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The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure

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Summary. Methods currently available for evaluating pollen quality in vitro include, (a) tests of germinability; (b) tests of the stainability of the vegetative cell contents; (c) tests for enzyme activity, and (d) the fluorochromatic procedure (FCR), which tests principally the integrity of the plasmalemma of the vegetative cell. Using germinability in vitro as a standard, a comparison has been made between histochemical methods of classes (b), (c) and (d) in application to various pollens, immature, mature, and treated in ways known to affect viability and membrane state. Predictably, the lowest correlation was obtained with tests of stainability. The highest was given by the FCR, which generally provided an excellent guide to potential germinability. The FCR procedure is subject to various limitations, however. (a) A high correlation between FCR and germinability can only be expected when mature, ripe pollen is used; with immature pollen, the FCR will predict excessively high potential germinability. (b) The FCR may also predict a higher potential level of pollen function than in vitro germinability when the germination medium is sub-optimal. In this situation, however, it will generally give a better guide to fertilising capacity. (c) The FCR is not a test of pollen viability. Like germinability in vitro, it can yield a negative score with pollen which is nevertheless capable of functioning. For example, false negatives will be obtained with some species if the pollen is not properly pre-conditioned by rehydration before testing, an important point in monitoring stored pollen. The paper includes a brief discussion of the rationale of pollen testing.

Key words: Pollen testing – Pollen germination – Fluorochromatic reaction (FCR) – *Lilium longiflorum* – *Narcissus pseudonarcissus* – *Helleborus niger* – *Primula vulgaris* – *Medicago sativa* – Lucerne – Alfalfa – *Lycopersicum esculentum* – Tomato

Introduction: the rationale of pollen testing

The need to test the functional quality of pollen arises in many contexts – for example, in assessing the fertility of parent plants and hybrids in genetical experimentation and plant breeding, in monitoring pollen state during storage, in research on pollen-stigma interaction, incompatibility systems and fertilisation, and, in more remote fields, in investigating the suspected hybrid status of individuals and populations in plant taxonomy and ecology. However, it is a familiar fact that the testing methods available often do not provide the kind of information required. The essential role of the angiosperm male gametophyte is to convey the two male gametes into the embryo sac, and any test which falls short of assessing the competence of the individuals of a given pollen population to achieve this must necessarily be less than ideal. The ultimate functional test ought therefore to be fertilising capacity as measured by seed set. To measure this usually involves a considerable investment of time, and, in addition, there may be difficulties in practice. Seed set may depend not simply upon fertilisation, but on the post-pollination development of the ovary, which in some genera depends on activation by pollen, and for this a minimum level of pollination may be required whatever the fertilising capacity of individual grains. Pistil receptivity is a critical factor, as emphasised especially by Walden and Everett (1961) in their valuable paper on the development of form of fecundation index ("K assay") for *Zea mays*. Further, as noted by Stanley and Linskens (1974), incompatibility reactions and

various forms of certation phenomena – perhaps unsuspected – determined by the female genotype, may interfere with the direct testing of pollen function, yielding false negatives for pollen which may be fully functional on another female.

Accordingly, simpler means for assessing pollen quality have been sought. These include tests of germinability and pollen tube growth in vitro, which have a considerable history, dating back for over a century to the work of various perceptive early investigators, including especially Van Tieghem (1869). During the present century, indirect methods not relying on observation of germinability but based upon staining procedures or enzymic and other tests have been introduced for the assessment of pollen quality, and currently a considerable battery of these histochemical methods is available. Their application and value have been discussed in various reviews, notably by Stanley and Linskens (1974), who list much of the earlier literature. An especially valuable aspect of the account given by these authors is the consideration they give to the theoretical appraisal of testing procedures and to the assessment of possible sources of error.

In the present paper we are concerned with a further assessment of one histochemical procedure, the fluorochromatic reaction (FCR), suggested some years ago (Heslop-Harrison and Heslop-Harrison 1970) as a relatively simple pollen-testing method relying principally on an evaluation of a critical property of the male gametophyte, namely the integrity of the plasmalemma of the vegetative cell. The theoretical basis for the reaction was discussed in the original paper, and may be summarised briefly as follows. As first demonstrated by Rotman and Papermaster (1966), certain non-fluorescent fatty acid esters of fluorescein freely enter living cells, where they may rapidly be hydrolysed to give fluorescein. If the cell has an intact membrane, the rate of escape of fluorescein, a polar molecule, is lower than the rate of entry of fresh substrate, so that for a period fluorescein accumulates in the cell, which accordingly becomes increasingly fluorescent. In application to pollen, the substrate adopted was fluorescein diacetate (FDA), and accumulation of fluorescein in the vegetative cell was regarded as testing firstly the presence of an active esterase, and secondly – and more significantly – the possession of an intact plasmalemma. The increase in fluorescence in an intact grain can usually be detected readily by eye using quite simple microscope equipment, or measured with suitable instrumentation.

