1953

235-Triphenyl tetrazolium chloride as a viability indicator of seeds of certain broadleaf species

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2,3,5 - TRIPHENYL TETRAZOLIUM CHLORIDE AS A VIABILITY INDICATOR OF SEEDS OF CERTAIN BROADLEAF SPECIES

by

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B. S. Montana State University, 1949

Presented in partial fullfillment of the requirements for the degree of

Master of Forestry

MONTANA STATE UNIVERSITY

1953

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Aug 17 1953
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ACKNOWLEDGEMENT

The writer wishes to express his great appreciation for the advice extended and for the great patience shown by Dr. C. W. Waters during the development and conclusion of this problem.

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INTRODUCTION

Seed testing has come to be recognized as a necessity both for the protection of the buyer as well as the seed dealer. As far back as 1897, rules were promulgated for seed testing. These have been revised and refined until at present there are but two sets of official rules recognized in the United States. One of these sets is that of the United States Department of Agriculture and the other is the official rules adopted by the Association of Official Seed Analysts of North America (Agricultural Handbook No. 30 1952). These sets differ on only a few minor points.

Seed testing provides information for planting purposes--amount of seed to use, amount of seed to purchase, establishment of equitable prices--for seed control work and for research problems. Seed must be tested by the merchant for the foregoing reasons and for proper labelling. The seed officials must test the seed to check the accuracy of the labels. Perhaps the primary objective is best stated by the Agriculture Handbook No. 30 of the Department Agriculture (1952). That is, "to provide accurate and reproducible information regarding purity, composition, rate of occurrence of noxious weed seed, and per cent of seeds that will provide normal plants under favorable conditions."

Tests are normally carried out at one of the federal or state seed testing stations located throughout the United States

or by a licensed private testing agency. Tests are generally carried out in sterile sand flats or on some of the other acceptable substrates such as germination blotters, paper towelling, soil, filter paper, cotton, and creped cellulose paper wadding. The tests are continued until germination ceases and the germination per cent is then computed. According to the Federal seed act (Agricultural Handbook No. 30 1952) a seed shall be considered to have germinated when it has developed into a normal seedling. Broken seedlings and weak, malformed, and obviously abnormal seedlings shall not be considered to have germinated.

In seed laboratory practice, germination is defined as the emergence and development from the embryo of those essential structures which, for the kind of seed in question, are indicative of the ability to produce a normal plant under favorable conditions. Germination is expressed as the percentage of pure seed of the kind under consideration which produces normal seedlings.

These germination standards and methods have been established basically for agricultural and vegetable seeds. However, they are highly applicable to forest tree seeds except for one basic difference found generally in this type of seed. The majority of agricultural and vegetable seeds are quick to germinate and require little or no pretreatment, as is the case with many tree seeds. As a matter of fact, pretreatment of vegetable seed may sometimes be detrimental as in the case of the common bean (Barton 1950 Eyster 1940) which loses vitality when soaked in water. Because

of their dormant type seed most of the tree species require from 21 to 120 days or more pretreatment. They must be stratified by one of the standard methods, i.e., by water soaking or being placed in a mixture of moist sand and peat moss for the appropriate lengths of time. This treatment must overcome one or more of the following causes for seed dormancy:

1. Impermeable seed coat
2. Physiologically unripe or freshly harvested seed
3. Dormant embryo
4. Immature embryo
5. Inhibiting substances

Therefore, testing tree seed by standard methods is often a long drawn out process. When the germination figures are finally obtained, one can not always be sure that they are correct since stratification periods are variable even in two lots of the same species and they may vary with age of seed, type of storage, moisture content of seed etc.

Mirov (1936) states that, "conventional methods of trial germination are unsuitable for forestry purposes. They give erroneous results...Seed should be handled in accordance with conditions under which it reproduces in nature." This is undoubtedly correct and would be desireable if it were possible.

The forest nurseryman should be able to arrive at dependable germination data quickly if he is, first, to purchase seed in an economical, businesslike manner and, second, if he is to plant

with any degree of assurance that he will have a full stand and yet one that will not stagnate because it was grossly oversown. Two examples may suffice to indicate the importance of a quick test. Recently a Forest Service nursery stocked some hundreds of pounds of pine seed (Pinus ponderosa Laws.) which, when tested gave a germination of 2 per cent. Certainly had there been a dependable quick germination test, this seed never would have been gathered and processed. A shelterbelt nursery purchased Nanking cherry seed (Prunus tomentosa), stratified it the usual 90 days and planted it, only to have a stand of approximately one tree per 50 feet or no stand at all. This has occurred two years in a row. The seed dealer is very dependable, the seed being imported and sold in good faith. However, the nursery wasted a considerable amount of time and money both in the purchase and the care of apparently worthless seed. A quick germination test could have saved both the nursery and the seed dealer considerable difficulty. It is also possible that somewhere in the stratification there could have been an error resulting in the loss of previously viable seed before it was planted. Here again a quick germination test would not only have saved the dealer embarrassment, but would have indicated to the nursery that an investigation into stratification methods was in order.

Seed whose viability is questionable after short periods of storage should not only be tested immediately after purchase, but yearly to indicate whether it is worth keeping and to indicate

changes in sowing rate because of decrease in vitality. Therefore, it is highly important, especially for the forester and tree nurseryman to have a germination test that is considerably shorter than the standard test and it must be one that will give dependable results even when stratification is ignored. A method that can be quickly and accurately applied in the field is also very desirable.

Some attempts at this type of testing are as follows and are all "indirect" methods as compared with the standard germination method which is considered "direct."

1. The cutting test

The seeds are cut open with a sharp knife or scalpel and by checking the presence of filled seed, color of embryo, odor, insect damage and presence of harmful fungi, the operator attempts to arrive at a rough germination estimate. (The cutting test can be sometimes very misleading, especially after storage, fumigation, or other disinfection (Mirov 1936).)

2. Flotation

The seed is floated on a container of water. Empty seed will float and it is assumed that those seeds which sink are viable. Other liquids, such as alcohol and various oils, have been used, but these are usually somewhat injurious and hence, not desirable or reliable.

