Use of Tetrazolium Salts in Determining Viability of Sugarbeet Pollen[1, 2]

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The increased use of stored pollen and cytoplasmic male sterility in sugar beet breeding necessitates determining the viability of pollen at the time of its use or at anther dehiscence. Stains commonly used for staining sugar beet pollen, such as acetocarmine or iodine, are not vital stains and hence do not differentiate viable mature pollen from mature pollen which has lost its viability. A stain specific for living mature pollen would be useful to persons working with stored pollen or pollen treated in any possibly lethal manner. Such a stain would also be of value in the classification of plants with different degrees of male sterility.

Tetrazolium salts, which are reduced to insoluble colored products (monoformazans or diformazans by action of dehydrogenase enzymes linked to respiratory processes, seem to offer possibilities for this purpose. The development of 2,3,5-triphenyl tetrazolium chloride (TTC) and its application to biology has been reviewed by Smith (8)[4]. According to Smith this chemical was first prepared by H. von Pechmann and P. Runge in 1894 and was found by R. Kuhn and D. Jerchel in 1941 to cause a red coloration in cells of yeast, bacteria and water cress. Lakon (2,3) in 1942 found it possible to determine germination percentage of cereal grains and corn by treating exposed embryos with TTC. He found that the percentage of those embryos stained red was not different from the percentage which germinated in a standard germination test. However, MacLeod (4) found that under a narrow range of conditions of grain moisture and temperature, TTC results grossly overestimated germination. This overestimation was due to the fact that seed germination was more sensitive to heat damage than was enzymatic activity.

TTC has been used in various tests on many types of tissues. According to Porter, Durrell, and Romm (7), the salt is an

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[4] Numbers in parentheses refer to literature cited.

oxidation-reduction indicator, and the development of the non-diffusible red color in a specific tissue is in general indicative of the presence of active respiratory processes.

Vieitez (9) in 1952 reported that a 2% TTC solution at 50°C provided a quick and reliable index of viability of maize pollen. However, Oberle and Watson (5) in 1953 reported that TTC stained to varying degrees certain fruit pollens known to be nonviable and concluded that the chemical was of no value as an indicator of germinability for peach, pear, apple, and grape pollens.

Other tetrazolium salts and derivatives have been formulated. Some of these have been found to be of value in the localization and quantitative measure of certain reducing enzymes. Pearse (6) found 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride to be rapidly reduced to a red monoformazan under aerobic conditions. He further found 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide to be rapidly reduced to a blue or purple diformazan.

The present study was conducted to determine whether certain of these newer tetrazolium salts are of value in determining the viability of sugarbeet pollen.

Materials and Methods

A series of eight tetrazolium salts were tested for their vital staining capacity of beet pollen. The eight salts were as follows:

1. 2,3,5-triphenyl tetrazolium chloride
2. tetrazolium blue
3. tetrazolium violet
4. tetrazolium red
5. nitro-blue tetrazolium
6. neotetrazolium chloride
7. 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
8. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide

Various concentrations of the salts were dissolved in distilled water. Salts 1 and 4 were readily soluble in cold water, 7 and 8 were soluble upon heating nearly to the boiling point and 2, 3, 5, and 6 were soluble upon being brought to the boiling point.

The salts were tested at the following four concentrations and three temperatures: 0.2, 0.5, 1 and 2% each at 20°. 35° and 50°C. The pollen was examined at intervals of 3, 5, 10, 15, 20, 30 and 40 minutes. Preliminary tests were made on pollen from

the stock beet A60-3 as pollen was most abundant on this population at the time of the study. All salts were first tested for vital staining capacity. Those salts which exhibited a vital staining ability were then used in determining the most effective concentration and temperature. The sugar beet populations used in these tests were 52-430 (inbred), 52-307 (inbred), 52-305CMS (cytoplasmic male-sterile inbred), A60-3 (stock beet), and 52-305CMS × A60-3. The population 52-305CMS × A60-3 was segregating for male-sterile, semisterile and fertile types. The semisterile plants were those with yellow shrunken anthers.

The plants used in this study were grown in the greenhouse during the winter of 1961-62. All pollen from the fertile plants was collected about 9 AM from anthers which had just dehisced. Anthers from newly-opened flowers of sterile and semisterile plants were used. Nonviable pollen from four sources was also tested using the vital staining salts at their most effective concentrations and temperatures. One source of nonviable pollen had been collected and stored frozen without humidity control for about 2½ years. This pollen had been previously determined to be ineffective for fertilization of cytoplasmic male-sterile plants. The other nonviable pollen sources were fresh pollen killed in 70% ethanol, fresh pollen heat-killed in an electric oven held at 80°C for 15 minutes and fresh pollen heat-killed by holding it at 110°C for 15 minutes.

