
I—The Use of Triphenyl Tetrazolium Bromide in Viability Tests of Rice Seed

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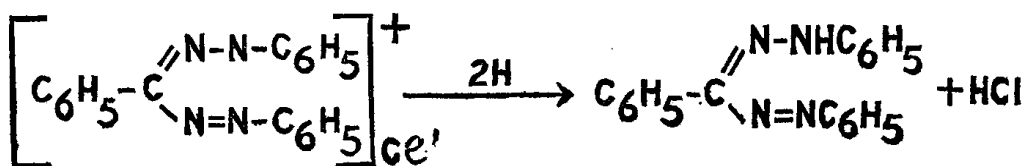
ESTIMATES of viability by germination tests, apart from taking 6-10 days and requiring elaborate equipment, are possible only with non-dormant seed. A simple and rapid method of determining viability, applicable even to dormant seed, by the use of Triphenyl tetrazolium bromide is described in this paper.

Many chemicals have been tried by various workers as seed germination indicators with varying success. A

topographical test of viable tissue in cereals using Sodium biselenite was originated by Lakon (1939). The toxicity of this reagent compelled him to reject it. Kuhn and Jerchel (1941) demonstrated that tetrazolium salts stained germinating seeds, fermenting yeasts, &c. Lakon (1942 *a*) proposed a method using 2, 3,—diphenyl—5—methyl tetrazolium chloride or 2, 3, 5,—triphenyl tetrazolium chloride. This method was based on the fact that on the gradual dying-off of the embryo,

the topographical spread of necrosis could be traced by the failure of dead tissue to stain. It was demonstrated

that this compound, which is colourless, was reduced by living cells into stable but non-diffusible red formazan.



Triphenyl tetrazolium
chloride (Colourless)

Triphenyl formazan (Red)

Cottrell (1948) found it possible to evaluate percentage germination rapidly by the use of tetrazolium salt. Porter, Durrel and Romm (1947) found that staining with 2, 3, 5-triphenyl tetrazolium chloride showed close agreement with the results of germination tests in some crops. Lakon (1949) summarizes his earlier work, leading to the prediction of the germinability of freshly harvested cereal seed by the use of tetrazolium. The work of Venkataratnam (1951) on rice is of special interest because of its relation to the investigations reported in this paper. He found that in rice and millets there was high correlation between the actual germination and the tetrazolium stain, and that the test was fairly reliable for monocotyledonous seeds.

scutellum, which would explain discrepancies with actual germination and growth, were not possible in this preliminary investigation. For the purposes of the present work, a seed in which at least the entire plumule and radicle, half the scutellum and the region between the plumule and the radicle stains is considered viable. (Vide Fig. 1a and b).

Materials and Methods

"Grodex" a proprietary brand of Triphenyl Tetrazolium Bromide was used in the investigation which forms the subject of this paper. The procedure adopted was similar to that advocated for maize by Lakon (1942 b). A detailed examination of the stained embryo was not made, and as such finer distinctions based on the distribution of stain in different parts of the embryo and the

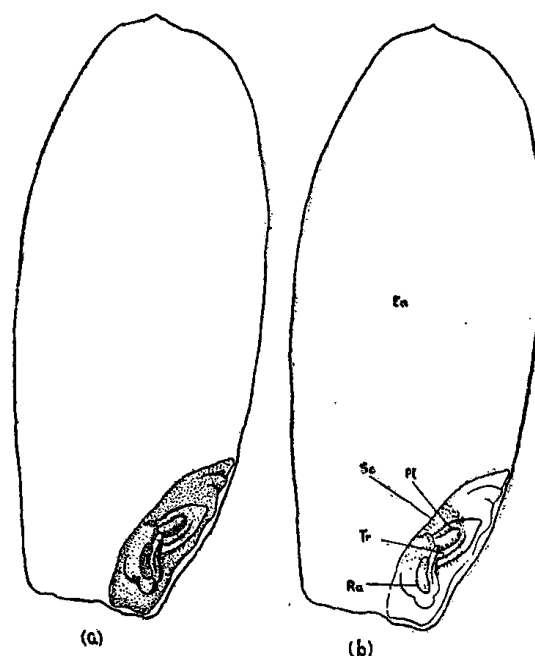


FIG. 1.—Germinable seed is taken to be that in which the stippled area is stained. (Median Longitudinal Section). En=Endosperm, Sc=Scutellum, Pl=Plumule, Ra=Radicle, Tr=Transition region between plumule and radicle.

