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Viability Test on Hevea *Seeds by the Tetrazolium Method*

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The tetrazolium test as a rapid method of estimating the viability of Hevea *seeds was investigated. Preliminary studies were to determine the practical preconditioning time and to investigate the optimal concentration, duration and temperature for staining. Seeds imbibed for 4h prior to staining resulted in light and irregular stain, while with longer preconditioning for 16h, the seeds were better stained. Seeds immersed in 1% 2,3,5-triphenyl tetrazolium chloride at 40°C for 2h gave the best uniform red staining of the embryo and endosperm. Using this staining technique, it was found that the results of the tetrazolium test closely correlated with those of the germination test for different quality seeds. The result of the tetrazolium test was obtained in 24h compared to the normal germination test of at least three weeks.*

The moisture content of a fresh *Hevea* seed is about 35%. The seed loses its viability within six days of exposure to sunlight and rain[1]. The rapid deterioration of seeds poses a serious disadvantage for field and nursery planting. Seed viability was previously only determined by the germination test.

The tetrazolium test has been found to give a prompt and reliable index of cereal seed viability[2]. The colourless 2,3,5-triphenyl tetrazolium chloride penetrates by diffusion into the cells of the imbibed seed, where it is reduced by the dehydrogenases present in living cells to form red, stable and non-diffusible formazan. This makes it possible to distinguish the red colouration of the living parts of the seed from the colourless dead parts.

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The tetrazolium test was developed more than twenty years ago for most forest tree species[3]. Porter *et al.*[4] were among the early workers who recognised the significance of the test for seed viability. Since then, the test has been used for various seeds including cotton seed[5], oats[6], rice[7] and *Brassica*[8].

At present, nearly all testing procedures have been developed for annual crop or temperate forest tree seeds. Except for the method developed for oil palm seed by Mok[9], testing procedures for the majority of tropical tree species are not available. There is therefore a need to evaluate this topographical tetrazolium test for other tropical species especially plantation crop seeds such as *Hevea*. *Hevea* seeds normally take two to three weeks to germinate. However, it is often necessary to know within a day or two the general condition of a seed lot. This is useful for estimating the correct amount

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of seeds required for field and nursery planting. This paper aims to investigate the possibility of using the topographical tetrazolium staining method as a rapid viability test for *Hevea* seeds.

MATERIALS AND METHOD

Seed Sample

Four thousand fresh clonal seeds (RRIM 600) were obtained from five-month-old capsules. The pods were harvested from *Field 31* of the Rubber Research Institute of Malaysia Experiment Station, Sungei Buloh.

Assessment of Staining Methods for Hevea *Seeds*

Hevea seeds were shelled and then soaked in water for 4, 16 and 18 hours. The endosperms of the soaked seeds were carefully bisected longitudinally to expose the cotyledons and the embryo axis. The endosperm halves containing the embryo axis and the cotyledons were immersed in 2,3,5-triphenyl tetrazolium chloride of different concentrations (0.1%, 0.5% and 1.0%) at different temperatures (air-conditioned room, 21°C ± 1°C, ambient room temperature, 28°C ± 2°C; ventilated oven, 40°C) for various durations (1 h, 2 h and 3 h).

The above combination which gave a total of twenty-seven treatments was repeated three times by dividing the seed samples into three seed lots. After termination of each treatment the tetrazolium solutions were decanted and the embryos were rinsed with distilled water prior to evaluation. The embryos were spread on petri dishes and kept moist throughout the period of evaluation. The different treatments were compared according to the degree of staining. The treatment that showed uniform carmine red staining of the cotyledon and embryo axis with minimum treatment time was considered to be practical. This was then chosen for further tests.

Simultaneously, a germination test was conducted to determine the actual quality of seeds from a five-month-old capsule. It was done in four replications of twenty-five seeds each divided into three seed lots.

Evaluation of Range of Staining Patterns

Three hundred seeds were tested six weeks after storage using the best method devised above. The range of staining patterns was observed and summarised as an evaluation chart.

Correlation of Staining Pattern with Germination of Different Quality Seeds

The accuracy of the viability test method was compared with the standard germination test. Fresh seeds and seeds stored for four and eight weeks were used so that the comparison can be carried out over a range of seed quality. For each treatment, twelve replicates of 100 seeds each were used for both the germination and staining tests. For the germination test, the seeds were sown in moist sand and evaluated twenty-one days after sowing.

RESULTS AND DISCUSSION

Assessment of Staining Method for Hevea *Seeds*

Presoaking the seeds for 4 h in water before staining was inadequate as only light patchy staining was observed irrespective of stain concentration, staining temperature and duration. Soaking for

16 h prior to staining resulted in more uniform staining. A longer presoaking period of 18 h showed no difference in staining pattern to that obtained with 4 h presoaking.

