THE USE OF TETRAZOLIUM CHLORIDE AS A MEANS OF DETERMINING SEED VIABILITY

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This is to certify that the

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The Use of Tetrazolium Chloride as a Means of Determining Seed Viability

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THE USE OF TETRAZOLIUM CHLORIDE AS A MEANS OF DETERMINING SEED VIABILITY

By

Joseph F. Delaney

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Joseph F. Delaney

A recent chemical test for determining viability in seeds involves the use of 2,3,5-triphenyltetrazolium chloride. Certain enzymatic systems of living tissue are capable of reducing the colorless solution of 2,3,5-triphenyltetrazolium chloride to a insoluble red precipitate called triphenyl formazan. The living tissue as a result appears bright red in color.

The test for viability in seeds is based on the fact that living seeds reduce the tetrazolium and become red while the dead seeds remain unchanged. Investigators to date have varied in their results. Some find the tetrazolium test agreed closely with germination tests especially with cereals and others have found wide variation in the comparisons of the two tests.

It is the purpose of this paper to contribute further information on the applicability of the tetrazolium test and to point out certain difficulties involved in both the mechanics of the method and interpretation of the staining patterns resulting in tests of certain seeds.

The tetrazolium test employed in this study was a modification of the Lakon Test. (1) The seeds were soaked in water overnight and sectioned through the embryo in a manner such that the radicle, hypocotyl, and plumule regions were bisected. The half seeds were placed in a 0.5% solution of 2,3,5-triphenyltetrazolium chloride for four hours at 30°C. At the end of the time they were removed from the solution and all embryos examined and classified according to staining. All tetrazolium tests were compared to standard germination tests.

In six of fourteen species of seed tested the percentage of embryos staining entirely in the radicle, hypocotyl, and plumule region agreed well with the percentage of germinating seeds. In three species no staining occurred in samples showing low germination. The staining patterns varied in intensity and amount of stained area. In other species no agreement between staining patterns and germination could be found.

Morphological features of certain of the seeds, such as hard seed coats and unsymmetrical embryos, made sectioning complicated and in some instances, impossible. Color of the embryo and dormancy of the seed also caused difficulty in comparison of the two tests.

In testing a series of elm seed samples having different viabilities, agreement between the tetrazolium test and germination was closed in three samples but differed widely in a fourth.

The tetrazolium test was found to be affected only by strongly acid or basic buffered solutions. A wide range of pH values from 6.6 to 9.0 resulted in similar staining patterns.

1. Lakon, G. Topographical tetrazolium method for determining germinating capacity of seeds. Pl. Physiol. 24:389-394. 1949

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INTRODUCTION

Seed investigators have long been interested in methods of measuring the germinating capacity of seeds without the necessity of a routine germination test. Though a germination test is probably the most accurate and most reliable test, it cannot be adapted to all circumstances. In many instances the time required to complete a germination test may make the results worthless. This is particularly true in determining the viability of dormant seeds. Many dormant seeds require several months of after-ripening before they will germinate under the proper conditions. Many times dealers in this type of seed cannot afford to wait through months of treatments when the seed lots are available and ready for planting. Events also arise frequently with non-dormant seed when the results of tests for viability are needed before germination tests can be completed. The investigations of Bennett and Loomis (3) and Goodsell (21) in determining freezing injury of seed corn are examples of such situations. As a result of such cases, many so called "rapid" methods of testing for seed viability have been devised.

LITERATURE REVIEW

Quick Tests in General

Probably the simplest of quick tests used by seedmen is to cut open the seed and examine it under a handlens. (19) Seeds are considered viable if the embryos are firm, plump and of good color. Abnormalities such as decayed, shriveled, rancid smelling embryos, empty or abortive seed etc. are considered non-viable. The results, however, are very general and many times very erroneous. (24)

Darsie, Elliot, and Pierce (7) based a viability test on the quantity of heat given off by germinating seed. They calculated the normal daily rise in temperature for a sample of highly viable seed. Any sample tested that had a rise in temperature lower than this normal was assumed to have a corresponding reduction in the vigor of that sample.

Waller (47) in 1901 attempted to determine viability in seeds by use of an electric current. Using an E.M.F. of 0.01 volt, he induced a current into the embryo and noted on a sensitive galvanometer the after-currents produced by the embryonic tissue. The characteristic after-currents of viable and non-viable seed were used as the basis for the test.

Fisk and Hibbard (14) in their method measured the electrical resistance of a volume of water in which the seeds had been soaking for a given period of time. The dead seed

permitted a greater outward diffusion of salts (electrolytes) than the live seed. Accordingly, a high resistance indicated high vitality and low resistance, low vitality.

The use of enzymatic activity for determining seed vigor has been reported by many investigators. The principle of the methods was based on the assumption that the activity of the enzymatic systems was directly related to viability of the tissue. Davis (8) utilized catalase activity as the basis for his method. He determined the ratio of the catalase activity of soaked seeds to that of dry seeds. If this ratio was greater than 1:1, they were considered to be highly viable. If it fell below 1:1, they were considered to be low in vigor. He stated that no definite conclusion could be drawn from the activity of dry seeds alone because the disappearance of catalase was found to lag behind the loss of viability. The soaking of the seed would rapidly disorganize the catalase activity of dead seed and permit true estimates of activity. Davis also used the phenolase activity as the basis for a similar test. (9) Mar (26) reported a method involving the amylase activity.

A method using excised embryos has shown promise as a rapid and accurate method of testing for viability. Flemion (15, 17) has reported good correlation between this method and germination with a wide variety of dormant and non-dormant seed. In her method excised embryos were placed on moist filter paper in Petri dishes. In noting their subsequent behavior, she could determine the viable from the

non-viable seeds. The viable seeds showed signs of development such as greening of the cotyledons, elongation of the radicle and hypocotyl, etc. The non-viable embryos rapidly deteriorated and became soft. If the embryos remained firm and did not show signs of development, they were classified as viable but low in vigor.