3. Embryo ratio method

This method has been suggested for coniferous seeds. The ratio between the length of the embryo and the total

length of the endosperm is determined. If the ratio is less than 80 per cent, the seed is unripe.

4. Color

With some species of trees the color of the seed is a rough indication of its viability, i.e., the seed coat should be shiny and brown in caragana (Caragana arborescens Lam.) and shiny and black in black locust (Robinia pseudoacacia L.).

These four methods of arriving at a germination estimate are so rough and inaccurate that they are only slightly better than no test at all.

A much more rapid, relatively direct method was pioneered by Tukey in 1928 (Reported by Baldwin 1942). This is the excised embryo test which has been explored considerably farther by Florence Flemion of the Boyce Thompson Institute (Flemion 1934, 1936, 1937, 1938, 1941, 1948). In this method, the embryo is excised and placed on moist filter paper. Within 2 to 10 days the nonviable seed will have disintegrated, and the viable seed will have turned green and began to grow. This method has been quite successfully used by Tukey and Flemion with many species of tree seed.

Relatively recently, certain chemical methods of seed testing have been tried. These fall into two general categories, the vital staining and the biochemical methods (Baldwin 1942).

The vital staining method depends on the property of the acid organic dyes (especially the aniline dyes) to penetrate dead tissue relatively quickly in low concentrations while they penetrate

live tissue very slowly and in high concentrations. According to Baldwin (1942), this group of chemicals was first tested by Neljubov in 1929. He found that indigo carmine in concentrations of 1:1000 to 1:2000 gave the best reaction.

Shirley (1935) stated that Shafer Safronova tested the following dyes:

Acid violet

Fast red dyes

Indigo carmine

Nigrosine

Safranine

Methylene green

Methyl violet

Bismarck brown

The method as used by Safronova is basically as follows. Indigo carmine was used in concentrations of from 1:200 to 1:5000 and a staining time of from 2 to 30 hours. Seed of yellow acacia (Caragana arborescens), Scotch pine (Pinus sylvestris L.) and Norway spruce (Picea excelsa Link.) were used. The best results were obtained with a concentration of 1:2000 with the seed left to stain for 2 hours for caragana and 3 hours for spruce. Staining standards were set and the results were very promising. Five hundred seed samples were run in two to three days instead of the conventional 18 days or longer. Shirley (1935) commented that this method showed promise for seed slow to germinate.

Biochemical methods of seed testing fall into two groups.

The first of these is based on the premise that the more viable the seed, the higher the amount of respiration and enzyme action. Two enzymes, oxidase and catalase have been used. Oxidase is measured by the usual biochemical tests. Catalase, an enzyme that is present in all living and some dead tissues, has been investigated to a greater degree. It has the property of releasing oxygen by decomposing hydrogen peroxide. Thus, it gives a more or less direct measure of respiration since the catalase activity has been shown to parallel the amount of respiratory activity. However, the most accurate determination using catalase action has been the separation of germinative ability into three rough classes by Schmidt (Reported by Baldwin 1942).

These are: "1. Good seed with high germinative power with a high initial catalase content and a sharp increase during the germination process.

"2. Indifferent seed of variable germinative power with a high initial catalase content but little or no increase following inhibition.

"3. Poor quality seed of low germination with low initial catalase content and no increase but frequently a decrease following wetting."

It would seem to the writer that such a classification is by far too general for use as an indicator of germinability where a specific determination is desired.

The second biochemical method takes advantage of the ability

of living cells to reduce or oxidize certain chemicals through respiration. The cell is then colored by the residue from this reaction.

According to Baldwin (1942) one chemical used for estimating the germination potential is phenol. Kondo and Takaheshi used this material and found that dark seeds germinated, but light seed did not.

Baldwin also lists the two types of metallic salts, the tellurides and selenite, as indicators. Hasewego used sodium telluride or tellurate and put this seed on a filter paper soaked with salt. He then barely covered the seed with water and allowed it to stand 48 hours. The seeds were then removed and the embryos excised. Dark embryos were viable while partly stained or light embryos were considered inviable.

Johnson (1947) reported that Eidmann tried the tellurium salts with little success on European seed and so turned to the selenium salts. The most successful was sodium biselenite ($NaHSeO_3$) and he found that this salt was changed by the plant cell to a red color. His method was to remove the seed coat wholly or partially to encourage penetration of the solution. Then the seeds were soaked 24 hours at room temperature and placed in a 2 per cent selenium solution for another 24 hours. The embryo was then removed and classified. His classification was broken down into 4 groups:

I Entire surface intensely colored

II Fully colored, but only part intensely

III Dull coloring - partially unstained on one third to two thirds of surface

IV Staining on one third or less of surface

Groups I and II equal the plant potential or plant per cent.
Groups I, II and III equal the germination per cent.
Group IV is inviable.

Johnston indicated that the selenium salts might prove very valuable in investigation work, e.g., in evaluating relative effects on seed vitality produced by different storage conditions.

One other salt of the oxidation type has been reported as promising for determining seed viability and vitality. This is the 2-3-5 triphenyl tetrazolium chloride, with which this paper shall be primarily concerned.

HISTORICAL

According to Mattson et al (1947), R. A. Dutcher, while on duty with the occupation forces in Germany in 1945, became acquainted with the work of George Lakon and was introduced to the use of the tetrazolium salts as a viability indicator. These salts were first prepared by Pechman and Runge in 1894. Apparently little was done with them until in 1941 when Kuhn and Jerchel reported that dilute solutions of 5-methyl, and 5-hendecyl-2,3-diphenyl tetrazolium salts stained yeast, garden cress and bacteria.