Only 21% of the seeds were stained (embryo axis and more than half of the cotyledon stained red) and classified as viable although the actual seed germination was 88% *(Table 1)*. There was no correlation between staining pattern of seeds presoaked for 4 h with germination percentage ($r = 0.3390$). This discrepancy was due to error in interpreting the staining patterns which were too light and irregular as a result of inadequate presoaking. Presoaking for 16 h and 18 h was adequate as the staining pattern was distinct and the percentage of seeds stained agreed closely with that of germination percentage. This supported the principle of tetrazolium staining that the embryo should be in the hydrated state before performing the staining test[10]. The 16 h preconditioning period was therefore selected as the most practical method of preconditioning *Hevea* before conducting the tetrazolium staining test.

Using the above optimal presoaking method, *Hevea* seeds reacted with varying staining intensities according to the temperature, concentration and duration of staining. The influence of temperature on the staining treatment was very distinct. Seeds stained at 22°C in an air-conditioned room were lightly coloured. A uniform and distinct red colouration was observed with higher temperature treatments. However, Smith[11] suggested that the biological reduction of tetrazolium was attributed to enzymatic action. At temperatures exceeding 45°C reduction of the tetrazolium chloride to formazan may not occur

TABLE 1. TETRAZOLIUM VIABILITY TEST COMPARED WITH PERCENTAGE GERMINATION OF *HEVEA* SEEDS BEFORE STAINING

Replicate no. (100 seeds each)	Viability of seeds from five-month-old capsules	
	Seed stained (%)	Germination (%)
1	11	92
2	7	91
3	14	91
4	26	93
5	25	91
6	34	92
7	10	72
8	18	91
9	36	92
10	32	85
11	15	70
12	24	90
Mean	21	88

owing to denaturation of the enzyme. The optimum temperature for staining *Hevea* seeds was therefore 40°C.

The most suitable working concentration for *Hevea* seeds was found to be 1% tetrazolium chloride. At lower concentrations staining was slow. As mentioned earlier the treatment with 1% tetrazolium solution at 40°C for 2 h gave the best stain. Prolonging the same treatment up to 3 h resulted in a dark-red colouration.

The treatment with 1% tetrazolium at 40°C for 2 h gave good even staining. Almost similar staining intensity was observed for treatment with 0.5% tetrazolium at 40°C for three hours. Hence, the former method of staining bisected seeds with 1% tetrazolium chloride for 2 h at 40°C after presoaking for 16 h was chosen as the standard method for future tests. Results of germination test show that seed batch collected from five-month-old capsules taken from the sample used on this staining test were of high viability with average mean germination of 92% *(Table 2)*. Seed staining results were therefore evaluated on the effect of staining treatment rather than the effect of seed quality.

Evaluation should be done immediately after treatment especially with fresh samples. Delay results in colour change which may cause wrong interpretation during assessment. With this method it would be difficult to accurately measure the viability of a large number of batches of seeds. The pretreatment process (decorticating, splitting of endosperm and staining) takes about 45 min for every 100 seeds and evaluation takes another 10 minutes. The accuracy of interpretation can be improved with experience and training.

TABLE 2. GERMINATION PERCENTAGE OF HEVEA SEEDS COLLECTED FROM FIVE-MONTH-OLD CAPSULES

Replicate no. (25 seeds each)	Seed batch			Average mean
	1st	2nd	3rd	
1	92 (23)	96 (24)	96 (24)	–
2	92 (23)	92 (23)	88 (22)	–
3	88 (22)	92 (23)	96 (24)	–
4	96 (24)	96 (24)	84 (21)	–
Mean	92	94	91	92

Figures within brackets indicate the number of seeds germinated twenty-one days after sowing.

Evaluation of Range of Staining Patterns

In formulating the criteria of seed viability, it is assumed that the portions of the embryo which are vital for the production of the seedling are the embryo axis, the cotyledons and the related food storage organs. When these parts react to the stain, the seeds are classified as viable[12]. *Hevea* seeds were therefore classified as viable when the embryonic axis and the cotyledons were stained red. Various staining patterns of the vital organs were observed when the seeds six weeks after storage were tested using the standard method developed. Deteriorated seeds did not stain evenly. The cotyledons of badly deteriorated seeds were also shrunken, flaccid and showed patchy areas of dark-red sediments. Seeds infected with micro-organisms commonly showed necrotic spots which appeared as dark-red patches after staining. Fred and Knight[13] also reported that tetrazolium stained micro-organisms dark-red. Healthy embryos on the other hand, were stained normal carmine red. Moore[14] proposed that a normal carmine red stain in the embryo and food storage organs should indicate seed viability and seedling vigour. Hence, in drawing up an evaluation chart for testing seed viability, the staining pattern, colour intensity and texture of the tissues (visually determined) must be jointly considered. Based on the above considerations and the range of staining patterns observed, an evaluation chart for *Hevea* was drawn up *(Figure 1)* as a guide for testing seed viability using tetrazolium chloride. The chart is also based on the assumption that if more than half of the cotyledon is unstained, the seed is considered non-viable since it will not have sufficient potential to mobilise its reserves for germination. Similarly, if the meristematic region of the root axis above the root cap tissues is unstained, it is also considered non-viable since further growth of the root is impaired.