As early as 1876, investigators turned to chemical tests as means of detecting viability. Dimitriewicz (10) used sulfuric acid on sectioned seed. He noted that the viable seeds turned a deep rose color in two to five minutes and non-viable seed required fifteen minutes or longer for the same reaction.

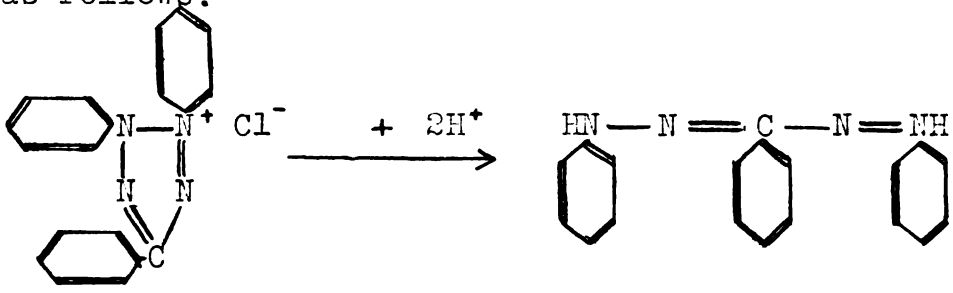
Gurewitsch (20) employed dinitrobenzene as the reagent in his method of staining viable seeds. Lesage (34) based his test on a distinct discoloration found in non-viable seed when treated with potassium carbonate.

Some of the more recent chemicals used to stain viable seed were sodium tellurate and sodium selenite. Hasegawa (26) reported that excised embryos stained dark indigo or black with sodium tellurate if they were viable. Browning or spotting indicated weak seed and lack of color was characteristic of dead seed. Eidmann (12) using sodium selenite, described a viable seed as staining a dark red with a solution of the salt.

Use of Tetrazolium chloride

The most recent chemical test showing much promise for determining tissue viability is the use of 2,3,5-triphenyl-

tetrazolium chloride. The preparation of the chemical dates back to 1894 when it was first prepared by Peckman and Runge.(40) The compound is one of the few chemicals that produces a color in its reduced state. When reduced, the readily soluble 2,3,5-triphenyltetrazolium chloride forms an insoluble red precipitate called triphenylformazan. The reaction proceeds as follows:



2,3,5-triphenyl-tetrazolium chloride

triphenyl formazan

Living tissue placed in a solution of 2,3,5-triphenyltetrazolium chloride reduces the salt and takes on a red color. This coloration is the basis of the test to differentiate between living and non-living tissue. The complete explanation of the cause of this reduction in living cells has not been determined. However, considerable research has been reported concerning systems in the living cells that may result in this reduction. Mattson, Jenson, and Dutcher (37) thought it might be caused by a dehydrogenase requiring coensyme I or II. They reported that glucose dehydrogenase plus coenzyme I, in the presence of its substrate, reduced the salt at pH 6.3. They found that many tissues such as the fleshy parts of apples, oranges, and grapes, the gill area

of mushrooms, carrot roots, white and sweet potato, young leaves, and the stigmas and ovaries of certain pollinated flowers, readily reduced the salt.

Dufrenoy and Pratt (11) found that the sites of reducing activity within the cells of sugar cane coincided with the location of oil droplets, whether in the cytoplasm near the plasmodesmata or between starch grains within the amyloplasts. The treatment of the sections with reagents for phosphatase and for phosphate showed that the sites of reduction of the tetrazolium salts were also sites of intense positive reaction for phosphate ions.

Kun and Abood (28) estimated the activity of succinic dehydrogenase in tissue extracts using tetrazolium chloride.

Fred and Knight (20) stained Penicilium chrysogenum with tetrazolium chloride at a pH of 7.2 to 7.4. They reported that the reaction was retarded at lower pH and virtually stopped at pH 6.0. Potassium cyanide in M/100 concentration inhibited the reaction. Sodium malonate, sodium azide, 2,4-dinitrophenol, sodium fluoride and iodoacetic acid merely slowed reduction at M/100 concentrations. These chemicals are known to affect specific enzyme systems in living tissue.

Jensen, Sacks, and Baldauski (26) applied tetrazolium in a test to determine the presence of certain dehydrogenases in plant tissue homogenates. They reported that many of these dehydrogenase systems requiring DPN and TPN (di and triphosphopyridine nucelotide) reduced the salt. They gave the redox

potential of tetrazolium as minus 0.08 at pH 7.0. The systems requiring DPN which they listed as reducing the salt were glucose dehydrogenase, alcohol dehydrogenase, lactic acid dehydrogenase, malic acid dehydrogenase, beta-hydroxylbutyric dehydrogenase, 3-phosphoglyceraldehyde dehydrogenase, and alpha-glycerophosphate dehydrogenase.

Isenberg, Odland, Popp, and Jensen (25) also used tetrazolium chloride to determine the effect of maleic hydrazide on the succinate, fumarate, malate and pyruvic dehydrogenase systems in onion plants.

Although the complete explanation of the reduction has yet to be determined, the literature to date indicates that many systems in the normal cell have the ability to reduce tetrazolium chloride.

