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Tetrazolium Chloride as an Indicator of Pine Pollen Germinability*)

By Stanton A. Cook and Robert G. Stanley

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Controlled pollination in forest tree breeding requires pollen of known germination capacity. Methods of determining pollen viability include germination in a hanging drop, in a moist atmosphere, on agar gel, or in a sugar solution (Duffield, 1954; Dillon *et al.*, 1957). Errors commonly arise in the application of these techniques because maximum pollen germination and growth is generally attained only when the grains are aggregated (Holubinski, 1945), or at optimal concentration of the pollen (Beams *et al.*, 1947). Furthermore, fungal infection commonly occurs when pollen is germinated *in vitro* longer than 24 hours. Thus, determination of pollen viability by germination is time-consuming and frequently unreliable.

In seed studies a rapid and simple test of germination capacity has been developed using 2, 3, 5-triphenyltetrazolium chloride (TTC). This colorless dye solution is reduced by dehydrogenase enzymes to an insoluble red formazan complex in living cells (Smith, 1951). Capacity to germinate is assumed to be correlated with the activity of these enzymes. However, contradictory results have been obtained when TTC is used as an assay of pollen viability. It was found to be unreliable in testing cultivated varieties of peach, pear, apple, and grape (Oberle *et al.*, 1953) but to be satisfactory in testing maize pollen (Vieitez, 1952). The dye methylene blue was used successfully on tests of germinability of coniferous and angiospermous pollen (Maurin and Kaurow, 1956). The purpose of the present study was to compare pine pollen viability as determined with TTC, with that obtained by germination on agar; also, to evaluate the potential use and value of TTC in tree breeding programs.

Materials and Methods

Crystalline 2, 3, 5-triphenyltetrazolium chloride, from Nutritional Biochemical Corporation, was prepared as a 0.5 percent solution with deionized water. Tetracycline hydrochloride and nystatin, sold as Mysteclin by E. R. Squibb and Sons, was made up in a sterile 4 percent solution. Both solutions were stored at 5° C. until used. A 1.5 percent solution of Difco Bacto-agar was prepared with deionized water and sterilized by autoclaving.

Pollen samples were selected from collections of the Institute of Forest Genetics, U. S. Forest Service, Placerville, California. These samples had been collected in 1957 and stored under standard procedures (Duffield, 1954) for 3 months.

The following procedure, modified from that developed in studies on maize pollen (Vieitez, 1952), was used to stain the pollen with TTC:

(1) Two lakes of two or three drops of 0.5 percent TTC solution were placed on a microscope slide.

(2) Sufficient pollen was sprinkled on these lakes to just cover the surfaces, (i. e., about one milligram of pollen per lake).

(3) A cover glass was placed over each lake, care being taken to exclude most air bubbles.

(4) The slide was then placed in a petri dish on glass rods over water.

(5) The petri dish was covered and placed in the dark in an oven at 55° C.

(6) After 90 minutes the slide was removed and wiped free of moisture.

Slides were examined with binocular microscope at × 100 magnification, using blue filtered reflected light to provide contrast. The two lakes were replicates of each sample. The mechanical stage was moved randomly, and the pollen grains in a predetermined sector of the field were counted to a total of 25. The stage was again moved at random and 25 more grains counted. Two hundred grains were counted in each lake, giving a total of 400 from each sample.

The pollen which was considered to be stained ranged in intensity from 5.0 R (ed) y/12 to 10.0 R (ed) 8/4 according to the Munsell *Book of Color* (1946).

Testing by Germination

The following procedure was employed to germinate pollen:

(1) (About 2 cc.) of agar solution was poured into a 5 cm. petri dish to uniformly cover the bottom.

(2) Three or four drops of Mysteclin solution were placed on the agar surface and evenly spread by rotating the dish.

(3) Approximately 3 mg. of pollen were distributed over the surface of the agar, care being taken to prevent the movement of the grains to the margins of the dish.

(4) The petri dish was then placed in an incubator at 29° C.

(5) Three additional drops of Mysteclin solution were added after 24 and 48 hours.