The range of methods now available for testing pollen in vitro is, then, as follows:

1 Tests of capacity to germinate and produce a normal pollen tube. These provide a direct and reliable assessment of viability, but do not necessarily produce scores closely correlated with fertilising capacity in all instances (Stanley and Linskens 1974). They depend critically on the ability of the investigator to formulate a suitable medium for obtaining consistent germination and tube growth, a requirement yet to be met in several economically important families.

2 Stainability tests. These assess the contents of the vegetative cell, with varying degrees of specificity for different constituents. Simple methods have been described for starch (I+KI) and other polysaccharides, including callose (periodic acid-Schiff procedure, aniline blue) and for chromatin and RNA and various undefined cytoplasmic constituents (acetocarmine, acetic orcein and similar stains; acridine orange). Various other general stains can give similar results, the principal criterion being whether they leave the exine sufficiently uncoloured for the stainability of the cell contents to be assessed.

3 Enzyme tests. These establish the activity of various enzymes of the vegetative cell, sometimes localising them in organelles. Most techniques have been based on the use of so-called redox dyes. Stanley and Linskens (1974) attribute the earliest use of the method to Sharadakov (1940), who took the enzymatic oxidation of benzidine as an indicator of pollen viability. The benzidine method has been used for various crop plants, e.g. by King (1962), but because of the health hazard associated with the substrate it has largely been abandoned. Stanley and Linskens (1974) also review comprehensively the applications of the most commonly used class of reagents, the tetrazolium group, which yield insoluble coloured formazans on reduction. Of this group, 2,3,5-triphenyl-tetrazolium chloride has been the most popular, and its value was investigated in detail by Cook and Stanley (1960).

4 The FCR procedure. This tests (a) for the presence of an active esterase, and (b) the integrity of the plasmalemma of the vegetative cell, as described above.

Methods classifiable under (1) we refer to here as germinability tests, and those under (2), (3) and (4) as histochemical tests. We may consider briefly the logic involved in the application of the methods in these classes. Germinability tests clearly have the potential for providing the best basis for predicting pollen performance. For the reasons already mentioned, however, the information they give is subject to two important limitations: they may give false positives, when factors associated with the female partner and not testable in vitro ultimately control the likelihood of successful fertilisation; and they may give false negatives, when the germination medium is sub-optimal, or when the test population has been too small to allow the identification of rare pollen grains capable, in a mass pollination, of effecting a fertilisation.

The histochemical tests have in logic one common characteristic: all are asymmetric in their predictive value. In each case a positive establishes the possibility, but not the certainty, of effective function, while in a properly designed test a negative should establish the certainty of non-function. With this in mind it may be seen that the value of each class of tests, and indeed of each test, is related to the minimal functional property it is capable of assessing. On a theoretical basis, then, the ranking in the order of increasing potential value might be, (2) stainability tests (a pollen grain devoid of essential contents can never function); (3) enzymatic tests (a grain lacking an essential enzyme cannot be expected to function, even if normally filled), and (4) the FCR procedure (a grain with a leaky membrane cannot hydrate to the point of normal germination, and therefore cannot function, even if normally filled and with an active enzyme complement in cytoplasm and organelles).

The histochemical tests obviously share one of the limitations of germinability tests, namely that the probability of identifying rare but critically important positives is related to sample size. On the other hand, they will in general avoid the more important limitation of germinability tests, that these will always yield false negatives when the medium is sub-optimal. Notwithstanding these considerations, germinability provides the most convenient and appropriate standard against which to assess the histochemical methods, and it has been used as such in the present work.

The aims here have been two-fold, (1) to examine further the proposition that the FCR provides principally a means of testing the integrity of the vegetative cell plasmalemma by following quantitatively the changes induced in the response by agents known to affect membrane properties, and (2) to compare the results obtained by the FCR procedure for pollens subject to various treatments, including the manipulation of water content, with those obtained from other testing methods, using germinability as the standard of measurement.

Materials and methods

Observation of fluorochromasia (FCR)

Test media were prepared by the rapid semi-empirical method described in the first paper (Heslop-Harrison and Heslop-Harrison 1970). Fluorescein diacetate was made up as a stock solution in acetone at 2 mg ml^{-1}. Immediately before use, dilutions were prepared by adding drops of the stock to 2 ml of a sucrose solution of a concentration appropriate to the species until saturation was reached as indicated by the appearance of persistent cloudiness. Pollen samples were dispersed in a drop of this medium on a microscope slide, and the fluorochromatic reaction allowed to proceed for the required period.