Besides enzyme studies, tetrazolium has been used in other capacities. Kuhn and Jerchel (27) found the salt stained viable cultures of bacteria and fermenting yeasts. Waugh (48) tested the stem tissue of trees and shrubs. Straus, Cheronis, and Straus (46) demonstrated the staining of neoplasms in mammalian tissues. Black and Kleiner (4) studied respiration in various tissues of mice using tetrazolium. Evans and Earle (13) found that the salt aided in studying cancerous growths in humans. The chemical has also been used in a quantitative colorimetric test for reducing sugars. (38)

Application to Seed Testing

Probably the most important application of the use of tetrazolium chloride has been in the field of seed testing. The method of testing seeds was first proposed by Lakon (30) in Germany in 1942. He reported a good correlation between this test and viability in testing seeds of oats, barley, wheat, rye and corn. Since his first investigation he has improved his method and has had it adopted by the German Seed Testing Stations as the official method of determining the germination capacity of cereals and maize. (33)

Lakon used a 1 percent solution of 2,3,5-triphenyltetrazolium chloride buffered between pH 6 and 7. He first soaked the grains of wheat, rye and barley in water overnight (six to eighteen hours). The embryos were then excised from the starchy endosperm and pericarp and placed in the tetrazolium solution, making certain that all the embryos were completely submerged. They were kept thus at room temperature for seven or eight hours. At the end of this time the embryos were removed and examined for staining. He classified as viable only those embryos that had stained red in the regions of the plumule and adjacent tissue bearing root primordia.

In testing corn, Lakon modified his technique. He bisected instead of excising the embryo. He also classified as viable all those corn embryos that had stained either completely or at least in the region of the shoot, including initials of the secondary radicles and the scutellum. He stated that as

long as the corn embryo could produce good secondary roots it would produce a normal seedling.

Porter (42) in his review of seed technology considered the tetrazolium test as being superior to the other rapid methods and having considerable practical application for a quick evaluation of certain kinds of seed, especially of the Gramineae. Porter, Durrell, and Romm (41) tested various samples of seeds of several members of the grass family, seeds of three leguminous species, and seeds of buckwheat and cotton. In a number of instances good correlation was obtained between the staining test and germination test. They encountered difficulty with hard seed in cotton. They also reported that it was impossible to section regularly through the epicotyl or plumule region of the Legumes. This difficulty nullified the test in determining the so called "baldhead" injury in this group.

Cottrell (6) reported close correlation between the tetrazolium test and germination tests of barley, wheat, peas, and vetches. She pointed out that the test was limited by the size of the seed being tested. Seeds of species such as mustard, turnip, etc. would not allow detailed examination, especially in the case of embryos that were extremely small as in the parsnip or folded as in the mustard.

Sheul (44) reported that the test was reliable for fresh seed but that with older seed (viability less than 60 percent) it was much less accurate. The resulting coloration ranged

from a carmine to a light pink in the viable seeds of barley and oats. He could not find a correlation between the light staining and slowness of germination.

Goodsell (21) employed the tetrazolium test in making early appraisal of minimal freezing damage to seed corn. He found that the test could be used directly if the moisture content of the grains was less than 49 percent when the freezing occurred regardless of the amount of freezing. If the moisture content was higher, the grains had to be dried prior to testing in order that the injured kernels might lose their ability to reduce the tetrazolium solution. He concluded that the delay in breakdown of the enzyme systems was responsible for the reduction in the grains having high moisture content.

Bennett and Loomis (3) also reported difficulty in applying this test to freshly frozen seed corn. They stated that the dead kernels slowly lost their ability to reduce tetrazolium but that after several days or weeks the Lakon test could be applied.

Flemion and Poole (18) tested a wide variety of dormant and non-dormant seed with the tetrazolium method, using their excised embryo method as a standard. They reported a significant correlation between the two methods but found large and frequent deviations in individual tests.

The results of the previous investigations in determining seed viability with tetrazolium chloride have varied. Some reported good corfelation with germination tests; others

have found a wide diversity of staining patterns that could not be correlated with germination. It is the purpose of this paper to contribute further information on the applicability of the tetrazolium test and to point out certain difficulties involved in both the mechanics of the method and the interpretation of the staining patterns resulting in tests of certain seeds.

MATERIALS AND METHODS

The majority of the seeds used in this study was gathered in the vicinity of Michigan State College, East Lansing, Michigan. The collected seed was dried at room temperature and stored in sealed containers in the laboratory until utilized.

Both germination and tetrazolium tests were carried out on all lots of seed. The germination test was used as a standard in evaluating the results of the tetrazolium test. As nearly all of the seeds used in this study had some type of dormancy, various treatments to overcome this dormancy were necessary before germination could take place. A summary of these treatments along with the references to the recommended treatments is given in Table I.

After the treatments used to break dormancy, the seeds to be germinated were placed in moist paper toweling and placed in a germinating chamber at a temperature alternating between 25°C for sixteen hours and 30°C for eight hours.

The tetrazolium test was a modification of the method used by Lakon. (32) The tetrazolium chloride was dissolved in distilled water and made up to a 0.5 percent solution. Since this chemical is light sensitive, the solution was stored in the dark.

The seeds to be tested with the tetrazolium solution were first placed in moist paper toweling and allowed to absorb

water overnight. They were then carefully sectioned with a sharp razor blade through the embryo. The cut was made to pass through the radicle, hypocotyl, plumule, and cotyledons. The first three regions were those considered by Lakon as important in estimating viability. Cottrell (6) reported that the cotyledons did not necessarily have to be stained entirely. She classified the seeds viable as long as some staining developed in the cotyledons along with complete staining of the other regions of the embryo.

However, not all the seeds in this investigation could be sectioned in a manner such that the cut surface passed through all regions of the embryo. The folded cotyledons in Acer and the offset radicle in Ailanthus, for example, make sectioning difficult. Plate A shows sketches of the various seeds studied and the dotted line indicates the plane of sectioning which proved most effective for the test. Due to the small size of the seed, a disecting microscope was employed in some cases to insure the cutting of the essential parts.

As the seeds were bisected, one half of each seed was placed in distilled water in a vial until one hundred seeds were cut. The water was then poured off and the seeds covered with a 0.5 percent solution of tetrazolium chloride. The vials containing the seed were placed in a 30°C chamber for four hours. At the end of this time the seeds were removed and the embryos examined for staining.