(6) After a minimum of 72 hours the petri dish was removed from the incubator, and the pollen samples were removed and examined.

Two loops of pollen from different places on the agar were removed and each was placed in a separate lake on the same slide. Thus, replicate samples were obtained which were similar to those used in the staining procedure. The same microscope and counting procedure was used as with TTC, except that white light was used instead of reflected light. When the pollen tube exceeded the maximum width of the grain it was considered to be germinated. No attempt was made to measure tube length.

Results

Inherent limitations of these procedures must first be recognized and evaluated if the results are to be meaningful. Probably the most difficult problems in the TTC assay are avoiding manipulative procedures which decrease coloration and deciding when pollen grains are actually stained.

The intensity of pollen staining was observed to decrease toward the margin of the cover slip and in regions of air

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bubbles. *Table 1* summarizes observations indicating that decreasing available air increases coloration of the pollen. These results are in agreement with those of OBERLE and WATSON (1953) who showed that pollen would not stain unless a cover slip was used, or the cover slip was ringed with vaseline. GONSE and YOTSUYANAGI (1955), FRIED and ZWEIBACH (1955) have shown that simple agitation of pollen in the dye solution also suppressed coloration. These results all indicate that a possible reason for pollen failing to stain a uniform red may be the inhibition of reduction of the TTC by air. To minimize this source of error in testing pollen viability with TTC, only grains in masses remote from faded, high oxygen areas, should be counted.

Table 1. — Effect of exposure to air on staining of *Pinus ponderosa* pollen with 2, 3, 5-triphenyl tetrazolium chloride

No.	Treatment	Observation
1.	On slide under cover slip in air	Center of mass red, edges colorless to pink
2.	In open watch glass in air	Slightly red
3.	In tube with air bubbled through	Colorless

In some cases the color was uniformly distributed over the grain, exclusive of the wings, in others it was concentrated in globules. One possible explanation for this phenomenon may be that the TTC is absorbed by the lipioidal components of the cell. BROWN (1954) reported that TTC is strongly absorbed by lipids; also, ungerminated pine pollen is reported to contain up to 14 percent fat (ELSER and GANZMÜLLER, 1931) In the present tests this aggregation of color appears to increase with time of exposure to heat. Maximum staining was reached after approximately 90 minutes at 55° C. *(Table 2).* Very little staining occurred in the first 50 minutes. The greatest increase in staining occurred after 60—75 minutes.

Table 2. — Effect of time on staining of *Pinus ponderosa* pollen with 2, 3, 5-triphenyl tetrazolium chloride at 55° C.

Pollen	Time (hours)	Percent $\bar{x}$	S. D.
A	1.5	79.6	± 2.3
A	2.0	75.1	± 3.9
A	3.7	77.6	± 5.3
B	1.5	75.5	± 5.5
B	2.0	77.8	± 5.9

In the germination tests fungal contamination was almost always present in varying degrees. Past experience using ultraviolet light as a fungicide indicated that any fungicidal pretreatment of the pollen would probably affect the viability of the pollen more than the fungus. Hence Mysteclin, a combination fungistat-bacteriostat, was used in very low concentrations. Fungi and bacteria were checked although not completely inhibited. High concentrations were avoided because of the possible affect on pollen germination.

TTC viability assays of pine pollen are compared with germination in *Table 3.* Since each sample represents pollen from a different tree, the procedure usually followed in collecting pollen, it is not justifiable to combine the data for all *Pinus ponderosa* pollen, i. e., samples 1 to 6. In evaluating this data the lack of replicates within each sample must be recognized. Germination diferences from tree to tree may frequently be greater than interspecific differences. Since a large number of grains were counted in each sample, the defect resulting from lack of replicate samples was partially overcome.