Lakon had substituted the tetrazolium salts for the sodium selenite previously used in his topographic method for determining germinability of seeds. He found that he was able to predict germinability of corn, oats, rye, wheat and barley by observing the embryo parts stained. The staining phenomenon is due to the formation of insoluble carmine red formazans upon reduction of the tetrazolium salts. This is believed to be an oxidation-reduction and the development of the non-diffusable red color is presumably an indicator of active respiration during which hydrogen radicals are transferred to the tetrazolium ion. Porter, Durrell and Romm (1947) repeated Lakon's work with corn and then continued with small grains, legumes, and sorghums. They found some difficulty due to the formation of a reddish coating on the cotyledons which had to be removed before examination of the seeds. They noted that the

presence of the abnormal (baldheaded) bean seedlings could not be determined, and hence, this system might not be applicable to all seeds. They were also careful to say, "Because measurements of germinability are not direct and are dependent on all factors affecting oxidation-reduction potential of the cells, careful standardization of the procedure will probably be necessary."

Mattson, Jenson and Dutcher (1947) synthesized the triphenyl- and 5-furfuryl-2,3-diphenyl tetrazolium compounds. They found that both were about equal in staining other tissues such as the fleshy parts of apples, oranges, grapes, the gill area of mushrooms, carrot roots, white and sweet potatoes, young leaves, stigmas and ovaries of certain pollinated flowers, bull sperm and the blastoderm of hens eggs. Serum of bull sperm and the chalazae of egg white also gave positive reactions. The authors determined that the reaction was not due to sugars, but was an enzyme reaction since tissue heated to 82 degrees C or higher lost its ability to react. They concluded that the reduction of the tetrazolium salts could not be considered a general test for life, but that it could be used in research involving tissue viability.

Flemion and Poole (1948) set up a series of tetrazolium salt tests to determine seed viability. They examined over one hundred different seed lots involving seventeen families and fifty eight species. The rapid viability tests (Flemion 1934, 1936, 1937, 1938, 1941, 1948) were used as the basis for comparison. The authors felt that due to the difficulties encountered in the

special treatment needed for standard germination tests of certain of the species, the rapid viability test would in all probability be more dependable. Whole seeds were soaked during the preliminary tests but spotty and insufficient staining often occurred. Excised embryos stained much more quickly and readily and so all staining was done by the excised embryo method. Seeds were treated for 2, 4, 6, 24 and 48 hours at 20 degrees C. Temperatures of 30 degrees C and 40 degrees C were also used. The 20 degree C temperature for a period of 24 hours was considered most satisfactory. Through statistical analyses, significant correlations were obtained in many cases, but individual tests often varied to a considerable degree. The authors said about the tests, "Before the tetrazolium technique used in this survey can be recommended, much more detailed work is necessary."

Bennett and Loomis (1949) found that a concentration of .05 per cent was best for testing freezing injury in seed corn. They determined that freshly frosted seed corn could not be tested as to definite germinability because the tetrazolium tests were uniformly high, but after several days the "dead" kernels apparently lose the ability to reduce the tetrazolium and this method could then be used to test germinability. However, they found that the severity of frost damage can be estimated within two hours of the frost instead of the customary two weeks.

Lakon (1949) rewrote his original article, in English, to clear up some "misconceptions." He states that tetrazolium[1]

[1] "Tetrazolium chloride" or "tetrazolium" will be used synonomously with 2,3,5-triphenyl tetrazolium chloride.

"practically eliminates the experimental error. Statistical processing of several thousand tests with 200 seeds each always shows a lower error than germination test with 400 seeds." He claims that the initiation of germination is neither necessary nor significant to the test, that even non after-ripened cereals can reduce the tetrazolium. The only reason for pre-soaking of seed is to facilitate removal of the embryo. Oat seeds were simply cut and put into tetrazolium. However, to hurry the reaction the seed can be soaked in warm water and then put into tetrazolium. This is then placed in an oven at 30 degrees C. Staining must be completed within 24 hours, otherwise microorganisms appear, react with the tetrazolium and mask the results.

Lakon lists the following advantage of tetrazolium in comparison with direct germination tests:

1. No great amount of equipment or space is needed. Tetrazolium tests can be accomplished in limited space with simple tools. The test is simple and conclusive.
2. The test is rapid, even when done on a large scale. Small cereals can be tested in 48 hours. Corn can be tested in one day (and if the information is urgently needed, the time can be shortened.)
3. The method provides reliable and exact results.

"Even in cases of impediments to germination, as in freshly harvested non after-ripened cereals which are not

immediately germinable, but possess inherent germinability, the eventual germination can be determined."

Thoneberry and Smith (1953) in studying the effect of triphenyl tetrazolium chloride on oat embryo respiration showed that it might cause significant alterations in respiratory metabolism. They indicated that these effects might be the basis for the tetrazolium toxicity. Therefore, it could affect the usefulness of the tetrazolium compounds as indicators of viability.

On (1952) working with coniferous seeds noted the very significant fact that the use of the embryo alone, as had been done in the past, tended to make germination determinations erroneous. He used both the endosperm and embryo, discarding a seed as nonviable if either embryo, endosperm, or both showed no coloration after 12 hours soaking in 1 per cent tetrazolium at room temperature, in the dark. On one occasion he predicted a 3 per cent germination on a recently processed lot of Ponderosa pine seed. Approximately ten months later his findings were substantiated by the Seed Testing Laboratory at Corvallis, Oregon, where the germination per cent was placed at 2.

Johnson Parker (1953) investigated the possibility of using tetrazolium as a viability indicator on Beech (Fagus sylvatica L.), various oaks, and some species of conifers. Like On, he used both endosperm and embryo to make his determinations on the conifers. He also found some significant correlations between the tetrazolium and the check.

Past work definitely indicates that the tetrazolium salts are reliable in part at least as viability indicators. However, much more work is necessary to build up a mass of information that can be used in determining a standardized process so that the germination tests can be repeated by several laboratories on the same seed sample with reliably close results.

EXPERIMENTAL

A. Species Tested

The three species used in these tests were selected because of difficulties in obtaining satisfactory stands of these species with any regularity while the author was assistant nurseryman at the Forest and Shelterbelt Nursery, Montana State University. All three are species with dormant seeds, are difficult to test by conventional germination methods, and are subject to rapid loss of viability in storage--thus making yearly germination tests almost mandatory for economical sowing.