TABLE I

TREATMENTS USED TO OVERCOME DORMANCY

Kind of Seed	Treatment to break Dormancy	Reference for treatment recommended
Acer negundo L.	moist paper toweling 5 C., 2 months	Chadwick (5)
Acer platanoides L.	moist paper toweling 5 C., 3 months	Chadwick (5)
Ailanthus glandulosa Desf.	moist paper toweling 5C. 30 days	U. S. D. A. (19)
Betula papyrifera Marsh	moist paper toweling 5 C., 2 1/2 months	Chadwick (5)
Catalpa speciosa Warder	moist paper toweling 5 C., 2 months	For.Res.Dig. (29)
Crataegus spp.	3 hrs. sulfuric acid, 3 hrs. washed in running water, 3 weeks at 25 C. 3 weeks at 5 C. in moist peat	Flemion (16)
Fraxinus pennsylvanica Marsh	moist paper toweling 5 C., 3 months	For. Res. Dig.(29)
Gleditsia triecanthos L.	1 hr. sulfuric acid, 10 min. washing in running water	Chadwick (5)
Maclura pomifera (Raf.) Schneid.	moist paper toweling 5 C., short period	For. Res. Dig.(29)
Pinus densiflora Sieb. & Zucc	moist paper toweling 5 C., 3 months	Chadwick (5)
Platanus occidentalis L.	moist paper toweling 5 C., 2 months	U. S. D. A. (19)
Robinia pseudoacacia L.	1 hr. sulfuric acid, 10 min. washing in running water	Chadwick (5)
Thuja occidentalis L.	moist paper toweling 5 C., 2 months	Barton (1)
Tilia americana L.	extract seed from pod, 20 min. in sulfuric acid, 2 hr. washing in running water	Barton (2)

Plate A. Sketches showing internal structure of the various seeds tested. The dotted line indicates the plane of sectioning for the Tetrazolium Test:

A. Acer negundo, 5X; B. Acer platanoides, 4X; C. Ailanthus glandulosa, 1X; C. Betula alba, 6X; E. Catalpa speciosa, 1.5X; F. Crataegus spp., 6X; G. Fraxinus pennsylvanica, 1X; H. Gleditsia triacanthos, 3X; I. Maclura pomifera, 9X; J. Pinus densiflora, 7X; K. Platinus occidentalis, 5X; L. Robinia pseudoacacia, 11X; M. Thuja occidentalis, 5X; N. Tilia americana, 10X; O. Ulmus americana, 7X.

Drawings reproduced from reference 19.

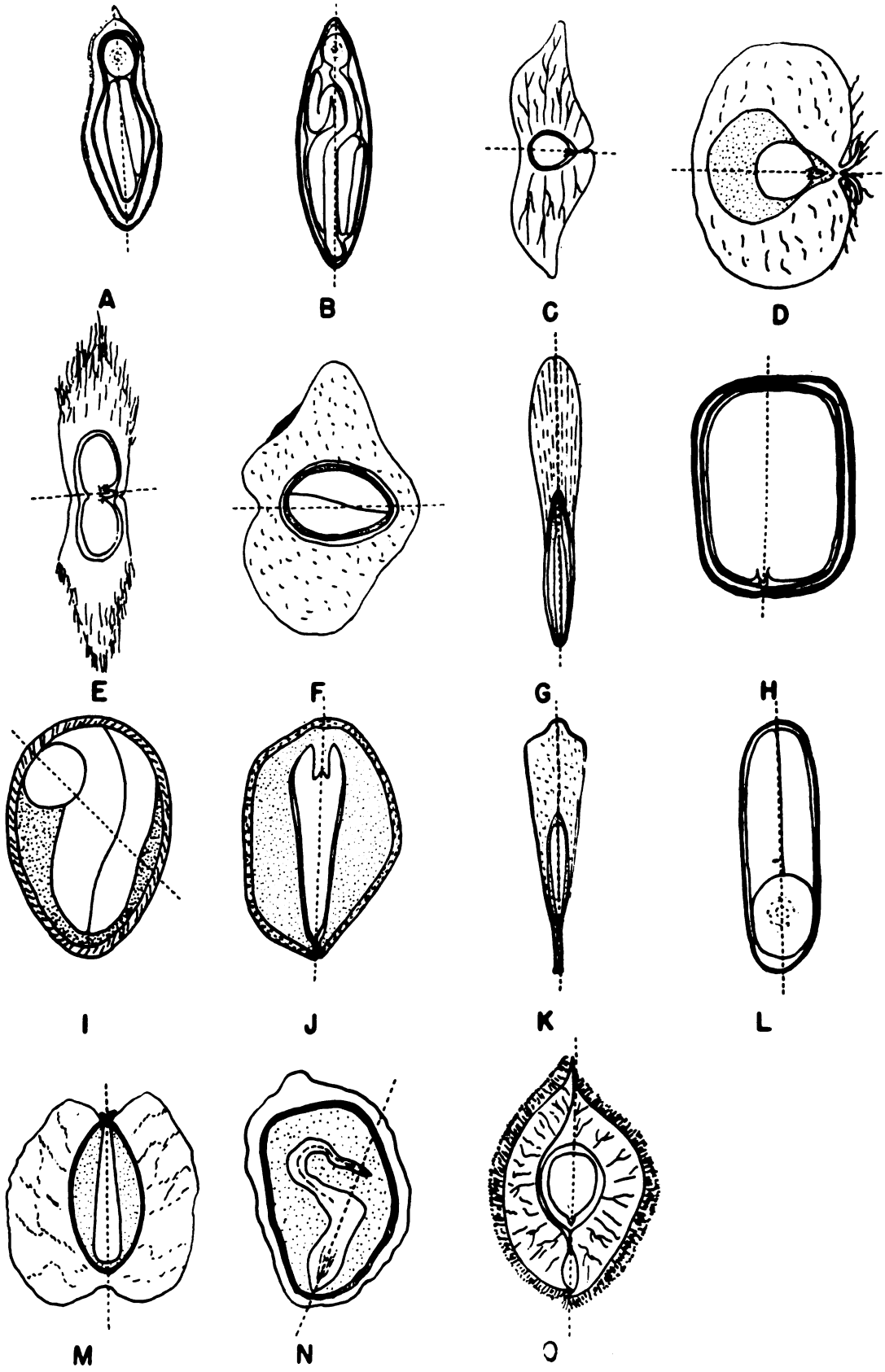


Plate A

RESULTS OF TESTS

A summary of the percentages of viability determined by germination and the tetrazolium test is given in Table II. The percent germination in all cases was based on the number of seedlings showing good development and normal in every respect. Those seeds that were hard, insect injured, diseased, decayed, or empty were classified as non-germinated.

