., 1984; Nepi and Pacini, 1993), but the requirement for fluorescent microscopy eliminated it as a field technique. The peroxidase enzyme reaction was eliminated because of lack of specificity and the fact that the substrate is a health hazard (Hauser and Morrison, 1964; Dafni, 1992; Kearns and Inouye, 1993).

Several of the viability tests chosen required incubation. We found for most tests that the desired temperature could be obtained in the field by using a dark plastic box with a clear plastic top placed in the sun. We were able to keep the box between 35° and 37°C without problems and found this gave excellent results. Moistened paper toweling was placed on the bottom to increase humidity. In cases where slides were to be incubated in the dark, the slides were covered within the box with aluminum foil. Each of the test solutions can be kept over several weeks without loss of activity. Solutions should be kept cool if the time in the field will be more than a few days in duration.

Tests Chosen

1. Pollen staining after incubation with 5-bromo-4-chloro-3-indoyle-β-galactoside (X-Gal-test)(see Trognitz (1991) and Singh, O'Neill and Knox (1985)). Pollen produces B-galactosidase soon after the reduction division of meiosis (Mascarenhas, et al., 1986). The X-Gal substrate is hydrolyzed to 5-bromo-4-chloro-indigo, a blue dye, in the presence of β-Galactosidase (Trognitz, 1991). Medium was made up as follows: a ratio of 1 mg X-Gal (dissolved in 50 µl N, N - dimethyl formamide) and 1 ml acetate buffer (50 mmol, pH 4.8). Pollen was incubated for 30 minutes in dark at 35-37º C in the medium.

2. Pollen staining after incubation with MTT (2,5-diphenyl tetrazolium bromide - also known as thiazolyl blue) (Khatun and Flowers, 1995; Norton, 1966). Norton (1966) found that MTT was the best of 12 tetrazolium salts tested.

We used a 1% solution of MTT with and without a 5% sucrose solution. Khatun and Flowers (1995) mention that the pollen takes the stain in 10-15 minutes, though Norton (1966) mentions incubating it for 30 to 60 minutes for color development. We incubated the grains for 30 minutes at 35-37° C and found that effective. The grains stain from red to black. Grains that are light red to red are considered viable; non-colored ones and black ones, non-viable. Norton (1966) suggests that the black ones are in the process of degeneration.

3. Pollen staining after incubation with Baker's solution (see Dafni, 1992). This stain tests for the presence of alcohol dehydrogenase. Similar solutions have been reported by Juncosa and Webster (1989) for other enzymes as well. The substrate consists of: 10 ml of 0.1 M phosphate buffer (pH 7.3-7.5) diluted 1 part buffer to 2 parts distilled water; enough nitroblue-tetrazolium (NBT) to give a slight yellow color; 6 mg of nicotinamide adenine dinucleotide (NAD); and 0.5-1 ml of ethanol (95%).

The slide may be kept for 15-20 minutes in a closed Petri dish (wet filter paper at bottom) to prevent drying and contamination after which the slide can then be allowed to dry under ambient conditions. After 30-60 minutes the slide can be inspected. An alternative is to incubate the slide (in the petri dish) at 35-37° C for 30 minutes. We used the latter technique for more uniform conditions among tests.

4. Pollen staining after incubation with isatin. This test indicates the presence of proline. Variations of this test have been reported by Palfi and Koves (1984), Palfi et al. (1988), Palfi, Gulyas and Rajki (1988), and Gulyas and G. Palfi-Deim (1991). Because this technique only works on those species with at least 1% content of proline in pollen dry matter, a test should be performed for "isatin positive" species first (Gulyas and Palfi-Diem 1991). The solution for the isatin positive test was made by dissolving 0.25g isatin in a mixture of 20 ml methanol and 0.8 ml glacial acetic acid. The grains should stain almost uniformly (98%) black if isatin positive. If not positive, no grains will be stained.

Isatin mixture was made by adding 5 ml concentrated acetic acid and 2 grams isatin to 200 ml of acetone. Although we used fresh pollen for our tests. pollen can be fixed and dried within 5 days of collection (the proline will remain). Pollen was placed on a microscope slide, then 1-2 drops of isatin mixture were placed on the slide and mixed with the pollen until the solvent evaporated. This was repeated a second time and then the slide was developed for color at 90° C for 12 minutes. For incubation in the field we used a small box, with an inserted thermometer, that could be heated over a small camp stove. Maintaining the temperature was not difficult because of the short incubation time. Afterwards the yellow stain around the periphery of the slide was removed before distilled water and a cover slip were added.