All the seeds used in these tests were from the seed stores of the previously named nursery.

1. Box Elder (Acer negundo L.)

The fruit of the box elder is a samara containing a single seed and has no endosperm. The embryo is not folded as is the case with the other members of the maple family but is bent somewhat in the shape of a closed horseshoe. It is a dormant seed of the dormant embryo type (U.S.D.A. Misc. Pub. No. 365 1949) and is usually stratified for 90 days in moist sand and peat moss before planting. An alternative pretreatment is 14-21 days water soak in cold running water.

Recommended length of germination tests is 50 to 60 days with a night temperature of 50 degrees F and a day temperature

of 77 degrees F. Seed lots 3 to 6 were all 1952 seed of Montana seed source picked by the author.

2. Green Ash (Fraxinus pennsylvanica var. lanceolata Borkh Sarg.)

The fruit of the green ash is also a samara containing a single seed. It has a relatively large endosperm with a small, straight embryo. It is a dormant seed of the dormant embryo type (U.S.D.A. Misc. Pub. No. 365 1949) and the suggested pretreatment is 60 to 90 days stratification in moist sand and peat moss at 41 degrees F. An alternate pretreatment is water soaking at room temperature for 10 to 21 days.

Recommended length of germination test is, for unstratified seed 60 to 90 days, or for stratified seed 40 to 60 days at room temperature.

Seed lot 21 was 1950 seed; lot 23 1951 seed; lot 101 and 102 1952 seed. All lots were North Dakota seed purchased from E. C. Moran, seed collector.

3. Wild or American Plum (Prunus americana Marsh)

The fruit of the wild plum is a one seeded drupe with a thick fleshy pulp. The "stone" is a bony, pitted, matured endocarp (U.S.D.A. Misc. Pub. No. 365 1949) enclosing the true seed which is covered with a brown membranous coat. It has an endosperm which according to Martin (1946) is not starchy, with an embryo that is small. It is a dormant seed of the dormant embryo type (U.S.D.A. Misc. Pub. No. 365 1949) complicated by the hard endocarp.

The suggested stratification is 150 days in moist sand and peat moss at 41 degrees F. The author has seen this time extended to 180 days without having excessive germination prior to planting.

The recommended length of germination tests is 60 days at 50 degrees F, after proper stratification.

Seed lot No. 1 was 1951 seed from Montana, picked by E. C. Moran; lot No. 2 was 1952 Montana seed, picked by the author; lot No. 3 was 1952 seed from North Dakota, picked by Moran; lot No. 4 was 1949 seed from North Dakota, picked by Moran.

B. Direct Germination Tests

These were conducted as checks in the green house in benches as is the practice when testing seed for the nursery. The substrate was sifted medium sand from river wash. Four hundred seeds of each seed lot were tested in 4 tests of 100 seeds each. Seedling emergence was recorded every 5 days for the duration of the tests and at their conclusion ungerminated seed was recovered and examined for its condition. The number of apparently healthy ungerminated seed is recorded (see Tables I to III) but not included in the germination estimate.

The germinative energy (Baldwin 1942) is the per cent of seed germinating in a given period under optimum conditions. The period is usually from 7 to 14 days and was a 14 day period for these tests.

The germinative capacity is the per cent of seed actually germinating irrespective of time. In this case the length of time

was 60 days for box elder and green ash and 90 days for the wild plum.

The box elder and green ash were pretreated 14 days in cold running water prior to planting. The wild plum was stratified for 150 days in moist sand and peat moss at approximately 40 degrees F prior to planting. The germination benches were both sub and top irrigated and covered with a translucent cover to conserve moisture. The results of the germination tests are recorded in Tables I to III.

C. Rapid Viability Tests

The rapid viability tests were conducted as further checks as suggested by Flemion (1934, 1936, 1937, 1938, 1941, 1948). Duplicate samples of 50 seeds each were used. Seed of the box elder and green ash were removed from the dry pericarp by opening it with a razor blade. The seed was sterilized in a 1:1000 solution of mercuric chloride (Lammerts 1942) and put on moist filter paper for 24 hours. At the end of this time the seed coats were removed and the embryos were excised and placed on moist filter paper in petri dishes at room temperature. Readings were possible at from 5 to 10 days and are recorded in Tables IV and V. Only germinative capacity is recorded since there seems to be no way of determining the germinative energy figure. However, total germination is the figure usually determined and in this case it is all that is required for the desired comparison.

The wild plum seed was removed from the endocarp with an ordinary claw hammer and a hinged, two-piece, inclined plane.

Table I

BOX ELDER GERMINATION TESTS (SAND)

Sample	# Seed	Germ. Energy %	Germ. Capacity %	No. of Apparently[1] Good Seed Recovered
Lot # 3				
1	100	34	40	9
2	100	31	33	2
3	100	65	68	6
4	100	71	80	4
Average	Lot 3	50.2	55.2	5.2
Lot # 4				
1	100	25	34	37
2	100	20	37	33
3	100	14	27	35
4	100	14	29	40
Average	Lot 4	18.2	31.7	36.2
Lot # 5				
1	100	16	40	47
2	100	20	37	57
3	100	30	48	48
4	100	23	39	51
Average	Lot 5	22.2	41.0	51.0
Lot # 6				
1	100	35	56	35
2	100	35	48	38
3	100	34	52	29
4	100	40	59	36
Average	Lot 6	36.0	53.7	34.5

[1] At the end of the germination period, the sand was sifted through a 2 mm. sieve. All seed was recovered and apparently good seed found by means of the cutting test. Criteria used were color, odor and firmness.