With the seeds that were sectioned and soaked in tetrazolium solution, a wide range of staining patterns resulted. Some stained a deep red throughout; others stained only partially and still others produced no stain at all. In order to reduce the number of types of staining patterns, arbitrary groups were set up based on the percentage of stain appearing in the entire region of the radicle, hypocotyl, and plumule. Those that were entirely stained were again subdivided into heavy, medium and light staining. Table III shows the percentage of seeds classed in the various groups.

To arrive at the percents of viability with the tetrazolium test, the staining groups were compared with the percents of actual germination. The comparison indicated in a general way that only those embryos staining entirely would be viable. Using this criteria for classification, six of the fourteen species of seeds compared favorably with germination. Those species were Acer negundo, Ailanthus glandulosa,

Fraxinus pennsylvanica, Gleditsia triacanthos, Maclura pomifera, and Pinus densiflora. Two other species, Acer platanoides and Catalpa speciosa, were classified slightly different to bring them in agreement with the percent germination. The slightly stained embryos of A. platanoides were not counted in the total and the three percent of the embryos of Catalpa speciosa that were placed in three-fourth stained were counted in the total viable. Explanation of these variations in classification will be given under the detailed description of the individual species.

As shown in Table II, comparisons using the described classification were not favorable in the remaining six species. Wide variations in the two tests are apparent. In order to point out these individual variations and characteristic features of the seed that may cause the variation, each specie is described separately.

TABLE II

COMPARISON OF THE MEAN PERCENT VIABILITY AS DETERMINED BY GERMINATION AND TETRAZOLIUM TESTS

Kind of Seed	Viability			
	Germination Test		Tetrazolium test	
	No. of Seed	Mean Percent	No. of Seed	Mean Percent
Acer negundo	400	57	400	51
Acer platanoides	400	59	400	65
Ailanthus glandulosa	400	96	400	97
Betula alba	400	6	400	0
Catalpa speciosa	400	96	400	92
Crataegus spp.	400	3	400	42
Fraxinus pennsylvanica	400	41	400	40
Gleditsia triacanthos	400	88	400	92
Maclura pomifera	400	100	400	99
Pinus densiflora	400	81	400	75
Platanus occidentalis	400	88	400	5
Robinia pseudoacacia	400	43	400	30
Thuja occidentalis	400	56	400	0
Tilia americana	400	18	400	79

TABLE III

PERCENTAGE OF SEEDS CLASSIFIED ACCORDING TO STAIN IN TETRAZOLIUM TEST

Kind of Seed	Completely stained			3/4 stained	1/2 stained	1/4 stained	No stain	Hard seed	Insect injured	Dried	Empty
	Heavy	Medium	Light								
Acer negundo	50		1				1		48		
Acer platanoides	12	53	21[a]	1			3				10
Ailanthus glandulosa	67	30					3				
Betula alba							9			91	
Catalpa speciosa		88	1	3[b]							8
Crataegus spp.	33		9				5			8	45
Fraxinus pennsylvanica	25	9	6		3	1	11		25	20	
Gleditsia triacanthos	92				2		2		4		
Maclura pomifera		99								1	
Pinus densiflora	41	25	9	1	1	2	1			20	
Platanus occidentalis	3	1	1		2		19			74	
Robinia pseudoacacia	29	1		2			1	67			
Thuja occidentalis							44			56	
Tilia americana	79			6		1	2		12		

[a] Not included in Total of Table II

[b] Included in Total of Table II

Acer negundo L.

The embryos were easily sectioned, although the folded cotyledons permitted only irregular portions to be tested. A large percentage showed insect injury. All except one percent of those considered viable were stained bright red throughout.

Acer platancides L.

The difficulty of sectioning this specie was similar to A. negundo. As was stated previously, the group classified as slightly stained was considered non-viable. The greenish color of the embryo interfered with the staining. This lightly stained group was a muddy brown color whereas the two viable groups were distinctly red.

Ailanthus glandulosa Desf.

The embryos were easily sectioned if removed from the pericarp. With pericarp still attached, difficulty was encountered in accurately bisecting the radicle. The embryos stained well and none with less than complete staining were classified as viable.

Betula alba Marsh.

The seeds were small and required magnification in order to section them properly. Germination was very low and the cutting operation with the tetrazolium test showed nearly all the seeds to be shrivelled and dried. Nine percent, however, did appear normal, having a cream color and firm consistency. None of this latter group showed any staining with tetrazolium.

A second tetrazolium test was made on the following year's crop and again no staining resulted. No germination test was made on this second lot due to the time required to after-ripen the seed.

Catalpa speciosa Warder

Removal of the pericarp also facilitated sectioning with this specie. As is shown in Plate A, the cotyledons are attached to the hypocotyl opposite each other. It was impossible to bisect either cotyledon and the growing points at the same time. The plane of sectioning passed through the radicle and hypocotyl at right angles to the cotyledons. The whole cotyledon that remained attached stained well nevertheless.

The staining in the embryos was more of a medium red than in the other species. The staining was complete except in a few cases in which the extreme tips of the radicle were not stained. This lack of staining was thought to be due to injury resulting from the cutting operation. Those having this slight unstained area were considered viable.

Crataegus spp.