The germinability of all pollen was tested on an agar-sucrose culture medium as described by Artschwager and Starrett (1). This medium contained 1.5% agar and 40% sucrose. The incubation period was 7 hours at 32°C.

It was found most convenient to drop the tetrazolium solution on the pollen grains on a glass microscope slide, mix slightly, and cover with a glass cover slip. The slides were then set aside in daylight until examined.

Results and Discussion

Four of the eight tetrazolium salts, 1,4,7 and 8, acted as vital stains on fresh pollen. The staining action of salts 1 and 4 was similar as was 7 and 8 except for their resulting colors. The deepest staining and most rapid reaction in salts 1 and 4 took place in 2% solution at 20°C. Most morphologically mature pollen grains were stained pink to deep red in 25 minutes. Salts 7 and 8 were most effective in a 0.5% solution at 35°C. After 5 minutes most morphologically mature pollen were stained pink to red by salt 7 and purple to deep purple by salt 8. A 2% solution of salts 7 and 8 did not stain. In general the staining by all salts was

more rapid as the temperature increased except at the 2% concentration where the threshold of activity was evidently exceeded. None stained in this concentration at 50°C. The reaction in salts 1 and 4 at all concentrations and temperatures was rather slow and not completely positive; light-pink and nonstained pollen were hard to distinguish. The reaction in salts 7 and 8, particularly 8, was more rapid and much more positive.

Morphologically mature pollen grains ruptured in solutions of salts 1, 4 and 8. Rapidity of the rupture increased with concentration and temperature. This rupture might be primarily due to the low osmotic concentration of the solution. The staining reaction was complete before any cell rupture occurred at any concentration of salt 8. In salts 1 and 4, however, rupture often occurred in unstained or only slightly stained cells. Cell rupture was not noted in salt 7, however, minute insoluble particles in the solution interfered with the observations. The staining reaction in salt 8 was the most positive followed in order by salts 7, 1 and 4.

The reaction in each salt was the same in all pollen-fertile populations tested. One of the semisterile plants produced about 9% morphologically mature pollen, which stained in the same manner as pollen from the pollen-fertile plants. The abortive pollen did not stain. All pollen from the cytoplasmic male-sterile plants and from all but one of the semisterile plants was abortive in appearance and was not stained in any of the solutions. It will be noted in Table 1 that the nonstaining portion of the fresh pollen from fertile plants ranged from 16.8 to 38.9%. This nonstaining portion consisted primarily of cells which had apparently aborted at an early stage of development.

These same pollen sources were tested for germinability on an agar-sucrose medium. After 7 hours of incubation the pollen tubes of the germinated pollen grains were up to 200 microns in length. The percentage germination varied somewhat with populations but even that of A60-3 was only 13.9. Low germination might be expected because in reviewing this subject Artschwager and Starrett (1) stated that N. Favorsky in 1928 had obtained poor germination of sugar beet pollen, not more than 30%. In their own studies they got pollen to germinate easily and abundantly, but they did not report actual percentages. Work summarized by Artschwager and Starrett (1) and this study indicate that there are additional unexplained factors affecting the germinability of sugarbeet pollen on the artificial medium used.

Optimum conditions for germination have not been accurately determined. Hence, the percentage germination of pollen on the culture medium is not likely to be a good direct measure of pollen viability.

The pollen known to be nonviable was tested in salts 1, 4, 7 and 8 at concentrations and temperatures which produced the most favorable reaction with fresh pollen. Salts 1 and 4 each caused a light-pink color in the 2½ year-old pollen, particularly in pollen grains near the periphery of the cover slip, while salt 7 stained red about 1% of the pollen which had been heat-killed at 80°C for 15 minutes. Salt 8 did not stain any of the types of nonviable pollen. None of the pollen germinated when incubated on agar-sucrose medium.

Since salt 8 was the only solution which resulted in no staining of pollen known to be nonviable, it was tested further on pollen exposed at room temperature for 3 and 8 hours.

According to Artschwager and Starrett (1) viability of pollen under Colorado field conditions does not extend beyond a day. They further reported that it often loses its viability in less than 3 hours when stored in a shallow glass dish in daylight at room temperature.

The results for pollen given such treatments as compared with fresh pollen are summarized in table 1 for salt 8.

