THE USE OF 2,3,5-TRIPHENYL-TETRAZOLIUMCHLORIDE AS A MEASURE OF SEED GERMINABILITY[1]

R. H. PORTER, MARY DURRELL, AND H. J. ROMM

(WITH ONE FIGURE)

Received June 13, 1946

Seed analysts have long been interested in methods of measuring the germinability of seeds without the necessity of a routine germination test, particularly when dealing with dormant seeds or with seeds requiring a long period for the completion of a test. Even with seeds that can be tested in a week or 10 days it is often desirable to know within a day the general condition of a seed lot. This is especially important in the fall season when it is necessary to know whether or not an early killing frost has caused damage to the seed crop of corn, sorghum, and soybeans.

For dormant seeds, especially the peach, apple, pine, Douglas fir, plum, hawthorn, European mountain ash, witch hazel, and *Rhodotypos kerrioides*, normally requiring a long period of after-ripening, it has been shown by BARTON (**1**), FLEMION (**6, 7, 8, 9**) and others that by excising the embryos and placing them on moist filter paper in Petri dishes at room temperature it is possible to determine their vitality within a period of 5 to 10 days. CROCKER and HARRINGTON (**2**), DAVIS (**3**), and LEGGATT (**13**) have published data indicating that the determination of the catalase ratio of dry and germinating seeds may serve as a measure of seed viability. MAR (**14**) has shown that the amylase activity of soaked oat seed is definitely correlated with germinability. LAKON (**10**) and others (**4, 5, 15**) have published data on the use of selenium and tellurium salts in solution as a means of determining the viability of a given seed lot by color reaction of the embryo. LAKON (**11, 12**) in 1942 compared his results by regular germination test of seeds of oats, barley, wheat, rye, and corn with those obtained by placing the cut seeds in a solution of 2,3-diphenyl-5-methyl-tetrazoliumchloride or 2,3,5-triphenyl-tetrazoliumchloride. The resultant staining of the viable embryos correlated well with his germination results.

Tetrazoliumchloride is colorless but forms carmine red formazans upon reduction. The salt is thus an oxidation-reduction indicator, and the development of the non-diffusible red color in a specific tissue is presumably an indication of the presence of active respiratory processes in which hydrogen radicals are transferred to the tetrazoliumchloride.

In 1945 a supply of 2,3,5-triphenyl-tetrazoliumchloride was obtained by officers of the Chemical Warfare Service in Germany, and Dr. Georg Lakon provided information about the testing program developed by him in which this material was used. Copies of publications, unobtainable in the past,

[1] Journal Paper no. J–1384 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project no. 86.

were received. With the assistance of the junior authors a series of tests was undertaken with seeds of corn (*Zea mays*), barley, (*Hordeum vulgare*), oats (*Avena sativa*), wheat (*Triticum aestivum*), buckwheat, (*Fagopyron esculentum*), cotton (*Gossypium herbaceum*), pea (*Pisum sativum*), rice (*Oryza sativa*), sorgo (*Holcus sorghum*), soybeans (*Soya max*), vetch (*Vicia sativa*), and Bahia grass (*Paspalum notatum*).

Procedure

The use of the tetrazoliumchloride test for corn, barley, oats, rye, and wheat as described by LAKON (**11, 12**) consisted of soaking seeds to be tested in water for several hours to permit absorption of water and initiation of germination processes. Following the soaking period each seed was bisected longitudinally through the center of the embryo so that each half had a part of the plumule and the radicle. One half of each seed was then placed in a Petri dish and a 1% solution of tetrazoliumchloride poured over the cut seeds until they were completely immersed. The dish was kept for 2 to 10 hours in a dark cabinet at room temperature. At the end of the immersion period each half seed was examined to determine the degree to which the parts of the embryo were stained carmine red. LAKON (**11**) stated distinct staining of the vital parts of the embryo was evidence of ability to produce a normal seedling. For cereal seeds he concluded that if both the embryo and the scutellum stained carmine red, the seed would be strongly viable. His criterion for seeds of corn (**12**) was unusual, for he did not consider the primary root in corn absolutely essential to the production of a normal seedling, especially if secondary roots were produced. His view coincides with that of PORTER (**16**) and also with a report of the Committee on Standardized Tests of the Association of Official Seed Analysts (**17**). LAKON (**12**) observed that in the region commonly referred to as mesocotyl, buds were produced on the sides of the region between the plumule and radicle and that in viable seeds these structures stained carmine red, developing into secondary roots. He concluded, therefore, that viable seeds of corn were those which have stained: (1) the entire embryo and scutellum, (2) all parts of the embryo and scutellum including the lateral buds in the mesocotyl region, but not the radicle, or (3) the entire embryo, but not the scutellum; he doubted that all seeds of this group were included among the normal seedlings. In general, it was his belief that a seed possessed stronger vitality if the scutellum stained.