The treatment to overcome dormancy with this specie was extreme. (see Table I) The hard outer seed coat which is impermeable to water was also difficult to section. The seeds were treated with sulfuric acid previous to sectioning in an attempt to facilitate the cutting operation. Many of the seeds were found to be empty. Of those having what appeared to be normal embryos, the majority stained either bright red

or a medium shade of red. Both colors were taken to indicate viability. The low percentage of germination may have resulted from an incomplete treatment to overcome dormancy. The comparison between the two methods of testing showed a wide variation in the estimate of viability.

Fraxinus pennsylvanica Marsh.

The seeds, once removed from the pericarp, were easily sectioned. The staining, however, varied considerably. As is shown in Table III, nearly all patterns were found in this specie. The staining in the cotyledons also varied from complete staining through a graded series to no staining whatever. The seeds taken as viable were those whose radicle, hypocotyl, and plumule region stained entirely regardless of the staining of the cotyledons.

Gleditsia triacanthos L.

Once treated with sulfuric acid as shown in Table I, these seeds readily absorbed water and were easily sectioned. The staining was bright red in all the seeds taken as viable.

Maclura pomifera (Raf.) Schneid.

The structure of this seed did not permit the bisecting of all parts. The radicle tended to wind its way along one side of the seed. The staining of this specie was pink throughout the cotyledons, hypocotyl, and plumule but had bright red radicles. The staining pattern was consistent throughout all the seeds.

Platanus occidentalis L.

The seed was rather small but could be readily sectioned under the disecting microscope. The majority of the embryos were dried and brown in color when cut. Of the embryos that appeared normal about one fourth stained with tetrazolium. This stained group was considerably lower than the percent of germination.

Robinia pseudoacacia L.

The seeds of this group were first treated with sulfuric acid and then allowed to absorb water. Many, however, remained hard and could not be sectioned without shattering the dry embryo. Those that swelled could readily be sectioned although the plane of sectioning passed between the cotyledons. As germination was carried out over a longer period, many of the hard seeds germinated after a time. With the tetrazolium test, only the seeds that swelled immediately were sectioned and tested.

Thuja occidentalis L.

The seeds were small but could be sectioned without difficulty under the disecting microscope. Many were dry on cutting but of those appearing normal, no staining resulted. A second lot of the same crop was tested but again no staining took place in those that appeared normal.

Tilia americana L.

Seeds were removed from the pericarps and treated with sulfuric acid. After soaking in water overnight, they were easily sectioned. The irregular cotyledons made the plane of sectioning difficult to determine. (see Plate I) The seeds of this specie, similar to those of Crataegus, required extreme treatment to overcome dormancy. In the germination test the large majority of the seeds not germinating were still firm at the end of the test. All the seeds classed viable in the tetrazolium test stained bright red throughout.

SPECIAL TESTS ON ULMUS AMERICANA SEED OF REDUCED VITALITY

Further experiments were undertaken to study the effect of the tetrazolium staining on seeds that were beginning to lose their viability. Most investigators have found that the tetrazolium test works well with seeds that are either of high vitality or very low vitality. The difficulty arises in estimating viability of seed lots that are somewhere between the two extremes.

An attempt was made to obtain within one species of seed, samples having a high, intermediate, low and no viability. The seed utilized for this study was Ulmus americana, due to the abundant crop that was available in East Lansing in the spring of 1950. The seed was gathered and dried at room temperature for five days. To obtain a graded series of viabilities, storage conditions were set up that would produce differential rates in the loss of viability. According to Steinbauer and Steinbauer (45), Ulmus americana maintained its viability up to nine months while stored under conditions of low moisture and low temperature. An increase in temperature or moisture content caused a dropping off in the germinacapacity of the seed.

In this study four sets of seed were placed in sealed containers over various concentrations of sulfuric acid. The concentrations selected were those calculated to produce relative humidities of the air in the containers of 10, 30, 60,

and 85 percent. (35) At these relative humidities the moisture content of the seed would vary from a relatively high moisture content to one fairly low. (39) The seeds were maintained at room temperature under these conditions for four months. At the end of this time both germination and tetrazolium tests were performed on all lots.

The results of the experiments are given in Table IV. The complete range of viabilities desired was not obtained since the maximum germination was 89 percent. The storage conditions did, however, result in a sliding scale of viabilities from 89 percent to 0 percent.

As in the previous studies, the degree of staining that corresponded to the percent germination was determined. Plate B shows the varied staining patterns resulting from the tetrazolium test of all lots. The letters found in groups 1, 2, 3, and 4 under the tetrazolium test of Table IV correspond to the staining patterns listed in Plate B. The sum of all the seeds having those types of patterns is the estimated viability of the tetrazolium test.

With certain combinations of patterns, the lots stored at 10, 30, and 85 percent relative humidity could be compared favorably but the lot stored at 85 percent showed great variation. Its lowest estimate was still higher than the germination percentage of the 10 percent storage.

TABLE IV

COMPARISON OF VIABILITY OF ULMUS SEEDS AS DETERMINED BY GERMINATION AND TETRAZOLIUM TESTS

Method of estimating Viability	Storage Condition Percent Relative Humidity			
	10%	35%	60%	85%
Germination	89	87	5	0
Tetrazolium				
Group 1 (A,B)[1]	88	47	0	0
Group 2 (A,B,C)	91	49	1	0
Group 3 (A,B,C,D)	89	49	1	0
Group 4 (A,B,C,D,E,F)	65	71	5	0

[1]Letters correspond to staining patterns shown in Plate B.