Exposure of pollen in daylight at room temperature apparently reduced its viability drastically. This is reflected in germination and staining percentages. Pollen neither germinated nor stained after exposure for 8 hours. There would appear to be a relation between germination and stainability.

Although pollen of A60-3 which had been frozen failed to germinate it is doubtful that this pollen was completely nonviable since sugarbeet pollen has remained viable for at least 4 months when stored at low temperatures[5]. Vieitez (9) reported that maize pollen was not stained by TTC after it had been cooled to 0°C and he referred to this as an "enzyme inhibitor treatment". Pollen storage studies indicate, however, that this would not be a permanent enzyme inhibitor treatment in sugarbeets.

Pollen in solutions of salts 1 and 4 was not stained when not covered by a cover slip. Pollen in salts 7 and 8, however, was stained whether covered or uncovered.

[5] Unpublished data of LeRoy Powers and J. W. Dudley in 1958 Sugar Beet Research Report, Sugar Beet Section, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture.

When the slides were prepared and immediately placed in darkness no staining of pollen was noted in salts 1 and 4. Salts 7 and 8 stained equally well in light or darkness.

After 2 days, solutions of salts 1 and 4 had lost most of their staining capacity. This change cannot be explained by a difference in pollen but could possibly have resulted from a pH change of the solution (not investigated). Salts 7 and 8 maintained their staining ability even after being in solution for 28 days. A slight black precipitate that appeared in salt 8 did not alter its effect.

When germinated pollen was placed in a solution of salt 8 the percentage stained was only slightly less than that recorded in Table 1 for fresh pollen. Hence, many pollen grains were stained but ungerminated. Among the germinated pollen grains the cytoplasm of both the pollen cell and tube was stained. Rarely were there individual pollen grains which had germinated but did not stain.

Table 1.—Germination of sugar beet pollen and development of purple color in a 0.5 percent solution of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide.

Population and treatment[1]	Germination (percent)	Purple color (percent)
52-307:		
None (fresh pollen)	9.1	64.3
Exposure 3 hours	0.0	0.0
Exposure 8 hours	0.0	0.0
52-430:		
None (fresh pollen)	12.3	61.1
Exposure 3 hours	0.1	6.8
Exposure 8 hours	0.0	0.0
A60-3		
None (fresh pollen)	13.9	83.2
Exposure 3 hours	0.2	7.5
Exposure 8 hours	0.0	0.0
Freezing 96 hours	0.0	20.6

[1] Fresh pollen was stained or incubated immediately after collection. Exposed pollen was stored in the collection dish in daylight at room temperature for the specified period. Frozen pollen was stored in a tightly corked container at –30°C for 96 hours without humidity control.

Under the conditions of the tests, salt 8 was the only one which did not stain known nonviable pollen. In addition this salt was the most rapid and positive in its staining action. It stained most rapidly when used in a 0.5% solution at 35°C. But since the reaction is rapid, leading to considerable cell rupture after 15 minutes, it is more convenient to use a 0.5% solution at about 20°C, which leads to a reaction equally as effective but slightly less rapid. This allows greater latitude in the period of examination, which is made most easily after 5 to 20 minutes.

After 30 minutes at 20°C, considerable cell rupture occurs accompanied by draining of the cytoplasm. The empty cells are somewhat difficult to discern from nonstained pollen cells.

This study indicates that 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide is useful as an indicator of pollen viability in sugar beets. However, there remains the possibility that a narrow range of conditions may exist in which pollen germinability is inhibited but enzymatic activity continues. If this were to occur it could lead to an erroneous conclusion using salt 8 as an indicator. Under the limited set of conditions in this study this possibility was not detected.

Summary

Studies were conducted in an attempt to find a tetrazolium salt which would rapidly and accurately determine the viability of mature sugar beet pollen.

Eight tetrazolium salts were tested for their staining capacity at concentrations of 0.2, 0.5, 1 and 2% and at temperatures of 20°, 35°, and 50°C. Positive results were obtained with four of the salts. Of these four the most positive and effective was 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide at a concentration of 0.5% at 20°C.

The percentage of pollen grains stained by this compound was related to the percentage germinated on artificial medium but was in all cases greater. It is believed, however, that all viable pollen was not germinated on the artificial medium.

The mature pollen grains assumed to be viable were stained an easily distinguishable purple to deep-purple color. Nonviable mature pollen and abortive pollen from cytoplasmic male sterile plants was not stained.

The results obtained indicate that a 0.5% solution of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide at 20°C provides a specific and rapid means of determining the viability of mature sugarbeet pollen.

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