Leaching media

The observations on the effects of leaching media were made on pollen of *Lilium longiflorum* L. cv. 'Ace', in greenhouse cultivation. Tests were carried out using five leaching media, (a) 10^{-3} M $Ca(NO_3)_2$ in 0.5 M sucrose (referred to hereafter as "Ca + Su"); (b) distilled water ("H_2O"); (c) 0.5 m sucrose in 0.02 M phosphate buffer pH 7.0 ("Su"); (d) as (c), but with 0.14 M NaCl ("NaCl + Su"); (e) 0.14 M NaCl ("NaCl"), and (f) 10% dimethylsulphoxide in 0.02 M phosphate buffer, pH 7.0 ("DMSO"). The choice of these media was based upon the anticipation of their likely effects on the vegetative cell plasmalemma, and the improbability of their affecting enzyme activity in any serious manner. The Ca + Su formulation approximates to a germination medium, but is sub-optimal in having a higher than necessary sucrose content and lacking boron; in such an environment the cell should retain an essentially normal state. Water, without osmotic buffering, may be expected to burst a high proportion of the grains. This effect should be minimised in the SU medium, but the absence of calcium, a circumstance known to enhance the loss of vegetative cell contents in liquid media (Dickinson 1967), may be expected to increase the permeability of the plasmalemma. The NaCl medium approximately conforms to the "physiological" or "normal" saline widely used as an extractant in pollen-allergy testing. Na^+ is well known to increase the permeability of plant cell membranes (Greenway and Munns 1980), and the medium may be expected accordingly to enhance leakage from the vegetative cell. The same is true of DMSO in the concentration employed (Delmer and Mills 1969).

The pollen was collected from freshly dehisced anthers, and samples of c. 20 mg leached with 1 ml of each medium in small tubes. The samples were dispersed first by brief agitation on a Vortex mixer. This treatment coagulates the pollenkitt, which can then be removed with a needle as a coherent, largely pollen-free, pellet. Leaching was carried out at laboratory temperature (24–25 °C) on a rotator at 24 rev min for periods of 10, 30 and 60 min, the timing of the start for each medium being adjusted so as to allow a standard period for the subsequent FCR reaction. For this, the pollen was sedimented by centrifugation at 1,000 × g, rinsed briefly in 0.5 M sucrose in 0.02 M phosphate buffer at pH 7.0, collected again by centrifugation and transferred to a drop of the FDA medium containing 0.5 M sucrose. The reaction was allowed to proceed for 25 min.

Fluorimetry

Microfluorimetry was carried out with a transmitted-light UV system using an Osram HPO 200 mercury-arc light source with Bausch & Lomb 7–37 excitation filter and T2 barrier filter, the light emission being measured with an EMI IP28 photomultiplier tube. The sensitivity of the system was adjusted at the beginning of each recording session so as to give a reading of 100 chart-recorder units with the most brilliantly fluorescing grain encountered in a preparation of unleached, control pollen, and this standard was maintained throughout all observations. Readings were grouped in ten grades corresponding each to 10 chart recorder units, grade 0 including the zero emission class. The samples were scanned in a series of horizontal traverses. The grains were located first under bright field visual illumination, and each was then framed with the diaphragm and its light emission measured individually with UV excitation until a total of 100–150 grains had been measured per sample. Where the treatment provoked a rapid leakage of fluorescein from the grains, the medium itself became fluorescent, and this was found to falsify the fluorimeter readings. When this happened, the original medium was replaced by fresh medium containing 0.5 M sucrose.

Pollen pre-treatment

For the comparisons of pollen germinability, FCR, and response to other standard tests following various pre-treatments known to affect pollen viability, observations were made on one monocotyledon, *Narcissus pseudonarcissus* L. (unknown cultivar), and two dicotyledons, *Helleborus niger* L. and *Primula vulgaris* L., all in garden cultivation. Effects of controlled dehydration and rehydration were examined using the pollens of *Lycopersicum esculentum* Mill. and *Medicago sativa* L.

For the tests on mature pollen, samples were collected from freshly dehiscing anthers and treated with minimum delay. Immature ("bud") pollen was collected by extracting the pollen mass from anthers of the required age and dispersing it immediately by vigorous agitation on a vibrator in the germination or other medium.

The pollen of *N. pseudonarcissus* was subjected to the various procedures as shed, and that of *H. niger* was first hydrated at 22–24 °C in an atmosphere of 80–95% relative humidity for 12 h. The pollens were tested without further treatment, and also after treatment (a) by immersion for 10 min in DMSO, 10% in 0.5 M sucrose, and (b) by heating in

the dry state at 60 °C for 30 min. In addition, tests were carried out on pollen extracted from a bud of *N. pseudonarcissus*, 4.5 cm in length, in which the perianth was still green but the pollen fully formed, estimated to be 2–3 days from anthesis.