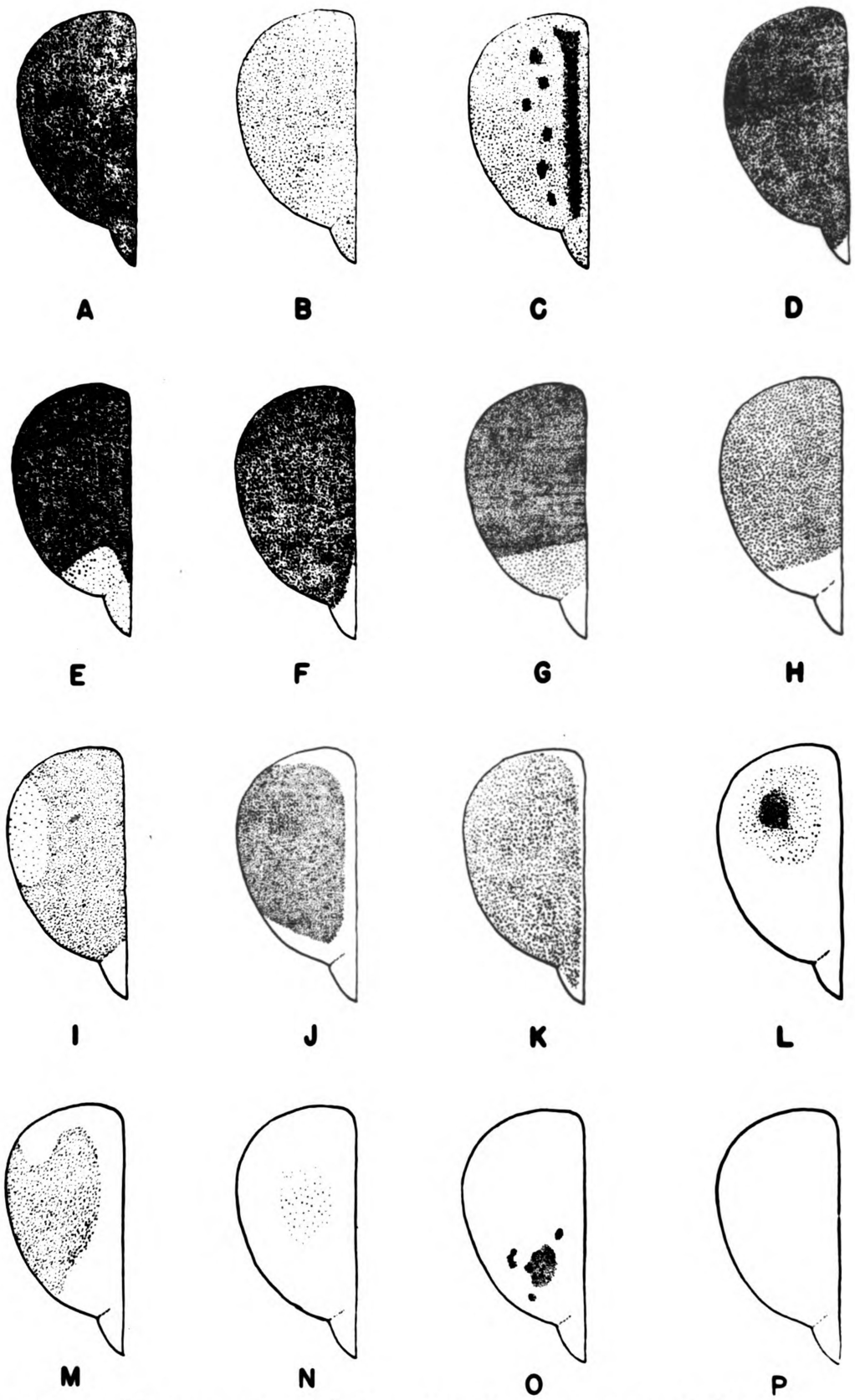


Plate B. Patterns of staining in the embryos of Ulmus americana. Intensity of stippling corresponds to the intensity of the stain. A. Maximum stain P. No stain 10X normal.

EFFECT OF pH ON TETRAZOLIUM TEST

Studies were made of the effect of pH on the staining patterns of seeds in the tetrazolium test. Previous investigators have buffered their solutions at various pH values. Lakon (33) used a solution buffered at pH 6 to 7. Goodsell (21) employed a solution as high as pH 13.21. Bennett and Loomis (3) reported that corn embryos stained well between pH 6.8 and 8.0 but tended to stain only faintly at lower pH values.

To study the effect of pH on the degree of staining, various buffered solutions of tetrazolium chloride were made up using Coleman Buffer Tablets. The range of pH was from 4.0 to 11.5 with increments of 0.5. Seeds of Linum usitatissimum (Flax) and Helianthus annuus (sunflower) were utilized for this study. The seeds were sectioned and placed in distilled water in Petri dishes. In order that all groups might receive the same treatment, the seeds as they were cut were placed one to a dish until all sixteen dishes had one half seed. The process was then repeated until all dishes had fifty half seeds. The water was rapidly poured off and the various buffered solutions of tetrazolium chloride were poured in, covering the seeds. The dishes were then transferred to a constant temperature germinator and maintained at thirty degrees centigrade in the dark for four hours. At the end of this time they were removed and the embryos examined for staining.

A summary of the staining produced at each pH value is given in Table V. The results showed that a uniform bright red staining occurred over a wide range of pH values from pH 6.5 to 9.0. The lower pH values resulted in a lighter stain and at pH 4.0 hardly any staining took place. At the higher pH range, the solution itself tended to turn a yellowish orange color. The staining in this upper pH range was very intense however. Even the decayed tissue turned a yellow color. With these two species, apparently only the strongly acid or strongly basic solutions interfere with the staining.