Triphenyl tetrazolium bromide has not shown any toxic properties. It is water soluble and colourless in solution but forms a red stain on the cut surface of living embryo tissue. Tetrazolium salt solution is light sensitive and so exposure for any considerable time, even to diffused sunlight, was avoided. It was found easier to dissolve the salt in water at 50°C. The seed to be tested were dehusked, soaked in water, and then cut longitudinally, bisecting the embryo medianally. One half of each seed was placed in a Petri dish and the tetrazolium salt in solution was poured over, until the seeds were covered completely. The dishes with the seeds, were then placed in the dark and after a lapse of time a count of stained seeds made.

Primarily it was necessary to determine the optimum method of application. Different periods of pre-

soaking the dehusked seeds in water (25°C) were tested. Periods less than 4-6 hours were found insufficient for softening the seed for uniform bisection. A pre-soaking period of 16 hours, commencing at about 4 p.m. was adopted as a most convenient working arrangement. Investigations of the suitable concentrations of the reagent and the staining periods were made.

Investigations of Technique

Seed of the pureline *Sulai* 301, of 3½ months duration, which was in storage for over 1½ months and in which its short dormancy period had fully expired, was used in this experiment. The actual germination percentage of the seed was found to be 98.3. The dehusked seeds were pre-soaked over-night in water (25°C) for 16 hours and the bisected seeds placed in Petri dishes, with the cut surfaces facing upwards.

Treatments —

(1) Concentrations of Tetrazolium solution	..	(a) = 0·1 per cent.
		(b) = 0·5 per cent.
		(c) = 1·0 per cent.
(2) Staining periods	..	(a) = 15 minutes
		(b) = 30 minutes
		(c) = 60 minutes
		(d) = 120 minutes
		(e) = 240 minutes

There were three replicates of the 15 treatment combinations.

Results—The analysis of variance of data transformed to the inverse sine scale appropriate to a binomial distribution is given in Table 1. Treatment means are summarized at the foot of the Table.

Table 1.—Analysis of Variance of Transformed Data

$$\theta = \text{Sin}^{-1} \sqrt{p}$$

		Degrees of freedom	Sum of Squares	Mean Square	Variance Ratio	F value at 0.1 per cent. point
Treatments	..	14	47,476.33	3,391.17	..	
Concentrations	{	2	4,589.31	2,294.66	..126.15***	.. 8.93
Staining periods	{	4	38,270.54	9,567.64	..525.98***	.. 6.25
Interaction	{	8	4,616.48	577.06	.. 31.72***	.. 4.69
Replicates	..	2	14.50	..		
Error	..	28	509.42	18.19	..	
Total	..	44	48,000.25			

*** Significant at 0.1 per cent. point.

Individual Means in Degrees

(Subscript denotes Mean Percentage)

Staining Periods Minutes	Concentrations			Means	Standard Error	Sig. Diff. at 5 per cent.
	0.1%	0.5%	1.0%			
15	.. 1.9 (0.3)	.. 3.8 (0.7)	.. 10.0 (3.3)	.. 5.2		
30	.. 12.6 (5.0)	.. 54.0 (65.3)	.. 68.5 (85.7)	.. 45.0		
60	.. 34.9 (33.0)	.. 76.5 (93.7)	.. 81.9 (98.0)	.. 64.4	.. ± 1.42	.. 4.12
120	.. 76.2 (94.3)	.. 82.9 (98.3)	.. 82.7 (98.3)	.. 80.6		
240	.. 85.4 (99.0)	.. 86.2 (99.3)	.. 85.4 (99.0)	.. 85.7		
Means	.. 42.2	.. 60.7	.. 65.7	..		
Standard error	.. ±	1.10				
Significant difference at 5 per cent.		3.20				

The two main treatments and their interaction attained significance at 0.1 per cent. point. All concentrations and staining periods differed significantly from each other. The regressions of staining percentage on concentration and period were marked.

It is evident from these results that 1.0 per cent. concentration with a staining period of 60 minutes is the most satisfactory, if time is an important consideration. The lowest staining period at which there was close agreement between the percentage stained and the actual germination was at this particular combination. If on the other hand, the chemical is in short supply a 0.1 per cent. concentration for a staining period of 240 minutes could be adopted. The relation of staining percentage to concentration and staining period is plotted 3-dimensionally in Figure 2.

