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SHORT COMMUNICATION

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A new procedure to asses pollen viability

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Abstract We tested pollen viability of eight species using four vital dyes, a new peroxidase test together with three other established methods (MTT, Baker's and X-Gal), to determine their potential to differentiate fresh pollen from pollen heated for 2 h and 24 h at 80°C (killed pollen) and compared the results with in vitro germination. We found that two of three dyes previously employed to determine viability also stained killed pollen, while the new peroxidase test and MTT did not. We suggest that the latter two are the best methods to test pollen viability, since they do not normally stain either killed or aborted pollen.

Key words Pollen germination · MTT · Peroxidase

Introduction

The need for assessing viability of pollen used in artificial pollination and in breeding experiments (Stone et al. 1995) is also important in the understanding of sterility problems and hybridisation programs (Gupta and Murty 1985), fruit breeding programs (Oberle and Watson 1953), and evolutionary ecology (Thomson et al. 1994).

Lately, a large variety of dyes have been used to test pollen viability, but few studies have tested the potential risk of these dye to stain killed pollen. The most common nuclear and vital dyes, which indicate the presence of cytoplasm or enzymes, respectively, used thus far (Alexander's procedure, acetocarmine, aniline blue in lactophenol, TTC, MTT, X-Gal) have recently been strongly criticised, as they also stain killed pollen (Käpylä 1991, Parfitt and Ganeshan 1989; Khatum and Flowers 1995; Sedgley and Harbard 1993).

Therefore, we pose two questions: (1) is the dye able to differentiate between fresh pollen and killed pollen and (2) is the dye able to differentiate between pollen that can germinate from that which can not? In this work, we try to answer the two questions by comparing three common vital dyes and a new peroxidase test with in vitro germination of fresh and killed pollen in order to test their reliability, and thus determine their potential efficacy as indicators of pollen viability.

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Material and methods

Plant material

In all cases fresh pollen was collected in the field from recently opened anther and brought into the laboratory. Depending on the amount of pollen per anther, we took either one anther for each sample of pollen or all of the pollen from one flower. Pollen was extracted and mixed on a microscope slide and then divided into three samples: (1) fresh pollen, (2) pollen heated to 80°C for 2 h (designated 2-h pollen), and (3) pollen heated to 80°C for 24 h (designated 24-h pollen).

Tests for viability

Four methods for staining, along with in vitro pollen germination, were used to test pollen viability.

1. Baker's procedure (Dafni 1992). This test detects the presence of alcohol dehydrogenase. The test solution consisted of 7 mg phosphate buffer/l0 ml H_2O) (pH 7.3–7.5); nitroblue-tetrazolium just to give a slight yellow colour; 6 mg nicotinamide adenine dinucleotide and 0.5–1 ml of ethanol (35%). The pollen grain was considered viable if it turned violet or pink.
2. X-Gal-test (Trognitz 1991; Singh et al. 1985). This test detects the presence of β-galactosidase. The test solution consisted of 1 mg X-Gal (5-bromo-4-chloro-3-indoyle-β-galactoside) dissolved in 50 µl N,N-dimethyl formamide and 1 ml acetate buffer (50 mmol, pH 4.8). The pollen grain was considered viable if it turned blue.
3. MTT (Khatum and Flowers 1995; Norton 1966). This test detects the presence of dehydrogenase. The test solution consisted of a 1% concentration of the substrate 2,5-diphenyl tetrazolium bromide (MTT or thiazolyl blue) in 5% sucrose. The pollen grain was considered viable if it turned deep pink or if it presented no colour but showed irregular black lines over its surface.
4. p-Phenylenediamine. This test detects the presence of myeloperoxidase. The test solution consisted of one vial peroxidase indicator reagent (Sigma 390-1), and 200 µl 3% hydrogen per-

Table 1 Viability percentage (mean±standard deviation) of fresh pollen (*fresh*) and killed pollen after 2 hours (2 h) and 24 hours (24 h) as determined by four vital dyes and in vitro germination.