Table II

GREEN ASH GERMINATION TESTS (SAND)

Sample	# Seed	Germ. Energy %	Germ. Capacity %	No. of Apparently Good Seed Recovered
Lot # 21				
1	100	6	29	1
2	100	2	26	2
3	100	5	32	0
4	100	7	24	0
Average Lot 21		5	27.7	0.7
Lot # 23				
1	100	0	18	1
2	100	1	18	1
3	100	0	22	2
4	100	1	17	0
Average Lot 23		.5	18.7	1.0
Lot # 101				
1	100	29	51	19
2	100	15	47	6
3	100	7	42	3
4	100	12	34	9
Average Lot 101		15.7	43.5	9.2
Lot # 102				
1	100	59	69	8
2	100	66	82	5
3	100	61	66	3
4	100	65	74	2
Average Lot 102		62.7	72.7	4.5

Table III

WILD PLUM GERMINATION TESTS (SAND)

Sample	# Seed	Germ. Energy %	Germ. Capacity %	No. of Apparently Good Seed Recovered
Lot # 1				
1	100	1	5	9
2	100	2	7	8
3	100	0	10	8
4	100	1	6	10
Average Lot 1		1.0	7.0	9.2
Lot # 2				
1	100	0	23	54
2	100	0	34	46
3	100	0	32	32
4	100	0	32	32
Average Lot 2		0.0	30.2	41.0
Lot # 3				
1	100	0	1	1
2	100	0	0	2
3	100	0	0	5
4	100	0	1	2
Average Lot 3		0.0	0.5	2.5
Lot # 4				
1	100	0	5	3
2	100	0	3	8
3	100	0	6	9
4	100	1	10	13
Average Lot 4		0.2	6.0	8.2

Table IV

BOX ELDER GERMINATION TESTS (RAPID VIABILITY)

Sample	No. Seeds	No. Empty Seeds	Germination Capacity %
Lot No. 3			
1	50	15	72
2	50	22	56
Av. Lot No. 3		18.5	64
Lot No. 4			
1	50	8	76
2	50	6	86
Av. Lot No. 4		7	81
Lot No. 5			
1	50	4	92
2	50	2	96
Av. Lot No. 5		3	94
Lot No. 6			
1	50	0	98
2	50	0	98
Av. Lot No. 6		0	98

Table V

GREEN ASH GERMINATION TESTS (RAPID VIABILITY)

Sample	No. Seeds	No. Empty Seeds	Germination Capacity %
Lot No. 21			
1	50	15	40
2	50	15	26
Av. Lot No. 21		15.0	38
Lot No. 23			
1	50	8	46
2	50	11	42
Av. Lot No. 23		9.5	44
Lot No. 101			
1	50	6	76
2	50	9	70
Av. Lot No. 101		7.5	73
Lot No. 102			
1	50	7	66
2	50	12	64
Av. Lot No. 102		9.5	65

The seed was placed between the two halves of the inclined plane and moved along its edge until only the center ridge of the seed protruded above the plane. The two pieces of the inclined plane were held closed so that the seed was clamped in place. The seed was then broken with the hammer. By allowing only the center ridge of the seed to protrude above the surface of the plane, a sharp blow with the hammer broke the endocarp only, leaving the seed uninjured. The seed was then sterilized with mercuric chloride solution (Lammerts 1942) and treated the same as the box elder and green ash (see Table VI).

D. 2,3,5-Triphenyl Tetrazolium Chloride Tests

Duplicate 50-seed samples were used for these tests as well. The three species were removed from the pericarp or endocarp as in the rapid viability tests and allowed to absorb moisture for 24 hours.

1. The box elder embryo was then removed from the seed coat and put into the tetrazolium solution to soak for 12 hours. At the end of this time the color had reached its full intensity and the germination per cent was read (Table VII).

2. The green ash was cut in half longitudinally with a razor blade. By using this method, the embryo and endosperm both could be observed. One half of each seed was placed on a sheet of filter paper saturated with the tetrazolium solution. The seed was allowed to remain on this paper for 12 hours. At the end of this time the color had reached its full intensity and germination per

Table VI

WILD PLUM GERMINATION TESTS (RAPID VIABILITY)

Sample	No. Seeds	No. Empty Seeds	Germination Capacity %
Lot No. 1			
1	50	0	12
2	50	0	14
Av. Lot No. 1		0	13
Lot No. 2			
1	50	0	78
2	50	0	72
Av. Lot No. 2		0	75
Lot No. 3			
1	50	0	0
2	50	0	6
Av. Lot No. 3		0	3
Lot No. 4			
1	50	0	20
2	50	0	18
Av. Lot No. 4		0	19

germination per cent was read (Table VIII).

3. Wild Plum

The seed coat of the wild plum was removed and the cotyledons split. The half with the radicle and plumule attached was placed in the tetrazolium solution and allowed to remain for 48 hours. At the end of this time the color had reached its full intensity and the germination per cent was read (Table IX).

If at any time it was impossible to read the germination at the end of the staining period, the seeds were placed in a 1:1000 solution of mercuric chloride (Lakon 1949) until the author was able to examine them.

PROCEDURE

A. Preliminary Tests

The literature (Porter et al 1947, Lakon 1949, Flemion and Poole 1948, On 1952, Parker 1953) generally agrees that a .01 per cent aqueous solution of the 2,3,5-triphenyl tetrazolium chloride gives the best staining reaction with uninjured seed. Bennett and Loomis (1949) found that a .05 per cent solution worked better than either .01 or .5 per cent but their work was done with frozen corn. The freezing injury may have caused a lowered respiration activity in the seed and therefore, a stronger solution was needed to get definite staining. However, since the present work was done with uninjured seed, it was decided to use only the previously tested strength of .01 per cent.

In order to determine the best pretreatment, each species was (1) soaked in water; (2) put between saturated filter paper in petri dishes; and (3) laid on the surface of saturated filter paper in petri dishes. Water soaking appeared more efficient with the plum, but the box elder and green ash responded better to being placed on the saturated filter paper.

Soaking periods of 12, 24, and 48 hours were tried. At the end of 12 hours it was found that the seed coat was generally too difficult to remove. At the end of 24 hours the seed had generally absorbed enough moisture to facilitate seed coat removal. Staining

took place readily after this length of time and since the 48 hour period did not appear to give any additional advantage, the 24 hour period was used in all tests.