Correlation of Staining with Viability

The main object of this study was the estimation of the correlation between potential germination and the tetrazolium test. In pursuance of this, and in the light of earlier observations, tests were conducted with six varieties of paddies of 3 to 4 months duration for determining the correlation coefficients. Seeds which were placed in solution with the cut surface down developed a stronger stain. Two categories have

accordingly been recognized. The de-husked seeds were pre-soaked for 16

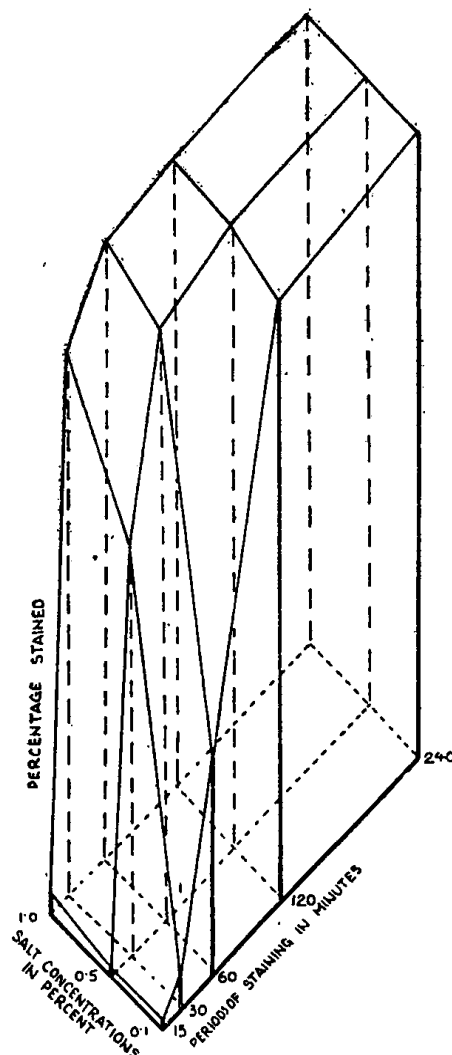


FIG. 2.—Three-dimensional graph showing the distribution of percentage stained in relation to salt concentrations and periods of staining.

hours, bisected longitudinally and then treated with a 0.1 per cent. solution for 240 minutes. The results are given below.

Table 2.—Correlation Coefficients between Potential Germination and Tetrazolium Test

<i>Position of Seed</i>	<i>r</i>	<i>S. E.</i>	<i>Differences between paired values</i>		<i>Sum</i>
			—	+	
Cut face up+ 0.994	..± 0.003	.. 41	.. 7	.. -34
Cut face down+ 0.992	..± 0.004	.. 0	.. 66	.. +66

The coefficients were significant at 0.1 per cent. point and indicate almost complete correlation. In order to ensure that viability is not over-estimated, the placing of seeds with the cut surface up should be preferred. The magnitude of the correlation establishes the fact that

germination tests can be replaced by staining tests with tetrazolium salt. The relation of percentage stained by the tetrazolium test (cut surface facing up) to actual germination is plotted in Figure 3.