Samples of the pollens of the two morphs of *P. vulgaris* were collected from 9 mm buds, and also from mature flowers immediately after the natural dehiscence of the anthers in a damp environment. The mature pollen was tested in the fresh state, and samples were also partly dehydrated by transfer after collection to a relative humidity of 5–10% for c. 12 h at 22–24 °C. A portion of the partly desiccated pollen was then subjected to the testing procedures immediately, while the remainder was rehydrated for 3 h before testing in a relative humidity of 90–95% at the same temperature.

The test procedures were as follows:

1 Germination. A basic liquid medium containing 2×10^{-3} M H_3BO_3 and 3×10^{-3} M $Ca(NO_3)_2$ in 0.45 M sucrose was used for all species. After dispersal in 1 ml medium on a vibrator, the pollen samples were maintained for the required periods in roller-tube culture at 24 °C. Pollen was regarded as having germinated normally when a tube of a length at least equivalent to the diameter of the grain had emerged.

2 FCR. The fluorochromatic reaction was carried out as described above.

3 Lactophenol-acid fuchsin. The reagent was made up as described by Sass (1964), with 0.1% acid fuchsin (Lamb) in equal parts of lactic acid, phenol, glycerine and distilled water. The pollen was dispersed in the slightly viscous medium, and scored as soon as a satisfactory colour contrast had developed.

4 Acetic-orcein. The stain was made up according to Darlington and La Cour (1950). Orcein (Lamb) was refluxed in 45% acetic acid to saturation, and the excess filtered off. The pollen samples were dispersed in the stain and warmed gently until the coloration was intense enough for a discrimination to be made between full and empty grains. Acetic-orcein staining yields essentially the same results as acetocarmine, with somewhat greater colour contrast between stained contents and the exine staining of unfilled grains.

5 Tetrazolium chloride. The procedure adopted was that of Cook and Stanley (1960), the reagent, 2,3,5-triphenyltetrazolium chloride (Sigma) being used at 0.5% in 12% sucrose. Pollen was dispersed in a drop of the medium on a microscope slide, and, as recommended by these authors, covered immediately with a cover-slip to exclude oxygen, which can inhibit the reduction of the dye. The preparations were incubated at 60 °C in a dark oven for up to 3 h before scoring, and only those grains in the central area of the sample were recorded.

In each test, 200–400 pollen grains were scored in a series of parallel traverses across the sample, the state of every grain in successive fields being recorded. In most cases no ambiguity arose in the scoring; the one instance of serious uncertainty arose in the application of the tetrazolium procedure to desiccated *P. vulgaris* pollen, discussed further below.

For the examination of the effects of dehydration and rehydration on pollen behaviour in *M. sativa* and *L. esculentum*, samples of fresh pollen were dispersed evenly on sheets of plastic film before exposure to treatment. Portions were then extracted for further treatment, or for germinability and FCR testing, by clipping off segments of the film; by this means uniform sampling was achieved throughout each experiment. For dehydration, the samples were exposed in air-tight boxes to 5–10% RH at 24 °C (*L. esculentum*) or 29 °C (*M. sativa*) for the required periods. Rehydration was carried out by exposure to c. 95% RH at the same temperature.

M. sativa and *L. esculentum* pollens were germinated on a semi-solid medium with 1% agarose (BDH), H_3BO_3 and $Ca(NO_3)_2$ each at 10^{-3} M, with 15% sucrose for the former and 12% for the latter.

Results

Response of the pollen of L. longiflorum to leaching agents

Without pre-hydration or other treatment, samples of the pollen of *L. longiflorum* gave germinabilities of 70–80%, and FCR scores of 80–85%. The effects on the FCR of the various leaching treatments given for periods of 10, 30 and 60 min are shown in Fig. 1. In Ca+Su the score remained unchanged after a slight initial diminution attributable to the early bursting of a small proportion of the grains. The response in the Su medium was essentially similar. In water, many grains burst immediately, and the proportion increased throughout. In the NaCl medium, the proportion of grains giving a positive FCR fell to zero within the hour. However, the incorporation of sucrose reduced the rate of decline substantially. After an initial slow fall in FCR score, the pollen samples in DMSO rapidly lost fluorescein retentivity, although within the period of the experiment the score did not actually decline to zero.