To determine the optimum length of time to leave the seed in the tetrazolium solution, times of 4, 8, 12, 16, 24, and 48 hours were tried. The maximum color appeared at the end of 12 hours with the box elder and green ash, while the plum took 48 hours to develop fully.

B. Staining Standards

According to Lakon (1949) the "topographical tetrazolium" method of estimating germinability is based on the gradual dying of the embryo. The spread of the necrosis can be readily seen through the "staining" properties of the tetrazolium. 2,3,5-triphenyl tetrazolium chloride is a colorless solution in its unreduced state, but is reduced or hydrated to a stable, insoluble, non-diffusable carmine red[1] formazan by a reaction in living cells. Necrotic cells do not react with the solution and therefore remain unstained.

With reference to the actual reaction in the living cells, Mattson et al (1947) states: "It is evident that it is an enzyme reaction since tissue heated to 82 degrees C or higher lose their ability to react. It is probable that the reduction is caused by dehydrogenase systems requiring coenzymes I or II."

Jenson et al (1951) also found that certain dehydrogenase

[1] Color designation according to Ridgeway's Color Standard and Nomenclature (Washington D. C., published by the author, 1912). Plate I.

systems (of corn) reduce tetrazolium salts.

Thoneberry and Smith (1953) showed that tetrazolium salts caused "significant alterations" in respiratory metabolism of oat seeds. They stated that this might be the cause of the toxicity of tetrazolium salts and so might affect its usefulness for testing seed. However, many of the previous investigators (Porter et al 1947, Flemion and Poole 1948, Lakon 1949, On 1952, Parker 1953) found the germination per cent as determined by the tetrazolium method to be actually higher than the direct germination tests. Hence it would seem that the supposition stated by Thoneberry does not hold true.

In order to determine what staining standards to use, germinating seeds of the species under consideration were selected and put into the tetrazolium solution. These were examined as to intensity of staining and amount of staining. Some of the germinating embryos were not entirely stained, but more generally staining was complete.

Finally, since no exact correlation could be determined between embryo staining and germination, a more or less arbitrary standard was set somewhat on the order of that of Flemion and Poole (1948).

Class I (equal to germinative energy). Entire embryo stained evenly. Endosperm also stained (if present).

Class II (Total Class I and II equal to germinative capacity).

a. Embryo fully stained, endosperm partially stained (if present).

b. Endosperm fully stained, embryo 3/4 or more stained.

c. If embryo alone, embryo 3/4 or more stained.

Class III (Nonviable)

a. Embryo less than 3/4 stained, endosperm partially unstained or completely stained.

b. Embryo completely stained, endosperm unstained.

c. If embryo alone, embryo less than 3/4 stained, no staining at all.

Since unstained areas indicate necrotic or dead tissue, it was felt that any such area, one fourth of the seed or more in extent, would indicate considerable weakness in as much as a necrotic area would only develop where vitality is initially low. In addition, in the long period of pretreatment, such an area would be an ideal host for the many seed destroying fungi. Therefore, such a seed would be an exceedingly poor risk. A seed with three fourths of the embryo or more stained would have a much better chance of survival through to germination. Two recent papers have appeared (On 1952, Parker 1953) where the authors have taken the total coniferous seed into consideration instead of merely the embryo as has been done in the past. Both have proceeded on the seemingly valid theory that a necrotic area in the endosperm would indicate a weakness since a fungus attack on that tissue would more

than likely spread to the embryo.

While the author realizes that the endosperm of gymnosperm seed and that of angiosperm seed arise from two different tissues, the function of both is the same, i.e., that of food storage (Strasburger 1921, Priestley and Scott 1938). It seems reasonable then to assume that if necrotic tissue in the endosperm of the coniferous seed is detrimental, it will also be detrimental in the broadleaf seed. Therefore, the germinability of both the plum and green ash was determined using the entire seed, i.e., embryo and endosperm. The germinability of the box elder which has no endosperm was determined by use of the embryo alone.

APPLICATION OF STAINING STANDARDS

The standards, as previously described, were applied to each test at the end of the appropriate staining period. Each sample was separated into classes and each class counted after all the seeds in the sample had been classified. Germination energy and capacity were computed only after all the samples had been classified in an attempt to avoid bias.

There was no difficulty in classifying the box elder as the entire group of samples stained readily with the carmine red color mentioned previously. The plum was a little more difficult, there being some minor variation in shade. The green ash was extremely difficult as there were many shades of color varying from a pale pink to a good carmine red. The author decided to follow the example of Flemion and Poole (1948) and ignore the varying shades, classifying the seed by the proportion of the embryo and endosperm stained. Apparently the intensity of color is somewhat determined by the vigor of the seeds for in the rapid viability tests, those seed lots that turned green and grew quickly were the lots that had stained the most intensely. The results of the tetrazolium tests appear in Tables VII to IX.

Table VII

BOX ELDER TETRAZOLIUM TESTS

Sample	Class 1[1]	Class 1 & 2[2]	Class 3[3]
Lot # 3			
1	74	80	20
2	64	66	34
Average	69	73	27
Lot # 4			
1	60	82	18
2	70	94	6
Average	65	88	12
Lot # 5			
1	88	94	6
2	96	100	-
Average	92	97	3
Lot # 6			
1	96	96	4
2	94	96	4
Average	95	96	4

1 Class 1 equal to germinative energy.
2 Class 1 & 2 equal to germinative capacity.
3 Class 3 equal to inviable seed.

Table VIII

GREEN ASH TETRAZOLIUM TESTS

Sample	Class 1[1]	Class 1 & 2[2]	Class 3[3]
Lot # 21			
1	30	48	52
2	36	48	52
Average	33	48	52
Lot # 23			
1	12	34	66
2	10	32	68
Average	11	33	67
Lot # 101			
1	40	64	36
2	42	66	34
Average	41	65	35
Lot # 102			
1	8	48	52
2	28	54	46
Average	18	51	49