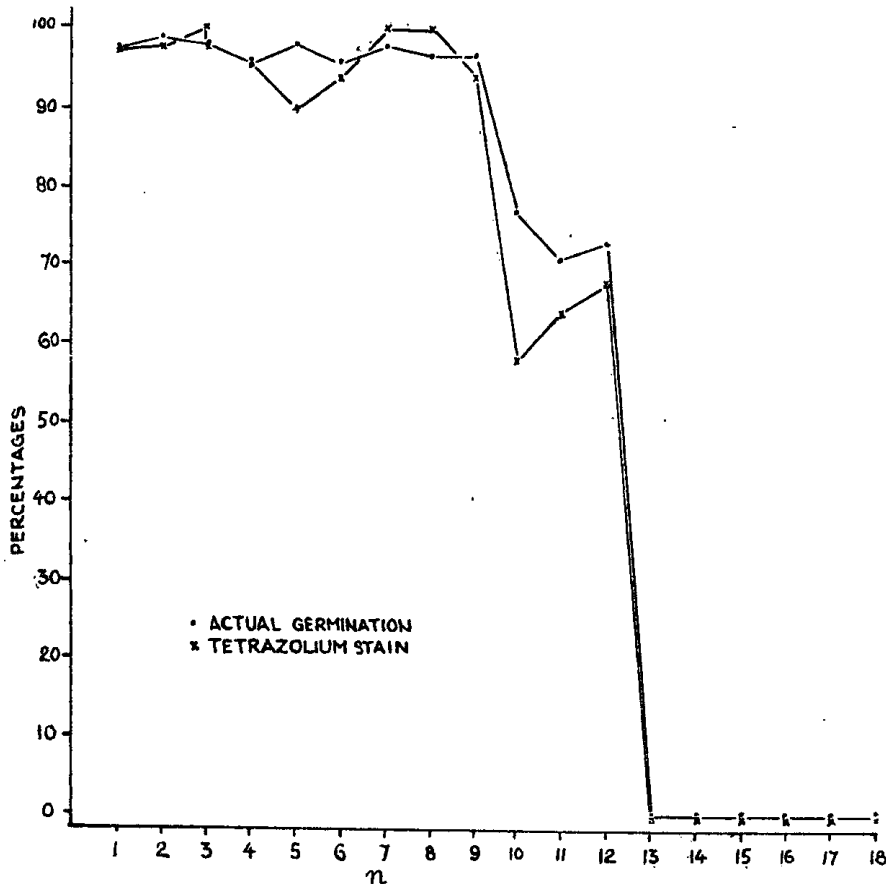


Fig 3.—Relation of percentage stained to actual germination in n paired observations.

Dormancy

One of the chief uses of this staining test with tetrazolium salt is that even in seed which is dormant, the eventual germinability can be predicted with certainty immediately after harvest. This fact is of immense value to the rice grower, particularly when dealing with late photoperiod-sensitive varieties (1952). The period of dormancy often exceeds six weeks, and normally

expensive processing and storage become necessary before suitability for use as seed can be determined. By the tetrazolium salt test the eventual germinability can be determined immediately after harvest, and grain unsuitable for seed purposes can be diverted to other uses.

Djalen, a 5½ months variety, relatively insensitive to the photoperiod, and *Panduruwi*, a 6 months variety, which is

highly sensitive, were tested for actual germination by standard laboratory methods at weekly and fortnightly intervals respectively from the date of harvest. Parallel tests with tetrazolium salt were set down four days after the former test commenced, as the peak

period for actual germination is on the fourth day. For the tests with tetrazolium salt, the dehusked seeds were pre-soaked in water for 16 hours and treated with a 0.1 per cent. solution for 240 minutes. The results are given in Table 3.

Table 3.—Corresponding Percentage Figures of Staining and Actual Germination tested at Intervals from Date of Harvest

<i>Djalén</i> (Harvested = 23.11.53)				<i>Panduruwi</i> (Harvested = 2.12.53)			
<i>Date Tested</i>	<i>Mean Percentage</i>			<i>Date Tested</i>	<i>Mean Percentage</i>		
	<i>Actual Germination</i>	<i>Stained</i>			<i>Actual Germination</i>	<i>Stained</i>	
27.11.53	21.7	100.0	..	8.12.53	0.0	100.0	..
4.12.53	46.7	100.0	..				
11.12.53	83.3	100.0	..	22.12.53	1.7	100.0	..
18.12.53	81.7	100.0	..				
22.12.53	93.3	100.0	..	5. 1.54	21.7	100.0	..
31.12.53	100.0	100.0	..				
8. 1.54	96.7	100.0	..	19. 1.54	50.0	100.0	..
14. 1.54	95.0	100.0	..				
22. 1.54	100.0	100.0	..				
29. 1.54	100.0	100.0	..				
3. 2.54	100.0	100.0	..				

The figures relate to seed that is progressively losing its dormancy but indicates the trend of the complete curve. Complete data relating to senescing seeds up to complete loss of viability are not yet available, but it is expected that the percentage stained will decline *pari passu* with percentage germination.

The effect of the stain on artificially killed seed is of interest in this connection. Seeds of *Sulai* 301, with an actual germination percentage of 98.3 and a corresponding staining percentage of 98.7, were killed by immersion in boiling water for 5 minutes and then tested

with a 0.1 per cent. solution of tetrazolium salt for 240 minutes. The staining percentage dropped to zero in the case of killed seeds.

Discussion

This technique of staining with tetrazolium salt can be widely adopted as a rapid means of determining the viability of rice seed. It should be of particular value to the extension worker who is obliged to purchase dormant seed immediately after the *Maha* harvest. Elaborate equipment is unnecessary. The only precision apparatus that may be out of the reach of some is a sensitive chemical balance. In such cases it should be possible to get the required quantity of the reagent packeted centrally. One gram of the reagent, which costs Re. 1.72, would be sufficient to test a number of samples. The application of the tetrazolium technique to other crops in this country is being investigated. Preliminary tests suggest that the technique may also be of value in testing bull spermatozoa.

Summary

The use of Triphenyl tetrazolium bromide as a germination indicator in rice seed has been studied, and a technique for general use is recommended.

The magnitude of the correlation coefficients establish the fact that germination tests can be replaced by staining tests with tetrazolium salt.

Even in dormant seeds, the eventual germinability can be accurately determined immediately after harvest.

The practical application of the tetrazolium salt test is discussed.

Acknowledgment

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