For each type of test the pollen was analyzed under 100-400x. Either random visual fields were assessed (total ~500 grains) or transects across the slide were examined until at least 500 grains were counted. In some cases there were fewer than 500 grains on the slide, in which case all grains were counted. When examining transects, a count was generated for each 100 grains encountered. If the variability of these counts was high, more pollen was counted (up to 1000 grains). The test was repeated from three to five times for each species.

In Vitro Germination

Pollen germination was done using a variation of the hanging drop method. Pollen was first hydrated by placing it for one hour on a glass slide inside a petri dish with moistened filter paper in the bottom. The germination medium used was a standard Brewbaker and Kwack (1963) mixture at 0%, 5%, 10%, 20%, 30%, 40%, and 50% sucrose equivalents as measured by a hand refractometer. The top of a 3.25 cm Petri dish was divided by lines into six pie sections and a 10 µl droplet of one of the solutions placed in the center of each section. The same solution was used to moisten filter paper on the bottom of the dish. Silicone gel was placed on the upper edge of the dish bottom. Pollen was placed in each of the droplets on the lid and

the lid was then inverted and placed on the bottom, thus being sealed with the gel. One dish was made for each of the sucrose concentrations.

Dishes were left at room temperature (20° C) for 24 hours. After this period, a droplet of Calberla's solution (Dafni, 1992) was added to the droplet on the lid and then the entire droplet was transferred to a microscope slide for counting. Slides could be kept in this state for several days if refrigerated. On the rare occasions when counting was not possible within a few days of harvest, the Petri dishes were placed in the freezer before addition of the stain. Slides made from five separate droplets were counted.

RESULTS AND DISCUSSION

Our review of methods (Dafni and Firmage 2000) showed that some methods previously reported to give inaccurate results (in that they only reflect stainability rather than viability) are still used frequently. In some cases tests were used after assessment of their accuracy for the species under study, but in other cases it appears that the test used was simply one that was handy or had been used in the past. No reports of inaccurate results were found for the four tests that we chose.

Data for the four field methods we compared include viability and germination data for 17 species (Table 1). The results show there is probably not one stain best in all cases, but that preliminary testing should be done on each species to be considered. However, the Baker's and MTT stains gave the most accurate results in 10 of the 17 species listed and at least as good as others in three additional species. The difference between the average percent viability as shown by the stain and that shown by germination (stain % - germ. %) was 1.14 for Baker's, -6.93 for MTT, 26.64 for X-Gal, and 42.49 for Isatin.

We found Baker’s solution stained the pollen quite darkly or left it clear in almost all cases. Although Dafni (1992) states the stain is dark blue, in some species there are a number of grains that stain a reddish color. Our comparative *in vitro* germination tests indicate these grains should be counted as viable also. Since our work with the species reported here, it was determined that for a number of other species, Baker's solution stains 100 per cent of the grains, and will even stain dead pollen (Rodriguez-Riano and Dafni, 2000; Firmage, unpublished data). Therefore it is less desirable as a starting point, although for some species it may still be a good choice.

MTT would be a good choice as a starting point for tests on a species under consideration. The relatively low incubation temperature was not a problem to accomplish in the field and in cases where it does present difficulty one can still perform the tests without heating. Our experience suggests the stain be left for longer periods of time (approximately 60 minutes) in such circumstances.

We found *Bellevalia flexuosa* pollen to germinate very quickly with full germination being attained within fifteen minutes. The pollen of this species began to germinate in the MTT, which does not kill the pollen as other stains do, and this gave us an indication of the color the viable grains were stained. As a further test we germinated *B. flexuosa* pollen grains in Brewbaker-Kwack solution with 5% sucrose for 15 minutes and then added stain. In both cases it was seen that the black and clear grains almost never germinated, whereas the red did. One must be careful in counting, however, as some of the grains stain a very dark red and at a quick glance can be mistaken for black ones. Our results agree fully with the suggestion of Norton (1966) that black stained grains are beginning to break down and are not viable.

Of the four tests, isatin results were only closest to the *in vitro* germination % for *Scilla autumnalis* (for which MTT was not tested) and *Mandrogora autumnalis*. For most species it either was not valid (the species not being isatin positive) or it greatly overestimated germinability. It was tested on several other species (*Ceratonia siliqua* L., Caesalpiniaceae; *Foeniculum vulgare* (L.) Aiton, Apiaceae; and *Inula viscosa* Miller, Asteraceae) for which only Baker's stain was available for comparisons. For the first two species the estimate averaged 24.5% above that for Baker's stain. In the case of *I. viscosa*, both stains gave an estimate of 97%. We believe isatin will in most cases overestimate the true viability of pollen. An additional disadvantage of using isatin is that the crystals tend to obscure unstained pollen, particularly if the grains are very small.

