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Evaluation of Seven Tetrazolium Salts as Vital Pollen Stains in Cotton *Gossypium hirsutum L.*[1]

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CYTOGENETIC studies involving translocations are aided by a simple technique of differentiating normal and semisterile or sterile plants. Routine cytological studies, though very conclusive, are time consuming and only a limited population of plants can be handled. In corn and some other crops it is easy to distinguish between normal and semisterile plants by the use of nonvital pollen stains but in cotton, pollen viability is not reliably estimated by the use of iodine or other nonvital stains. Tetrazolium salts offered a promise of success since their utility in seed germination tests in several crops had been demonstrated. Sarvella and Johnson (6) reported the success of two tetrazolium salts as vital stains for cotton pollen but these findings were not supported by the results of Tiranti.[3] Therefore, the present study was undertaken to evaluate several tetrazolium salts at differing concentrations and for differing periods of staining in an attempt to find a suitable vital stain that would give a reliable measure of pollen viability in cotton.

The development of 2,3,5-triphenyl tetrazolium chloride (TTC) and its application to biology has been reviewed by Smith (7) and by Roberts (5). The basis of the reaction is reduction of the soluble colorless triphenyl tetrazolium salt to the insoluble red formazan, which in turn gives red or deep purple color in living tissues. Viability is measured in terms of reducing activity or red coloration.

Mattson et al. (3) reported the potentialities of tetrazolium salts as a test reagent for living materials such as the fleshy parts of apples, oranges, and grapes, the gills of mushrooms, carrot roots, and stigmas and ovaries of certain pollinated flowers. Vietez (8) reported that a 2% TTC solution at 50° C. provided a quick and reliable index of viability of maize pollen. Sarvella and Johnson (6) reported success with TTC in testing cotton pollen for sterility, although they observed some light colored pollen grains in their tests. Hecker (1) also obtained positive results with 3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide in a concentration of 0.5% at 20° C. in testing viability of sugarbeet pollen. However, Oberle and Watson (4) found that TTC stained to a varying degree certain fruit pollen known to be nonviable and concluded that the chemical was of no value as an indicator of pollen viability in peaches, pears, apples, and grapes. Tiranti[3] also could not find any relation between the staining reaction of TTC and sterility in cotton plants heterozygous for translocations.

MATERIALS AND METHODS

It was intended initially to test the following salts for their vital staining capacity of cotton pollen: 2-3,5 triphenyl tetrazolium chloride (TTC); tetrazolium red, potassium tellurite, tetrazolium blue, tetrazolium violet, neotetrazoleum chloride, and 2p iodophenyl-3p Nitrophenyl-5-phenyltetrazolium chloride-2,3,5 triphenyl tetrazolium chloride. Tetrazolium red and potassium tellurite were found to be soluble in cold distilled water. The rest of the salts were soluble in water only when brought to the boiling point. Only TTC and tetrazolium red exhibited satisfactory staining capacity and good differentiation of stained and unstained pollen. Tests were continued with these two and the others were discarded.

The different concentrations of TTC used were 1%, 2%, 4%, 6%, and 8% in 60% sucrose solution. Cotton pollen ruptured in all concentrations of sucrose below 50%. The stocks used were inbred plants of Upland cotton, *Gossypium hirsutum* L., and a heterozygous translocation (Z2588–1515–1040TT × Z2886) grown in the greenhouse during winter 1962–63.

The staining procedure found to be satisfactory was evaluated on backcross progenies of the heterozygous translocation grown in the summer of 1963 at the Agronomy Farm. A 2% concentration of TTC in a 60% sucrose solution was used for staining and the slides were prepared at different times during the flowering period from June 6 to August 8.

A drop of the solution was placed on a glass slide and pollen from freshly picked flowers were shaken into the drop by tapping with a needle. Anthers were shaken to insure that a relative amount of sticky or immature pollen would be included in the drop. Excess pollen on the slide was wiped away. Pollen grains in the drop were agitated with a needle for 30 seconds to get uniform immersion in the stain. Care was taken to avoid air bubbles in the drop especially when placing the cover slip on the slide. Preparations were then stored at room temperature away from direct sunlight. Pollen counts were made from 2 to 24 hours after staining.

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[3] Tiranti, I. N. A cytogenetic study of *G. arboreum* L., *G. hirsutum* L. ring of six chromosomes and some plant characters, isolated from these species. M.S. Thesis, Texas A&M University, 1963.

RESULTS AND DISCUSSION

To determine the ideal concentration, a test was run on all concentrations and pollen counts were made after 18 hours of staining. The results are given in Table 1.

There was a marked difference in the staining efficiency of solutions with different concentrations. The 4% solution showed comparatively better staining and better differentiation of colorless pollen grains from Upland cotton and the heterozygous translocation than other concentrations. There were also fewer light-colored pollen grains both from Upland and translocation in 4% TTC as compared to other concentrations. The translocation stock was markedly different from inbred Upland stock in total nonviable pollen grains.

Since the 8% TTC solution was observed to be less reliable than the other concentrations, this concentration was dropped and 1% solution was added in the following tests to find the lowest concentration of the chemical for practical use. The results are given in Table 2.

The 2% and 4% TTC were almost equally efficient in extent and uniformity of staining and much better than 1% and 6% TTC. Even after 22 hours, there were many light-colored grains in the latter concentrations and there was such a great variation in color development that classification of pollen grains into different classes was difficult. The 2% and 4% TTC solutions were observed to be promising and were continued in further studies.