Figure 1 is based upon an assessment of individual pollen grains as giving either a positive or a negative FCR, and it gives no guide to the distribution of fluorescence intensity in the population or of its change with time. This is given in Fig. 2, which illustrates the results obtained using four of the leaching treatments for the same time intervals. The light emission from

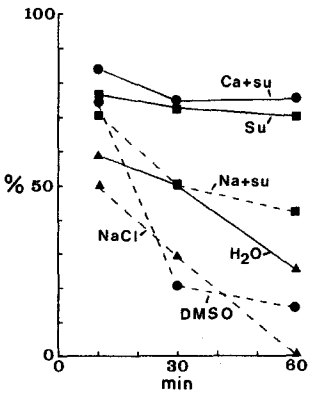


Fig. 1. Percentage showing positive FCR in samples of the pollen of *Lilium longiflorum* exposed to various leaching agents over a period of 60 min. Treatments as in the text; 100–150 grains per sample

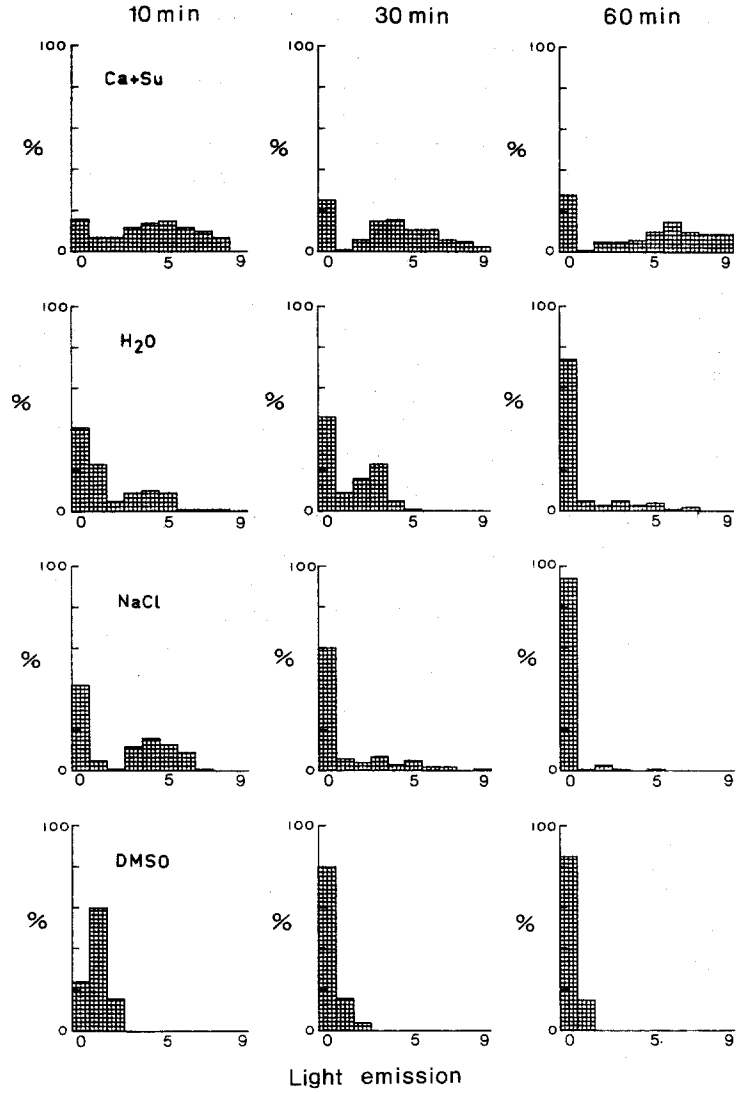


Fig. 2. Frequency distributions of light emission following FCR in samples of the pollen of *Lilium longiflorum* exposed to various leaching treatments for the periods shown. Light emission measured on a 10-class scale as in the text, where the treatments are also detailed. 100–150 grains per sample

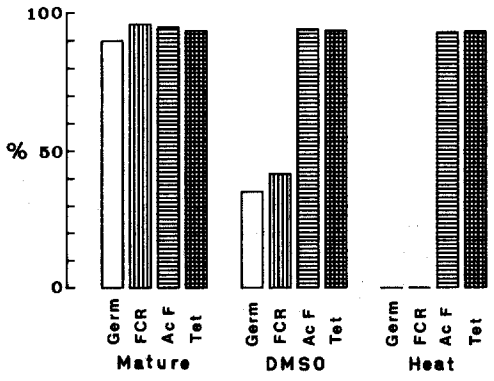


Fig. 3. Comparison of germinability (*Germ*), fluorochromatic reaction (*FCR*), acid fuchsin (*Ac F*) staining and the tetrazolium reaction (*Tet*), expressed as percentages giving a positive response, in pollen samples of *Helleborus niger* freshly released from the anther, and after exposure to heat and DMSO as detailed in the text

individual grains is here shown on a ten-point scale, established as described in the Methods section.

The distributions show a number of significant features. In all cases, the zero category is the modal one. In the Ca+Su medium, the population excluding the zero category shows a mode in the region 4–6, with a considerable scatter of light-emission values. However, there was little difficulty in this experiment with lily pollen in distinguishing the 0 and 1 classes by eye. This would not necessarily be the case with other species, a point to which we will revert.