Species	Test	Fresh	2-h	24-h
Calycotome villosa				
	Baker's	88.60±4.49	42.57±12.75	51.67±12.26
	X-Gal	99.20±0.85	95.67±4.01	78.03±8.99
	MTT	97.03±2.27	11.33±17.19	0.00±0.00
	p-Phenylenediamine	97.13±1.93	0.00±0.00	0.00±0.00
	Germination	90.07±3.15	0.23±0.81	0.00±0.00
Colchicum steveni				
	Baker's	83.69±11.06	81.77±9.79	74.23±14.31
	X-Gal	98.63±1.86	99.11±1.47	97.03±3.52
	MTT	72.34±12.74	7.40±10.94	0.03±0.17
	p-Phenylenediamine	79.10±17.18	20.95±27.45	0.00±0.00
	Germination	73.86±20.57	2.54±5.44	0.00±0 00
Crocus hyemalis				
	Baker's	87.28±11.33	68.00±11.93	66.60±8.68
	X-Gal	92.56±9.05	85.48±9.93	81.32±9.21
	MTT	61.20±15.07	15.24±31.08	0.00±0.00
	p-Phenylenediamine	69.92±15.55	0.28±0.98	0.00±0.00
	Germination	28.68±22.50	0.04±0.20	0.0±0.00
Cyclamen persicum				
	Baker's	87.76±6.44	0.00±0.00	0.00±0.00
	X-Gal	97.16±2.50	39.12±48.91	0.00±0.00
	MTT	94.16±4.22	0.00±0.00	0.00±0.00
	p-Phenylenediamine	94.36±4.11	0.00±0.00	0.00±0.00
	Germination	89.40±8.18	0.08±0.00	0.00±0.00
Iris palaestina				
	Baker's	97.92±1.50	96.48±2.52	96.24±2.30
	X-Gal	98.72±1.10	97.40±2.33	0.00±0.00
	MTT	97.56±1.85	0.16±0.47	0.00±0 00
	p-Phenylenediamine	92.40 ±17	0.56±1.29	0.00±0.00
	Germination	85.96±6.19	0.00±0.00	0.00±0.00
Narcissus tazetta				
	Baker's	67.92±9.86	66.00±10.73	61.60±12.11
	X-Gal	72.28±11.68	77.08±12.90	34.68±22.35
	MTT	70.00±12.07	68.00±10.95	44.56±9.22
	p-Phenylenediamine	70.24±10.86	30.72±11.32	0.00±0 00
	Germination	60.12±10.69	13.12±7.96	0.00±0.00
Oxalis pes-caprae				
	Baker's	66.40±7.78	63.80±6.00	61.27±8.86
	X-Gal	–	–	–
	MTT	59.00±6.78	0.12±0.44	0.00±0.00
	p-Phenylenediamine	55.04±15.59	0.00±0.00	0.00±0.00
	Germination	42.76±12.67	0.00±0.00	0.00±0.00
Romulea phoenicia				
	Baker's	97.96±1.81	97.32±2.41	95.28±1.93
	X-Gal	98.84±1.37	98.96±1.27	94.40±2.86
	MTT	95.20±3.86	0.00±0.00	0.00±0.00
	p-Phenylenediamine	92.68±4.69	0.00±0.00	0.00±0.00
	Germination	83.56±10.34	0.00±0.00	0.00±0.00

oxide (1:9, 30% hydrogen peroxide and phosphate buffered saline solution pH 7.4) added to 50 ml Trizmal 6.3 dilute buffer prewarmed to 37°C prepared by mixing Trizmal 6.3 buffer concentrate (Sigma 90-3 C) with deionized water 1:9. The solution can be kept in the refrigerator for about 15–20 days without loss of potential activity. If during this time the solution turned from light brown to very dark brown or black it was discarded. This solution was always kept and used in the dark. To stain pollen grains, we took a small amount of the solution and warmed it at 37°C about 10–15 min. The pollen grains were considered viable if they turned totally black.