Table 3. — Comparison of the number of pollen grains stained by TTC with the number germinating on agar for sixteen samples involving seven pine species

Sample number	Species	No. stained out of 400	No. germinated out of 400	χ^2 [1]
1	*Pinus ponderosa*	305	318	1.23 •
2		288	281	0.30 •
3		79	134	19.36
4		321	321	0.00 •
5		287	254	6.22
6		*276*	*311*	7.84
		$\sum_1^6$ 1,556	1,619	
7	*Pinus sabiniana*	176	311	96.65
8		256	342	48.98
9		302	259	11.03
10		263	350	52.82
11		*330*	*339*	0.74 •
		$\sum_7^{11}$ 1,327	1,601	
12	*Pinus resinosa*	375	382	1.20 •
13	*Pinus rigida*	173	229	15.68
14	*Pinus nigra*	187	170	1.46 •
15	*Pinus luchuensis*	341	140	210.64
16	*Pinus longifolia*	*311*	*307*	0.11 •
		$\sum_1^{16}$ 4,270	4,448	

[1]) x^2 computed according to (SNEDECOR, 1955, p. 198). —
•) Significant at the 5 percent level.

Data in *Table 3* indicate that in 7 of the 16 samples *(P. ponderosa* No. 1, No. 2, and No. 4; *P. sabiniana* No. 11; *P. resinosa, P. nigra,* and *P. longifolia),* the number stained did not differ significantly from the number germinated. In six of the nine others where they differed significantly, the staining test underestimated germinability. Where the number colored exceeded the number germinated, the chi-square test generally showed no significant differences at the 5 percent level. However, where there were significant differences, the chi-square values were relatively low, i. e., 6.22 for *P. ponderosa* No. 5, and 11.03 for *P. sabiniana* No. 9. The anomoly which *P. luchuensis* presents may be the result of our inexperience in germinating pollen of this exotic species.

The totals provide a crude measure of the results. No attempt was made to compare these summations statistically, since as explained above, each sample represents pollen from a different tree collected under different conditions. However, of 6,400 pollen grains grown on agar, 4,448 germinated; of 6,400 grains stained with TTC, 4,270 displayed a red coloration. The 4 percent difference in these methods indicates that fair agreement can result when large samples are utilized.

Discussion

To evaluate the significance and potential use of this rapid technique, an understanding of the object and technique of pollen germination *in vitro* in relation to tree breeding is required.

In laboratory studies a variation of ± 10 percent between replicate 200-grain pine pollen samples is not uncommon. However, usually only 50 or 100 grains are counted in field laboratories. The object of such deter-

minations is merely to be certain a fairly viable pollen is being used, i. e., above 40—50 percent viability, or to select the most viable from two or more available pollen lots. For these general purposes, the simple TTC test is probably entirely adequate if the pollen is fresh and the indicated percent viability is above 50 percent. Where these conditions are not met, then it is probably best to germinate all samples, or at least germinate representative samples.

In the present studies, germination percent was generally greater than the percent obtained by staining. Estimation of germinative capacity by growing pollen is more direct and valid, provided the number tested is representative of the actual sample from which it is taken and optimal germinating conditions are used. By the indirect TTC method, as shown above, if the pollen is of low germinative capacity, there is no way of knowing if the statistic obtained is equal to, more than, or less than the potential maximum. Lack of optimal germination conditions may possibly explain those TTC results which exceeded the germination data. It is conceivable that pollen of *Pinus luchuensis*, for example, may require a nutrient supplement or some special conditions for growth. In this and similar cases, the TTC test could be used to check and improve germination procedures until the best possible results are achieved. Likewise, the lower viability values obtained by TTC could be due simply to faulty use of the reagent. Perhaps different and better testing procedures can be found than those employed here; or another dye such as methylene blue or oxytetrazolium may give more valid results than TTC. An initial report by WORSLEY (1959) indicated that methyl green may be superior to TTC for determining gymnosperm pollen viability. But, another shortcoming of all staining tests is that inferences regarding pollen tube length can not be made.

In pine breeding we usually pollinate by brushing or blowing large quantities of pollen on receptive cones, hoping that the egg cell in the archegonium will be fertilized by a sperm nucleus from one grain. We are unable to measure the ability of pollen to provide sperm which can assure successful fertilization. Thus, an assay as crude as the TTC color reaction of fresh pollen may in most instances provide as valid an index of successful capacity to set seed as the more laborious germination procedures presently used.

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