Lakon's procedure was followed with the exception that the concentrations of the solution used were ½%, 1%, and 2% for two kinds of seed, and the length of time for presoaking and immersion in the tetrazolium salt solution was varied for some kinds of seed.

Seed samples used were tested in the Iowa State College Seed Laboratory. In all cases a germination test was made by approved methods to compare with the color test.

This paper reports the results obtained with the use of tetrazoliumchlo-

ride as a staining solution of the embryos of viable seeds in which germination processes have been initiated.

Results

Corn

For the initial studies with corn a sample with a known germinability of 98% under favorable conditions was selected. Seeds were soaked in water at room temperature from 5:00 P.M. to 8:00 A.M. the following day. Two hundred of the soaked seeds were then cut longitudinally through a median line perpendicular to the embryo side. Care was taken to split the plumule and radicle. One half of each kernel was saved and the entire set of 200 halves covered with a 1% solution of the tetrazoliumchloride. The Petri-dish container was immediately placed in a dark cabinet at room temperature. At the end of 15 minutes a faint pink coloration was noticeable in the embryo region of many seeds. At the end of 2 hours the entire number was examined and 99% showed bright carmine red, not only in the embryo including the plumule and radicle, but also in the scutellar region. The following day 400 more seeds of the same lot were soaked overnight and dipped in boiling water for 5 minutes. Two hundred were then sectioned, covered with the tetrazolium solution, and kept for 8 hours in the dark. No embryos changed color. The remaining 200 seeds were planted in sand, and none germinated. This preliminary test indicated that the tetrazolium solution was capable of differentiating at least between seeds that were nearly 100% germinable and those that were 100% non-viable. Several additional lots of seed corn produced in 1945 were tested with the same solution with results as shown (table I). At the same time a ½% solution was used on one lot as well as a 1% solution. There was no noticeable difference in the results obtained.

TABLE I

COMPARATIVE DETERMINATION OF SEED CORN VIABILITY BY APPROVED METHODS OF GERMINATION AND BY STAINING REACTION WITH 1% SOLUTION OF TETRAZOLIUMCHLORIDE

Sample no.	Staining		Viability in sand	
	No. of seeds	Coloration	No. of seeds	Germination
		%		%
5	200	90.0	200	90.0
22	200	90.0	200	89.5
23	200	86.5	200	90.5
39	200	22.5	200	23.5
40	200	9.0	200	7.0
1036	400	75.0	400	70.0
1072	400	86.2	400	82.0
1127	400	99.0	400	98.0
1169	400	85.0	400	81.0
1223	400	99.2	400	99.0
1228	400	97.5	400	90.0
1253	400	75.8	400	69.0
1298	400	90.8	400	90.0

Small grains, sorghum, buckwheat, and legumes

Samples of barley, oats, wheat, sorgo, buckwheat, popcorn, peas, soybeans, and vetch were tested with tetrazolium solution in much the same manner as described for corn. The soaking period, both in water and in the solution, was varied. The seeds of small grain, sorgo, popcorn, and buckwheat were cut medianly through the long axis of the embryo. The seeds of pea, soybean, and vetch were cut between the cotyledons, bisecting the radicle longitudinally. The data (fig. 1) show that for 3 lots of barley, 2 lots of oats, wheat, buckwheat, soybean, and popcorn there was a close relationship between the percentage of stained embryos and of normal sprouts produced in the ordinary laboratory germination test in sand (table II).

The tests with sorgo indicated that the color reaction might not be entirely reliable for the seeds of this plant; however, more tests are necessary before proof can be established. Sorgo seed would probably respond in a manner similar to that of corn and cereals. A careful study of the degree of coloration of the embryo, as well as time of soaking in the solution, may result in a refinement of the method.

Seeds of legumes require different treatment than those of grasses. For example, a long period of soaking in the chloride solution results in a coating of reddish stain over the entire flat surface of each cotyledon, and the stain must be rubbed off before the radicle can be examined. Furthermore, there is no indication as to the condition of the epicotyl in peas and vetch seed or of the plumule in soybean seed, primarily because it is difficult to split the plumule or epicotyl longitudinally in the sectioning process. To determine by this method which bean seeds would produce "baldhead" seedlings is practically impossible. The primary root emerges early in the germination of legume seeds and before the epicotyl or plumule shows much growth. This condition may prevent any practical use of the staining method as a means of determining the germinability of such seeds. Further investigations are needed before conclusions can be drawn.