5. The in vitro germination test used the hanging drop method (Shivanna and Rangaswamy 1992) with various sucrose solution concentrations (0%, 5%, 10%, 15%, 20%, 30%, 40% and 50%) with 2×10^{-3} M H_3BO_3 and 6×10^{-3} M $Ca(NO_3)_2$ added. Dishes were left at room temperature (20°C) for a maximum of 24 h. Pollen grains were considered to have germinated when pollen tube length was greater than or equal to pollen diameter. For each species, we recorded germination at the optimal sucrose solution.

All pollen viability tests were conducted by incubating the pollen in the medium for 30 min at 37°C. The process of staining using both X-Gal and p-phenylenediamine, was conducted in the dark. We used five replicas per sample, and five random groups of 100 pollen grains each per replica. We used a light microscope with either ×160 or ×400 magnification, depending on pollen size.

Species tested were: *Calycotome villosa* L. (Fabaceae), *Colchicum steveni* Kunth (Liliaceae), *Crocus hyemalis* Boiss et Blanche (Iridaceae), *Cyclamen persicum* Miller (Primulaceae), *Iris palaestina* (Baker) Boiss. (Iridaceae), *Narcissus tazetta* L. (Amaryllidaceae), *Oxalis pes-caprae* L. (Oxalidaceae) and *Romulea phoenicia* Mout. (Iridaceae). All of were collected locally on Mount Carmel during fall and winter 1998–1999.

Results and discussion

The results of germination using the four dyes are shown in Table 1. Three groups of dyes are apparent; those that always or almost always stained killed pollen in all the test species; those that stained killed pollen in some test species but not consistently across all species and those that never stained killed pollen.

Dyes in the first group (Baker's and X-Gal) always stained killed pollen, although sometimes only the 2-h pollen: only the Baker's stained killed pollen faster than fresh pollen, and X-Gal stained killed pollent at the same rate as fresh pollen. Although the colour with X-Gal was always lighter in killed than in fresh pollen, it was impossible to differentiate between fresh and killed pollen. In the case of *O. pes-caprae*, all pollen (fresh and killed) burst and there were no differences between them. Sedgley and Harbard (1993) found that X-Gal positively stained more than a third of the killed polyads in some *Acacia* species. Therefore, we recommend that Baker's and X-Gal not be used to test the pollen viability.

The second group (MTT) showed many different colour tonalities and sometimes the very dark pink pollen was difficult to distinguish from the black. In addition, MTT seldom stained killed pollen, although when it did the stain was always lighter than with fresh pollen. Therefore MTT should be used with caution, taking into account the species being tested. Parfitt and Ganeshan (1989) found that, in the case of some *Prunus* species, heat-killed pollen was intensely stained.

Use of p-phenylenediamine was the most reliable method to distinguish between fresh and killed pollen (Fig. 1), since killed pollen almost always turned greyish-brown, which strongly contrasted with the colour of fresh pollen; more importantly, it never stained aborted pollen. The p-phenylenediamine reaction was quite consistent across species and this contradicts the results of King (1960), who used another peroxidase. The only variability was in *I. palaestina*, in which most of the fresh pollen burst and the rest stained black with a very narrow halo around the pollen, while the killed pollen (2-h and 24-h) did not burst and the halo was much larger. In *C. persicum*, fresh pollen turned very dark brown with black spots, but fresh pollen could be distinguished from killed pollen. In the remaining species, fresh pollen colour was always black. In *I. palaestina* and *C. steveni*,