The H_2O and NaCl treatments produced similar trends, with a fall in the FCR score of approximately 50% in the first 10 min, followed by a progressive further loss through the 1 h period of the experiment. The trend with the DMSO treatment differed, in that the loss of retentivity was less in the first 10 min of

leaching, increasing thereafter throughout the 1 h leaching period.

Assessment of testing procedures

The principal objective of the trials was to assess the results produced by the range of testing procedures with pollens of different sources subjected to various pre-treatments, taking germinability in vitro as a basic standard of comparison. It is convenient to consider the first three species separately.

Helleborus niger (Fig. 3). With the mature, fresh pollen, FCR, acid fuchsin stainability and tetrazolium reaction correlated well, the three histochemical procedures each giving a slightly higher positive score than germinability. The DMSO and heat treatments diminished neither the acid fuchsin nor tetrazolium score. DMSO reduced germinability and FCR score appreciably, but roughly in step. Heat reduced both germinability and FCR to zero.

Narcissus pseudonarcissus (Fig. 4). With fresh mature pollen, a reasonably good relationship was observed between germinability, FCR score and tetrazolium reaction; acid fuchsin staining gave a somewhat higher score. The responses with immature ("bud") pollen are noteworthy. This pollen was incapable of germination in the standard medium in which mature pollen from the same clone germinated freely, but high positive scores were produced by all three of the histochemical procedures. Heat did not affect acid

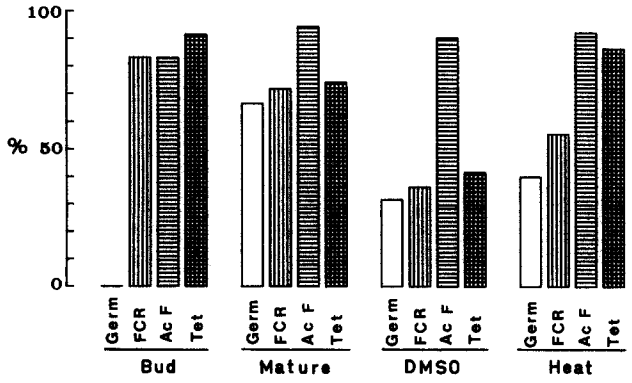
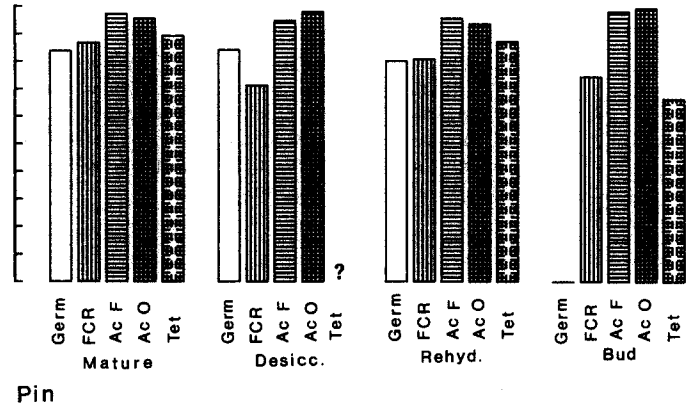


Fig. 4. As Fig. 3, *Narcissus pseudonarcissus*. This comparison also includes a sample of immature pollen extracted from a bud collected before anthesis



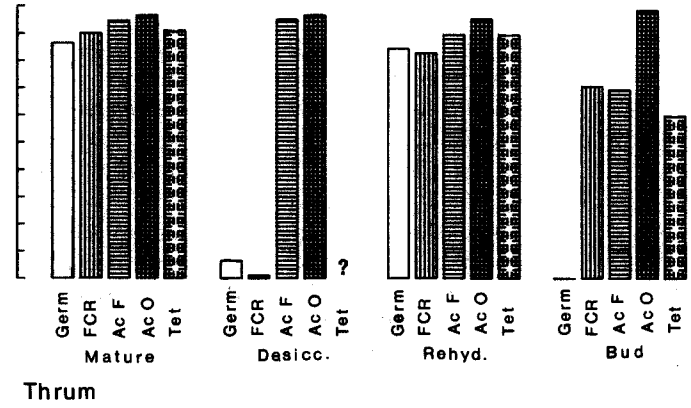


Fig. 5. Comparison of germinability (*Germ*), fluorochromatic reaction (*FCR*), acid fuchsin (*Ac F*) staining, acetic orcein (*Ac O*) staining and the tetrazolium reaction (*Tet*), expressed as percentages giving a positive response, in pollen samples of the pin and thrum morphs of *Primula vulgaris*. The mature pollen was collected on a humid day; samples were tested immediately, and after partial dehydration and subsequent rehydration as detailed in the text. The scores for pollen samples from a bud collected before anthesis are also shown

fuchsin staining or tetrazolium reactivity, but diminished both germinability and FCR score. DMSO was without effect on acid fuchsin staining, but in this instance it reduced germinability and the FCR and tetrazolium scores.