Table IX

WILD PLUM TETRAZOLIUM TESTS

Sample	Class 1[1]	Class 1 & 2[2]	Class 3[3]
Lot # 1			
1	0	2	98
2	0	8	92
Average	0	5	95
Lot # 2			
1	56	76	24
2	46	72	28
Average	51	74	26
Lot # 3			
1	0	0	100
2	0	0	100
Average	0	0	100
Lot # 4			
1	2	10	90
2	2	22	78
Average	2	16	84

1 Class 1 equal to germinative energy.
2 Class 1 & 2 equal to germinative capacity.
3 Class 3 equal to inviable seed.

DISCUSSION

Before considering the experiment, it seems advisable to examine the tetrazolium salts as viability indicators in view of the requirements of any new germination method before it can be accepted. As previously stated, the primary objectives of a germination test (U.S.D.A. Agricultural Handbook No. 30 1952) are "to provide accurate and reproducible information regarding purity, composition, rate of occurrence of noxious weed seed, and per cent of seeds that will provide normal plants under favorable conditions."

Purity, composition, and rate of occurrence of noxious weed seed would still have to be determined by the standard methods. Sampling techniques would probably remain the same, perhaps with some refinement to make them more applicable to taking samples directly in the field from small lots of seed.

As far as obtaining "accurate and reproducible information" is concerned, the tetrazolium, as any new method would, will have to be tested and retested and standards will have to be set and examined for each kind of seed, so that every germination laboratory will examine the stained seeds in the same light. The greatest obstacle in uniform reading of the tests would seem to be that of developing exact color standards. Since the seed with high vitality seems to react more strongly to the solution than that with low

vitality, some type of experimental work will more than likely have to be done to determine the shade of color when the seed vitality is strong enough to react with the chemical, and yet too weak to make a plant.

Porter et al (1947) made the statement that since the measurement of germinability with tetrazolium salts was not direct, but depended on all the factors affecting the oxidation-reduction potential of the cells, careful standardization of the procedure would be necessary. Flemion and Poole (1948), Bennett and Loomis (1949), Thoneberry and Smith (1953) all indicated that tetrazolium had good possibilities as a seed viability indicator, but it needed careful standardization of technique.

Lakon (1949) was the only author who suggested that tetrazolium was a better indicator of germinability than the standard germination tests. He said that statistical processing of several thousand tetrazolium tests showed that the error was always lower than the standard germination tests. He apparently had been able to interpret the staining more accurately than anyone else thus far.

The most difficult requirement to fulfill seems to be that of determining the number of "normal plants" that will grow. In preparing seeds for this experiment, the author has encountered abnormal seed which probably would account for some abnormal seedlings. These could be discarded before staining, and could thus be accounted for. However, some seed for example, the common bean

probably cannot be accurately tested by the tetrazolium method because there is no way of determining the number of "bald headed" seedlings that will occur (Porter et al 1947).

It would seem then, that before the use of 2,3,5-triphenyl tetrazolium chloride can be officially accepted as an indicator of seed germinability, each species would have to be thoroughly examined for staining standards and detection of abnormal seed.

However, for testing tree seed, where many species are dormant and pretreatment is extensive, this method of testing could be very valuable. It would simplify the present germination tests and more important would do away with the uncertainty that develops due to the pretreatment periods. The test is simple and rapid enough so that an individual could determine the germination per cent of a given lot of seed within a matter of twenty four to forty eight hours and could then order the proper amount of seed or adjust the price of the seed so that it would be on an equitable basis as far as the seed collection is concerned, a sample of seed could be collected in the field one day. The seed then tested and within a period of approximately three days field collecting could be done on a large scale with the knowledge that the seed is viable and worth collection. Much more must be done along this line to determine what effect moist, freshly picked seed would have on the tetrazolium solution and if the germinative capacity of this seed would change after drying and storage. A lot of coniferous seed recently tested by Dr. C. W. Waters showed a very high tetrazolium

germination rate. The same lot germinated very poorly in the sand flat test. Further investigation showed that many of the embryos were green. This indicated that perhaps the seed was picked too early and kept in a moist warm place where it continued to develop. The tetrazolium was applied when the seed had already passed from dormancy through the first stages of germination. When the lot was allowed to dry and was stored for a short while, the embryos died and the germination test was a failure.

The author tested freshly picked box elder seed, finding extremely high germination percentages, both with the tetrazolium and rapid viability tests. The same seed lots were stored dry for approximately six months and then retested. The viability in this case was only lowered two to three per cent.

Hence, the handling of the seed before the test is extremely important, and information as to time of picking and storage conditions perhaps should accompany the sample to be tested and should be considered when germination per cent is computed. How this could be done is beyond the scope of this problem but it must be kept in mind when considering the application of these tests.

For the past few years the Forest Nursery has had difficulty in obtaining satisfactory stands of the three species selected for this experiment. The seed has been pretreated as recommended and planted on appropriate dates for this area. In some cases the stand has been so heavy that it stagnated or was drastically thinned to prevent stagnation. In other cases the stand was extremely thin,

but the following year there was extensive delayed germination. In other instances the stand has been extremely thin with no germination the second year. These facts point not only to failure of germination tests to establish a sound basis for the correct sowing rate, but also to a possibility that the accepted standard in stratification may not be applicable in this area.

The author felt that the place to begin investigating these difficulties was with the seed and germination tests. At first there was some question of the amount of time that would have to be devoted to embryo excision and other seed preparation necessary to the tetrazolium tests. Different techniques were tried and those described selected as the easiest and quickest. With practice fifty seed samples of each species could be prepared in one half hour to forty five minutes and the total period covered by the longest test was seventy two hours. At the end of three days, then, germination figures could be obtained.

In order to have a comparison for the results of the tetrazolium tests, both sand germination and the rapid viability tests were used. Although the germination tests in the sand are generally accepted as the standard, it seemed better to use the rapid viability test as the standard in this case. Flemion (1948) found that there was a close correlation between the rapid viability tests and actual germination tests. It would seem that the tetrazolium and rapid viability tests both should be higher than the sand tests because there is no question of improper or incorrect pretreatment.