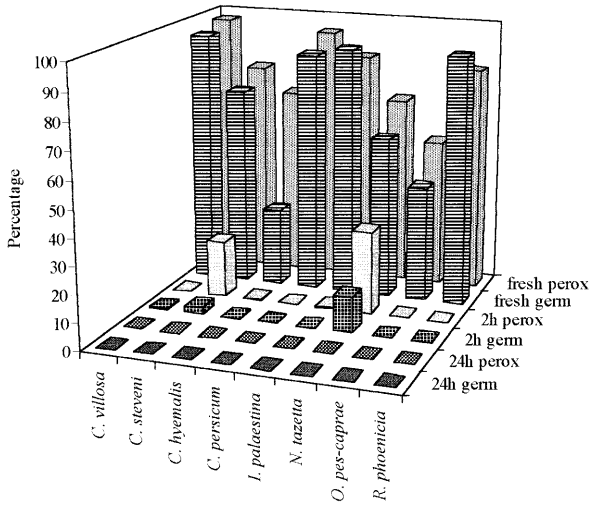

Fig. 1 In vitro germination (*germ*) and pollen viability using p-penylenediamine (*perox*) on fresh pollen (*fresh*) and killed pollen after 2 h and 24 h for the studied species

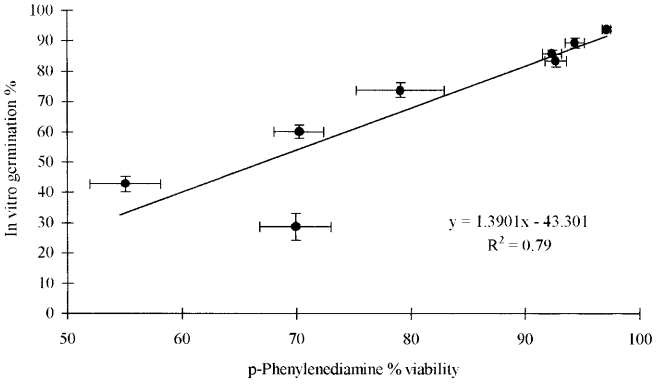

Fig. 2 Linear regression between in vitro germination and viability using p-phenylenediamine on fresh pollen

it was difficult to distinguish 2-h killed pollen from fresh pollen.

Germination percentage was always very high, from 60.1% in *N. tazetta* to 90.1% in *C. villosa.* There were two exceptions: One was *C. hymalis*, in which the number of pollen grains that germinated was very low (28.7%) and germination was accompanied by numerous morphological anomalies. This was also reported in *C. sativus* by Chichiricco and Grilli Caiola (1982), who relate the low germination to triploidy. Many anomalies were also found in the pollen tubes of *Iris*. The second exception was *O. pes-caprae* (42.8%), where about 40–50% of pollen grains aborted. These aborted pollen grains were easily distinguished from fresh pollen grains by the peroxidase. In Israel this species is reproduced only vegetatively and has high rate of pollen abortion (Dafni, personal observation).

Out of the four dyes tested, only MTT and p-phenylenediamine showed a high correlation with in vitro fresh pollen germination, and, in addition, they did not stain

either killed or aborted pollen. The variability of colour tonalities and the possibility of staining killed pollen (2-h and 24-h) by MTT in some species may sometimes make it difficult to use this dye to differentiate between fresh pollen and killed pollen. In the case of p-phenylenediamine, there was a statistically significant linear regression with in vitro germination with fresh pollen (F=22.78, P<0.01; Fig. 2) and with 2-h pollen (F=25.44, P<0.01, r^2=0.81).

Dyes were generally used by many researchers without any type of control. As a result of the present study we recommend using some type of control (such as killed pollen) to check the potential of the dye to test pollen viability before using it. If it stains killed pollen, then it must be avoided. The next step should be to test the potential capability of the dye to stain non-germinated pollen at different ages, because sometimes the use of vital dyes is unsatisfactory, since, as some authors have commented (Sedgley and Harbard 1993), enzymes that the vital dyes are testing for remain intact in older pollen. Based on our results, we suggest introducing the viability test with p-phenylenediamine as another routine for testing pollen viability. This test could also be used in the field and the results examined after 30 min.

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