X-Gal was more accurate than the other tests for *Colchicum steveni* and *Nicotiana glauca* (for neither of which MTT was available). However, in both cases the count still did not reflect the germination results very accurately. X-Gal stained more than 90% of the pollen in all but two species. It is our experience that this test, like TTC, generally overestimates germinability by a significant margin. We tested this stain on germinated *B. flexuosa* pollen in the manner mentioned above for MTT. Very few of the germinated grains stained, indicating that β-galactoside is apparently used or broken down in the germination process. However, of the grains that did not germinate (n=118), 81% still stained bright blue.

The present study shows that when pollen viability field tests are desired, prior testing of several stains on the species in question should be done, using an in vitro germination test as a comparison. The MTT stain is recommended as having a high likelihood of good correlation with *in vitro* germination tests.

It is clear that no one test is suitable for testing viability in all species. It is also apparent that some of the stains that have been suggested most often over estimate viability. For field tests, the MTT test for pollen viability is a good choice with which to start. Care should be exercised in the selection of a stain and adequate testing needs to be done before relying on the data obtained from any given test.

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Tables

Table 1. Percent stainability of pollen (±SE for tests run) as determined by four different methods and in vitro germination % (with % sucrose) for 17 species found in Israel.

Species	Baker's	Isatin	MTT	X-Gal	Germ. & % Sucrose
Anchusa undulata L. (Boraginaceae)	27.9±9.5	98.7±1.3	59.4±6.8	97.8±1.2	70.5±2.7 10%
Bellevalia flexuosa Boiss. (Liliaceae)	67.7±12.9	*	69.9±6.3	97.2±0.5	72.7±3.9 5%
Clematis cirrhosa L. (Ranunculaceae)	94.3±1.1	NT	96.0±0.8	95.1±1.5	72.6±8.7 10%
Colchicum steveni Kunth (Liliaceae)	61.5±3.9	**	NT	95.0±1.1	78.5±7.1 0%
Crocus hyemalis Boiss & Blanche (Iridaceae)	78.9±3.4	94.1±1.3	32.0±0.8	91.2±5.9	18.2±4.2† 20%
Cyclamen persicum Miller (Primulaceae)	71.5±3.7	*	NT	98.1±0.02	65.9±1.2 10%
Gagae chlorantha (MB.) Schultes et Schultes fil. (Liliaceae)	88.2±3.4	95.9±0.3	59.5±2.5	94.5±0.9	63.2±1.8 10%
Mandragora autumnalis Bertol. (Solanaceae)	90.2±7.0	78.1±7.9	81.4±4.2	98.4±0.4	66.0±3.4 10%
Mercurialis annua L. (Euphorbiaceae)	3.2±1.5	*	68.8±2.1	96.4±1.9	68.9±1.5 20%
Narcissus tazetta L. (Amaryllidaceae)	82.8±2.6	*	47.8±3.2	97.8±0.1	79.5±2.7 10%
Nicotiana glauca Graham (Solanaceae)	64.6±0.5	90.0±1.8	NT	89.8±1.6	77.5±2.4 20%
Ophrys israelitica Baumann & Künkele (Orchidaceae)	54.4±2.1	*	**	**	NT
Reseda alba L. (Resedaceae)	86.2±1.8	86.8±1.8	89.6±1.4	98.0±1.0	NT
Rosemarinus officinalis L. (Lamiaceae)	81.7±2.7	90.4±2.2	62.1±3.8	82.7±2.8	62.6±0.4 20%
Scandix iberica MB. (Apiaceae)	100	**	92.5±2.1	**	NT
Scilla autumnalis L. (Liliaceae)	95.5±1.4	85.3±8.2	NT	96.7±1.2	85.2±1.7 10%
Scrophularia rubricaulis Boiss. (Scrophulariaceae)	61.9±4.8	65.4±14.6	78.9±2.5	96.1±0.9	72.6±2.9 10%

* Not isatin positive; ** Stain vs. unstained too difficult to determine; NT = not tested

† We consider this to be artificially low and that proper germinating conditions have not been met. Germination % observed on 90 stigmas was 87.6±0.02.