The seed is exposed to fungus infection only a fraction of the time it is when tested for the sixty to ninety day period as is usual with the sand method. Also, both the green ash and box elder should have diurnally alternating temperatures varying approximately twenty degrees. Since the sand tests were run in a green house where such temperature alternation was impossible, another factor leading toward a lowered germination rate was introduced.

Baldwin (1942) has published a chart showing the allowable variation between samples (Table X). The sand tests generally compare favorably with this table, but there are some instances where the variation is too high. It is assumed that some of these instances may have been influenced by one or more of the factors previously mentioned.

Both On (1952) and Parker (1953) used endosperm as well as embryo to determine viability of coniferous seeds. Although the endosperm of the broadleaf species arises from different tissue than that of the coniferous species, the function of both is to store food presumably used by the seed during germination. Therefore it seemed advisable to use the endosperm as an aid in determining germinative capacity on the basis that if the food supply is infected or destroyed, the embryo will be handicapped, and as a result might not make a plant. It would seem then that the tetrazolium test would be lower than that of the rapid viability test which uses only the embryo. The tests of the green ash, which was the one species of the three used with a significant amount of

endosperm should then have been lower than the rapid viability test. This was the case except with one seed lot. This evidence, while indicative is not conclusive, but in view of the basic premise the author feels that the endosperm should be included when testing such seed.

In a comparison of the results of the three different tests in regard to germinating capacity (Table XII) the tetrazolium and rapid viability tests compare very favorably, the mean variation between the mean of the samples running as follows:

Box Elder	5.2%
Green Ash	10.7%
Wild Plum	4.5%

The largest single variation (twenty four per cent) occurred in the green ash, which is better than expected in view of the work done by Flemion and Poole (1948) where a high variation of seventy per cent was found.

The variation between the tetrazolium and direct germination tests ran from fifty six per cent to five tenths of one per cent. The low occurred however in a lot of seed that was inviable for all practical purposes.

In spite of the lack of available information, the tetrazolium tests were used two years ago to adjust the sowing rates of different lots of green ash seed. The predicted variations were, in all cases, relatively successful, e.g., sowing rates were raised or lowered a recommended per cent. Where this was done, the stand was relatively even and where it was not done, the stand was very

Table X

TOLERANCE PER CENT
(Allowable Variation Between Samples)
(Baldwin 1942)

Mean Germination Energy Per Cent	Tolerance Per Cent
90 and above	6
80 - 89.5	7
70 - 79.5	8
60 - 69.5	9
Less than 60	10

Table XI

COMPARISON OF TETRAZOLIUM AND DIRECT GERMINATION TESTS (GERMINATIVE ENERGY)

Lot	Tetrazolium	Direct Germ.	Difference
Box Elder			
3	69.0	50.2	+18.8
4	65.0	18.2	+47.8
5	92.0	22.2	+69.8
6	95.0	36.0	+39.0
Green Ash			
21	33.0	5.0	+28.0
23	11.0	0.5	+10.5
101	41.0	15.7	+25.3
102	18.0	62.7	-44.7
Wild Plum			
1	0.0	1.0	-1.0
2	51.0	0.0	+51.0
3	0.0	0.0	0.0
4	2.0	0.2	+1.8

Table XII

COMPARISON OF TETRAZOLIUM, DIRECT GERMINATION, AND RAPID VIABILITY TESTS (GERMINATIVE CAPACITY)

Lot	Tetrazolium	Direct	Diff.	Rapid Viability	Diff.
Box Elder					
3	73.0	55.2	+17.8	64.0	+9.0
4	88.0	31.7	+56.3	81.0	+7.0
5	97.0	41.0	+56.0	94.0	+3.0
6	96.0	53.7	+42.3	98.0	-2.0
Green Ash					
21	48.0	27.7	+20.3	38.0	+10.0
23	33.0	18.7	+14.3	44.0	-11.0
101	65.0	43.5	+21.5	73.0	-8.0
102	51.0	72.7	-20.3	65.0	-14.0
Wild Plum					
1	5.0	7.0	-2.0	13.0	-8.0
2	74.0	30.2	+43.8	75.0	-1.0
3	0.0	0.5	-0.5	3.0	-3.0
4	16.0	6.0	+10.0	19.0	-6.0

uneven. The lots of seed tested in the experiment have all been planted and it is interesting to note that a good stand has been obtained in the box elder, which agrees with the tetrazolium and rapid viability tests rather than with the direct germination test. The plum and green ash have resulted in poor stands in areas where the poor seed was planted, and in good stands where the good seed was planted. In the areas where the sowing rate was increased as recommended the stands are relatively good except with one lot of the green ash. With the assistance of the nursery foreman, it was determined that this lot was planted deeper than the rest. This would most certainly have had a detrimental effect on seed with the lowered vitality as was previously suggested of lots 21 and 23 of the green ash.

Although the field applications of the tetrazolium tests have been promising and the rapid viability tests of germinative capacity compare well with those of the tetrazolium, the author can only conclude that the tetrazolium salts are well worth testing, but until the technique of interpreting the color reaction is more highly developed, it cannot be accepted as reliable.

However, it would seem advisable to continue this investigation. One additional step seems to be suggested in view of the present result, that being the testing of the seed both before it is placed in stratification and after the period of stratification has been completed. This appears adviseable in order to determine what differences, if any, will result from the pretreatment of these dormant seeds.

SUMMARY

Three species of broadleaf tree seeds of the dormant type have been tested with 2,3,5-triphenyl tetrazolium chloride to determine their germinative capacity by the staining method. Where it was present the endosperm as well as the embryo was used in making this determination. Direct germination tests in sand and the rapid viability germinative tests have been used as comparisons. The tetrazolium tests compare favorably with the rapid viability tests, but both are considerally higher than the direct germination tests as might be expected. Much more information must be gathered to determine effect of shade of staining and to prepare staining standards for interpretation of the stained seeds. Field planting of lots of seed tested seems to favor the suggested changes in sowing rate which were determined by the tetrazolium method.

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