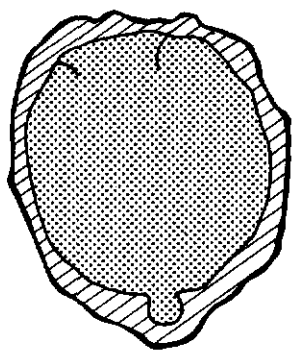
Correlation of Staining Pattern with Germination of Different Quality Seeds

The tetrazolium chloride test on the viability of three different quility *Hevea* seeds was carried out using the standard method developed and the evaluation chart. The results showed that the percentage of viable seeds given by the tetrazolium test was consistently higher than the actual germination percentage *(Table 3)*. This appears to confirm Moore's[15] results which also showed that in most cases the tetrazolium test gave higher values than standard germination, especially in seeds with high moisture content.

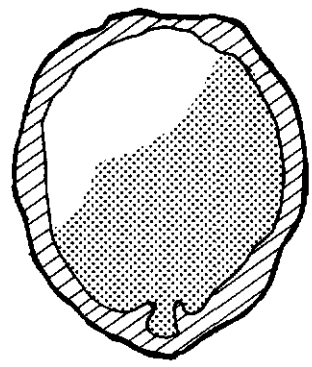
From the three groups of different quality seeds a mean difference of not more than 6% was observed between the percentage seed stained and germination percentage. Although this slight disparity exists between the two tests, the topographical tetrazolium test was found to be positively and significantly correlated with the normal germination test ($r = 0.9852$, significant at $P = 0.001$). This establishes that the tetrazolium test can be used as a rapid method for determining the viability of *Hevea* seeds.

CONCLUSION

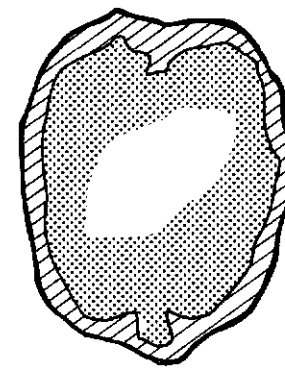
The topographical tetrazolium test is a practical and simple method for rapid assessment of the viability of *Hevea* seeds. The test result can be obtained within 24 h compared to the normal germination test period of at least three weeks. This involves staining the seeds in 1% 2,3,5-triphenyl tetrazolium chloride at 40°C for



Complete staining of cotyledon and root axis.

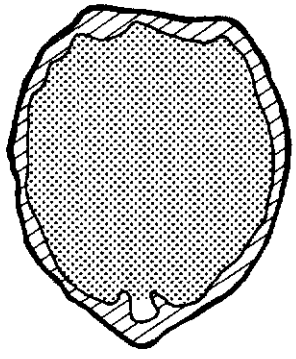


Complete staining of root axis and more than half of cotyledon.

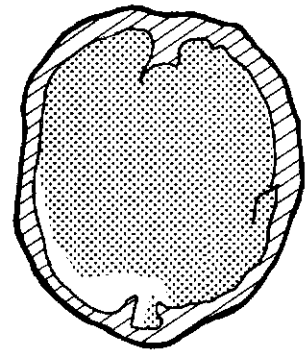


Minor discolouration of root axis, more than half of cotyledon stained.

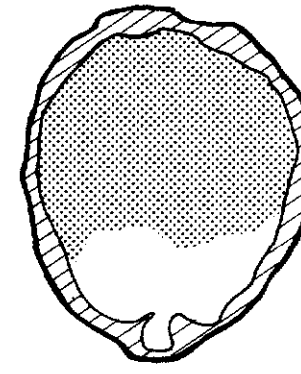
Viable seeds



Distal end of root axis unstained.



Partial staining of root axis and lower portion of cotyledon unstained.



Root axis and lower portion of cotyledon completely unstained.

Non-viable seeds

Figure 1. Viable and non-viable Hevea *seeds after staining in 1% tetrazolium chloride.*

TABLE 3. RESULTS OF COMPARATIVE TESTS BY TETRAZOLIUM CHLORIDE STAINING AND STANDARD GERMINATION FOR *HEVEA* SEEDS

Replicate no. (100 seeds per replicate)	Viability of seeds 2 days after collection		Viability of seeds after 4 weeks storage		Viability of seeds after 8 weeks storage	
	Seed stained (%)	Germination (%)	Seed stained (%)	Germination (%)	Seed stained (%)	Germination (%)
1	95	90	72	64	40	34
2	93	88	71	62	38	36
3	93	87	73	67	36	30
4	85	77	76	66	45	37
5	90	85	52	52	39	35
6	80	70	76	77	47	44
7	92	90	76	62	44	40
8	84	82	72	69	52	50
9	88	86	80	75	40	36
10	78	70	80	77	33	25
11	75	68	68	56	41	35
12	76	75	80	74	38	32
Mean	86	81	73	67	41	36

two hours. A *Hevea* seed can be evaluated as viable when its embryo axis and at least half of the distal portion of the cotyledons are stained red. The staining results though consistently higher correlated well with the germination results.

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