Primula vulgaris (Fig. 5). The comparison here included both morphs, the pollens of which were known from earlier work to differ greatly in water economy and other properties (Shivanna et al. 1983). With mature pollen of each morph, a good correlation was observed between germinability and the histochemical tests, which here included acetic-orcein staining. Again, immature pollen could not be germinated in the standard medium, although all four tests gave positive values. A comparison between the morphs suggests that acid fuchsin staining may be slightly more discriminating than acetic orcein, if correlation with FCR and tetrazolium reaction is taken as a standard for comparison. The comparisons between the partly desiccated pollens of the two morphs are especially significant. As observed in earlier work (Shivanna et al. 1983), the treatment has no more than a small effect on the germinability and FCR of mature pin pollen, while decreasing both indices dramatically with thrum pollen. It was found impossible to obtain a convincing estimation of the tetrazolium reaction with desiccated pollen of the two morphs; within the test period all grains acquired an intermediate coloration falling short of that taken as positive in the other assessments. Pin and thrum pollen rehydrated after the partial desiccation behaved essentially in the manner of fresh pollen in respect both to germinability and the histochemical tests.

Medicago sativa and *Lycopersicum esculentum* (Fig. 6). The observations on *P. vulgaris* provide an example of intraspecific variation in the response of pollen to partial dehydration, and the comparison of *M. sativa* and *L. esculentum* illustrates a similar kind of difference between species. FCR score and germinability for these pollens tested immediately after dehydration for various periods of time and after an interval of rehydration are shown in Figs. 6 A, B. The two species evidently differ considerably in their behaviour. Dehydration for 48 h has virtually no effect on FCR nor germinability in *M. sativa,* and the behaviour is not affected by the rehydration treatment. With *L. esculentum,* germinability and FCR fell steeply after 12 h dehydration, but both recovered to some extent with rehydration. However, the significance of the results with both species lies in the fact that the FCR here provided a faithful index of germinability with all treatments.

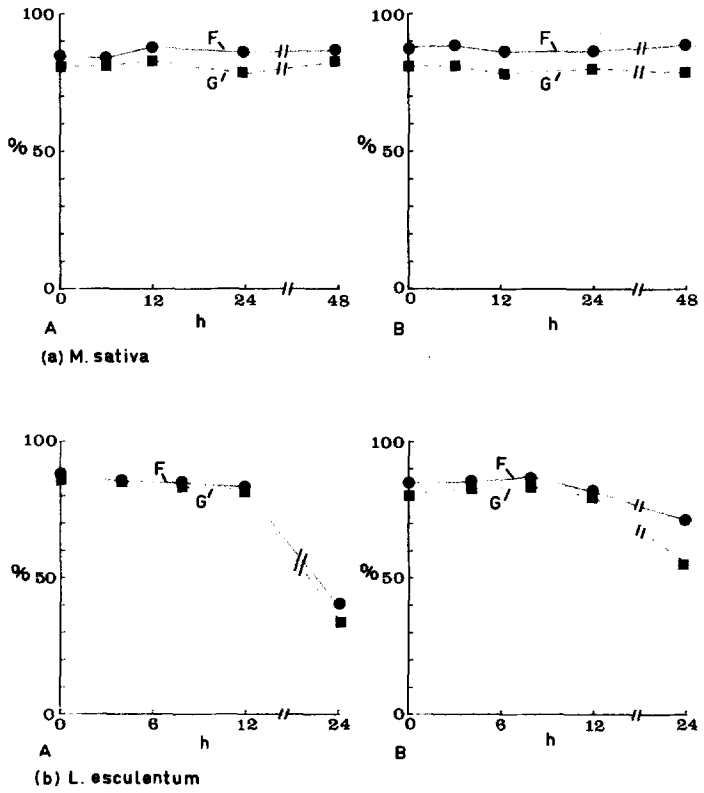


Fig. 6. a Comparison of germinability (*G*) and fluorochromatic reaction (*F*) in pollen samples of *Medicago sativa* exposed to 5–10% RH at 29 °C for the periods shown (*A*) and exposed for the same periods to dehydrating conditions and then rehydrated for 1 h at c. 95% RH at the same temperature (*B*). **b** As in **a**, *Lycopersicum esculentum* pollen, dehydrated at 5–10% RH at 24 °C (*A*), and rehydrated for 30 min at c. 95% RH at the same temperature (*B*)