To determine how soon after staining reliable differentiation could be observed, pollen counts were made on the same slides at different intervals. The results are given in Table 3.

Color development starts after about a half hour of staining; however, uniformity of its development varies in different concentrations of stain. Even after 2½ hours, the color development was not uniform in any treatment.

Table 1. Pollen color development after 18 hours of staining in different concentrations of TTC.

Treatment	Percent pollen grains (Average of 3 counts)					
	Upland cotton			Heterozygous translocation		
	Red colored	Light colored	Colorless	Red colored	Light colored	Colorless
2% TTC	95.13	3.18	1.69	82.33	8.47	9.30
4% TTC	98.87	0.16	0.96	92.90	1.04	6.05
4% TTC Red	97.06	1.62	1.32	91.27	1.25	7.48
6% TTC	94.22	3.55	2.23	91.75	--	8.24
6% TTC Red	94.58	4.57	0.85	76.15	18.15	5.09
8% TTC	98.75	0.62	0.62	69.91	18.64	11.44

Table 2. Pollen color development from Upland cotton after 22 hours of staining in different concentrations of TTC and TTC Red.

Treatment	Red colored, %	Light colored, %	Colorless, %
1 % TTC	93.90*	1.35	4.74
2 % TTC	97.03	1.27	1.70
4 % TTC	97.14	1.59	1.27
6 % TTC Red	95.32*	2.80	1.87

* Color development not uniform; many medium to light colored.

Table 4. Frequency of cytologically analyzed plants in different nonviable pollen classes.

Cytological analysis	Percent nonviable pollen				Total plants	Mean
	0-4	4-5	5-6	6-50		
24II + IV	0	2	2	60	64	13.796±7.584
26II	6	9	4	0	19	4.131±1.259

After 5 to 6 hours, the color in pollen grains had developed to a stage that semisterile plants could be differentiated, especially those plants which had high sterility. The same slides after 23 hours showed a uniform decrease in the light-colored class and good differentiation of nonviable pollen grains. Flowers picked at the end of the growing season and those checked for pollen viability 4 to 5 hours after picking showed an increasing amount of nonviability.

In the backcross progenies of the heterozygous translocation stock, 83 plants were analyzed cytologically, and the pollen grains from the same plants were tested for viability with a 2% solution of TTC in 60% sucrose. The data are given in Table 4.

The mean pollen viability of normal and heterozygous plants was significantly different. Pollen abortion in heterozygous plants varied between 4.348% and 48.286% with a mean of 13.769±7.584. In normal 26II plants the range was between 0.5% to 6% with a mean of 4.131±1.259. It was difficult to differentiate normal plants with 26II of chromosomes and showing 4 to 6% colorless pollen from semisterile plants with high pollen viability.

These studies agree with those of Sarvella and Johnson (6) in that 2 to 4% solutions of TTC or TTC Red in 60% sucrose solution can be satisfactorily used to test the viability of pollen. The presence of light-colored pollen grains, quite distinct from colorless, is unexplicable for the present, unless actual germination tests of such pollen are carried out. However, for practical purposes it might be useful to study the slides after 24 hours of staining rather than after 6 hours, because the longer the slides are kept the greater is the uniformity in color development. No deterioration in slides was observed after 24 hours. Tiranti[3] used 50% sucrose solution of TTC in his studies. Low concentration of sucrose might be responsible for the negative results he obtained, since the majority of pollen grains were observed to rupture in 50% sucrose solution in the present study.

It was observed that the pollen had a tendency to move towards the edges of the coverslip, when it was lowered. It might be worthwhile to try TTC with glycerol jelly as suggested by Marks (2) to avoid this discrepancy.

SUMMARY

Studies were conducted to find a tetrazolium salt which would rapidly and accurately determine the viability of mature cotton pollen, to differentiate between normal and heterozygous translocation stocks.

Table 3. Pollen color development at different intervals in various concentrations of TTC and TTC Red.

Treatment	Percent of pollen grains in different classes (Average of 2 counts)											
	Upland (Gossypium hirsutum)						Heterozygous translocation					
	1½ hr. after staining			5-6 hr. after staining			1½ hr. after staining			5-6 hr. after staining		
	Red colored	Light colored	Colorless	Red colored	Light colored	Colorless	Red colored	Light colored	Colorless	Red colored	Light colored	Colorless
2% TTC	55.27	43.72	1.01	99.31	0.17	0.52	89.33	5.11	5.56	95.03	0.68	4.29
2% TTC Red	55.49	42.89	1.62	97.70	0.31	1.99	88.97	3.26	7.77	90.18	0.59	9.23
4% TTC	94.06	4.20	1.74	97.79	0.17	2.04	81.98	10.95	7.07	90.79	0.64	8.57
4% TTC Red	91.01	4.96	4.03	96.34	0.84	2.82	80.51	8.63	10.86	84.13	1.50	14.37

Positive results were obtained with the use of 2% and 4% solutions of tetrazolium chloride (TTC) or tetrazolium red in 60% sucrose solution, at room temperature. The normal pollen grains start staining red to deep purple in about 1 hour and a rough estimate of pollen viability can be made after about 6 hours of staining; however, for reliable results, it is best to wait until a uniform color is obtained. Semisterile translocation plants with high pollen viability (93–96%) may be difficult to distinguish from normal on the basis of pollen counts alone. Such plants should be studied cytologically.

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