TABLE V

STAINING IN SEEDS OF FLAX AND SUNFLOWER AT VARIOUS pH VALUES

pH Values	Flax	Sunflower
11.5	solution in slightly yellow-orange in color	solution slightly yellow decayed areas yellow
11.0	very deep red in live tissue	very deep red
10.5	solution slightly yellow	solution slightly yellow
10.0	tissue deep red, solution clear	same as Flax
9.5	deepening of red color	same as Flax
9.0	deep red in living tissue, dead areas yellow	staining becoming deeper than lower pH values
8.5	bright red	slightly darker red than pH 8.0
8.0	" "	bright red
7.5	" "	" "
7.0	" "	" "
6.5	" "	" "
6.0	slight degree lighter	" "
5.5	lighter than pH 6.0	" "
5.0	light pink	slight degree lighter
4.5	faint pink	lighter than pH 5.0
4.0	very faint or none	noticeably less, approximately 1/3 that of pH 7.0

DISCUSSION

The experiments reported in this paper were carried out with the intention of determining patterns of staining that would yield a reliable estimate of viability. The results of these experiments have failed to show any general type of staining that could be used. With six of the fourteen species in the assorted list of seeds and three degrees of viability of the elm seed, the staining of the entire region of the hypocotyl, plumule, and radicle, either light or dark could be used in good agreement with results of germination tests. With the remaining species, variations of this type of staining had to be used as an index of viability. For instance with Acer platanoides, the seeds thought to be lightly stained had to be eliminated to bring the estimate of viability in accord with germination. No adjustment could be made in the elm sample stored at 35 percent relative humidity. With this sample the embryos that stained entirely far outnumbered the seeds actually germinating.

Similarly with the samples of Betula alba, Platanus occidentalis, and Thuja occidentalis, no combination of staining patterns could bring the estimate of viability to correspond with germination.

The two species, Crataegus spp. and Tilia americana, showed wide variation in comparisons of the two tests. They

illustrate a difficult problem in comparing a test such as the tetrazolium test to the standard germination test. Both of these species of seed are known to be extremely dormant. (18, 2) They require not only scarification with sulfuric acid but long periods of after-ripening before germination can take place. The reported germination of these species in this paper was probably far below their potential germination. They also illustrate the usefulness of the tetrazolium test, if properly interpreted, in investigations of dormancy in seeds. The germination test would seem to indicate that seeds of these species were almost worthless and yet the tetrazolium test if reliable shows them to be potentially high in viability.

As shown earlier, many investigators have reported good correlation between the results of germination and tetrazolium test for viability with seeds of cereals. The fault in applying this test to other types of seeds such as those used in this study may be in the standard of comparison utilized. Flemion (18) used her so called "rapid" method of testing seeds in which she extracted the embryos and noted their behavior on moist blotters. This method has not been used widely due probably to the difficulty of extracting the embryos. Many difficulties were encountered using the standard germination in this study as a standard of comparison. Most of the seeds tested had some degree of dormancy. Whether the treatments completely overcame dormancy is uncertain. In the case of Tilia americana and Crataegus spp. it is very doubtful.

Besides the variation in staining and the interference of dormancy, other difficulties were encountered. The proper sectioning of the seed prior to treatment with tetrazolium solution constituted a problem. Porter et al. (41) found that seeds of Legumes could not be sectioned regularly through the plumule and consequently the injury known as "baldheads" could not be detected. The "baldhead" condition in Legumes classifies the seed abnormal according to the Official Rules of Seed Testing. (43) The seeds of Maclura and Tilia, for example, as shown in Plate A offered a problem in sectioning all regions of the embryo.

The size of the seed as pointed out by Cottrell (6) would probably limit the type of seeds to be tested by tetrazolium chloride. She doubted if seeds the size of parsnip or the folded condition in the small mustard seeds could be properly sectioned and observed for staining in all essential regions.

Hard seeds such as were present in the Robinia samples in this study present a problem. They sometimes remain in the "hardseed" condition for many weeks before they become permeable and swell. The sectioning operation shattered the dry embryos. Some correction factor will have to be introduced to compensate for these dry hard seeds.

Another problem noted by Flemion (18) and also noted in this study was the factor of color in the embryo. Most embryos tend to be white or cream colored and the red staining of the tetrazolium chloride produces a good contrast. But in

samples such as Acer platanoides, the green color of the embryo interfers with the red staining, especially when the staining is light.

Nevertheless, the tetrazolium method even in its present state has many advantages over the other rapid methods of testing seed. It has been shown to indicate a fair evidence of viability. This plus the fact that it can easily be carried out, makes the test of great value to nurserymen and dealers in all types of seed. The test can be performed over a wide range of pH values at ordinary room temperature. It does not require elaborate equipment. Light, however, does interfere with the test.

But before this method can be used with accuracy in tests of all types of seed, more detailed studies must be made on the seeds that are low in vigor. Tests based on enzymatic activity, such as the tetrazolium test, all have the inherent problem of these low vigor seeds. In considering the tetrazolium method, Jenson et al. (18) felt that the lack of stain with the tetrazolium does indicate a loss in viability but that the positive stain does not necessarily indicate viability. It has been shown in the review of literature that many systems in the living cell reduce the salt. It may be possible, especially in lightly stained seeds, that the reduction is the result of an enzymatic system yet to be inactivated in an embryo that, for all practical purposes, can be considered dead.

SUMMARY

1. The Lakon Test for seed viability using 2,3,5-triphenyl-tetrazolium chloride was performed on a variety of sectioned seeds. The resulting staining patterns were compared to standard germination tests.
2. In six of the fourteen species of seed tested the percentage of embryos staining entirely in the radicle, hypocotyl, and plumule region agreed well with the percentage of germinating seeds. In three species no staining occurred in samples showing low germination. The staining patterns varied in intensity and amount of stained area. In other species no agreement between staining patterns and germination could be found.
3. The morphological features of certain of the seeds such as hard seed coats and unsymetrical embryos, made sectioning complicated and in some instances, impossible.
4. Color of embryo and dormancy of the seed also caused difficulty in comparison of the two tests.
5. In testing a series of elm seed samples having different viabilities, agreement between the tetrazolium test and germination was close in three samples but differed widely in a fourth.
6. The tetrazolium test was found to be affected only by strongly acid or basic buffered solutions. A wide range of pH values from 6.6 to 9.0 resulted in similar staining patterns.

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