Discussion

The results reported here with the pollen of *L. longiflorum* help to characterise the FCR reaction further, and illustrate some additional features of the effects of treatments which have a destructive influence on the membranes of the vegetative cell. In the Ca+Su medium the FCR remained at a high level throughout the treatment. This implies that the integrity of the vegetative cells was conserved, a circumstance that would in the normal application of the test be taken to mean a high level of viability and potential germinability. No germination was recorded during the period of exposure to the medium, illustrating the point noted in an earlier paper (Shivanna and Heslop-Harrison 1981) and mentioned in the earlier discussion, namely that a close correlation between FCR and germinability can only be expected when the optimum medium for the species in question has been identified. The Su medium similarly conserved the integrity of the vegetative cell membranes; again the medium is unsatisfactory for germination, but bursting is minimised because it provides an osmotic balance with the pollen, as is shown by the contrast with the water-suspended sample. The NaCl medium induces a still quicker leakage of the fluorescein released intracellularly, evidenced in this instance by the development of a fluorescent halo around individual grains; the effect is reduced to some extent by the incorporation of sugar at a level providing an osmotic balance with the vegetative cell. Buffered DMSO, while also enhancing fluorescein leakage, did so less rapidly. The proportion of grains scorable as positive remained over 70% for the first 10 min of leaching, although, as the light emission frequency distribution of Fig. 2 shows, no grains fluoresced with an intensity higher than grade 2. With continued exposure, the proportion of grains showing measurable fluorescence diminished. Informative controls, not included in the present series of experiments, would have been provided by testing DMSO media with osmotically balancing sucrose, with calcium, and with sodium chloride.

The light emission histograms of Fig. 2 have some implications for the application of the FCR procedure as a practical means for testing pollen quality. In the usual application, scoring would be done visually on a binary scale, pollen grains being classed as "+", fluorescent, and "–" non-fluorescent. A decision has therefore to be made about every grain. However, the distributions are mostly continuous, so the attribution of some grains at the low end of the emission scale may be difficult. In fact, in the case of the large pollen grains of *L. longiflorum* the problem is not severe, although an element of arbitrariness may be involved when the frequency distribution is not as conspiciously bimodal as it is in the Ca+Su populations of Fig. 2. The difficulty is likely to be greater in species with smaller pollen grains, particularly where the exine is autofluorescent and thick in relation to the total diameter of the grain, as for example in some Compositae. Fortunately most crop species, including Gramineae, Solanaceae, Leguminosae and Cruciferae, do not present this problem, and scoring can be carried out readily enough once it is appreciated that the "+" grain, as for example in some Compositae. For the FCR procedure and that a cut-off level has to be established at the low end of the scale and adhered to consistently throughout a recording session.

Comparison of the results in all of the tests shows that the staining procedures provide the poorest guide to germinability. The tendency throughout is for an over-estimation of the potential, false positives being given for immature pollen, heat and DMSO treated pollen, and for pollen rendered temporarily incapable of germination by partial desiccation. The result is wholly predictable from a consideration of the theoretical basis of staining procedures, and the conclusion that these are essentially worthless as predictive indicators for germinability is in conformity with that reached by Janssen and Hermsen (1976) in their study of pollen fertility in *Solanum* species and hybrids.

The results obtained with the tetrazolium procedure in the present series of trials may be taken as indicating a slightly better correlation with germinability, but again the test produced many false positives, and in application to partly desiccated pollen of *P. vulgaris* it proved essentially inoperable. Again there is some agreement with the observations of Janssen and Hermsen (1976); although these authors concluded that the enzymic method used in their tests, oxidation of benzidine, was not useful for their material, their recorded results show that the method did in fact give a somewhat better guide to potential germinability than the staining procedures.

Inspection of the histograms of Figs. 3, 4 and 5 and the curves of Fig. 6 shows that much the best guide to germinability is given by the FCR procedure. In all the trials with mature pollen, the correlation between FCR score and germination in vitro is extremely high. As in several of the earlier trials (Shivanna and Heslop-Harrison 1981) the FCR often – but not invariably – tends to over-estimate germinability by a small margin. However, the principal deviation appears with immature pollen, extracted from the bud before normal anthesis. In *N. pseudonarcissus* and in both morphs of *P. vulgaris* such pollen was incapable of germination, but invariably gave a high FCR score. We have verified this result with the pollen of other families, and consider that the principle it illustrates will be a general one. Our interpretation is that readiness-to-germinate is

achieved during the final ripening of the pollen in the anther, during which period no doubt the optimal amounts of osmoticum in the vegetative cell are attained and the appropriate degree of dehydration brought about (Linskens and Kroh 1967); predictably, the FCR as a test principally of membrane integrity cannot measure these parameters.

It is worth emphasising again that the FCR is not a test for viability. Pollen subject to desiccation may fail both to give a positive FCR or to germinate; yet it may be "viable" in the sense that it is capable with appropriate post-treatment of recovering both FCR score and germinability. The fact is important in the use of the FCR for monitoring pollen in storage; a negative score cannot be taken as an indication that the pollen is inviable, any more than failure to germinate in culture in vitro.

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