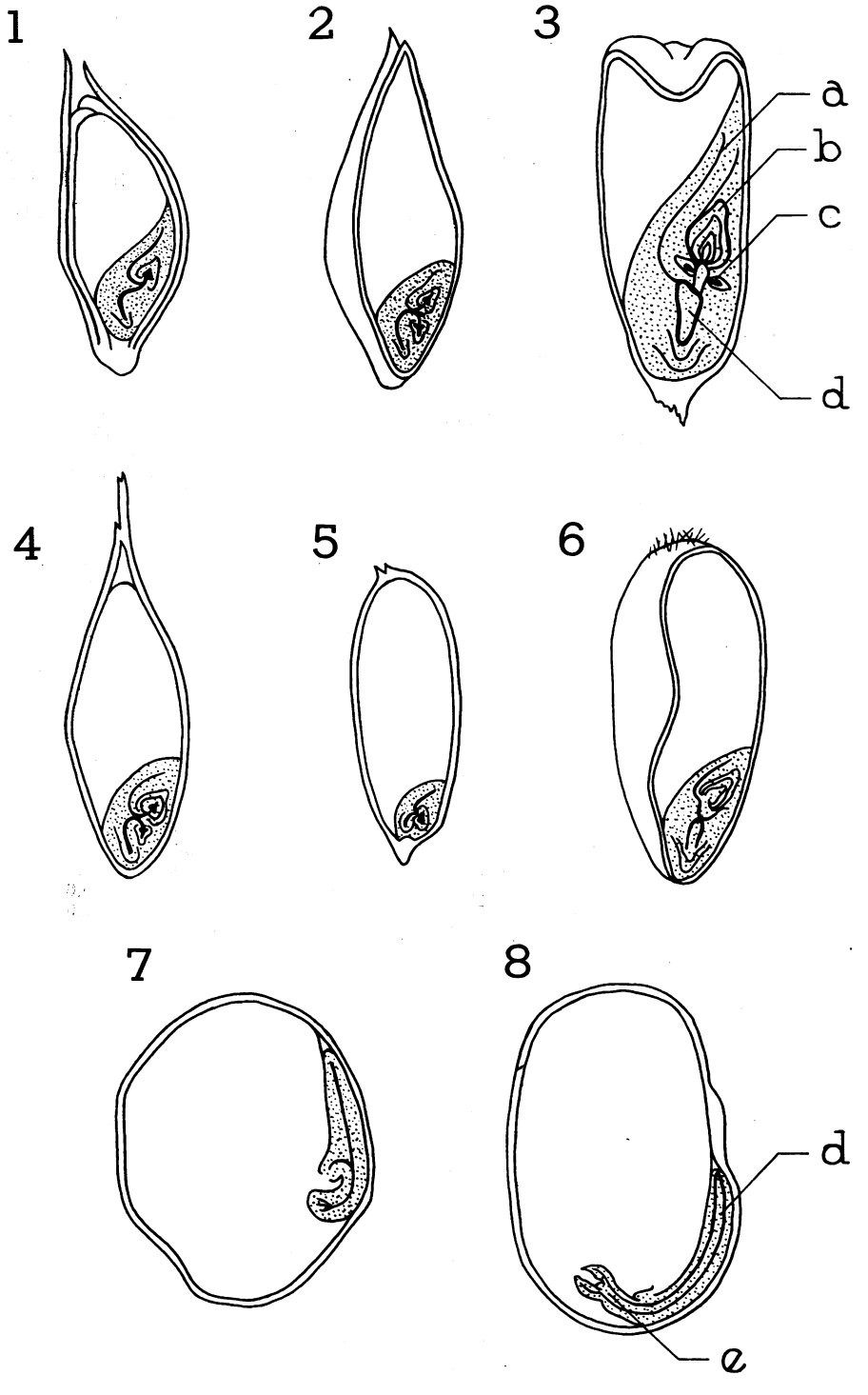
Fig. 1. Longitudinal sections of seeds showing area (heavy black or shaded portions) that stained carmine red when soaked in a ½% solution of 2,3,5-triphenyl-tetrazoliumchloride: *a*, procambial strand; *b*, coleoptile; *c*, adventitious root; *d*, primary root; *e*, plumule. Figure 1, 20×; figure 6, 12×; others, 6×.

1, 2, 4, 5, 6. Sections of Bahia grass, barley, oat, rice, and wheat seeds. Only those which showed the entire embryo and scutellum with carmine red stain were considered viable.

3. Corn kernel—seeds which stained carmine red in the scutellum and plumule regions down to and including the lateral root buds were considered viable. The stain was usually present throughout the scutellum and embryo.

7. Pea cotyledon—a section cut between the cotyledons and through the radicle. Those with a carmine red radicle were considered viable although no evaluation of the condition of the epicotyl could be made.

8. Soybean cotyledon—a section cut between the cotyledons and through the radicle. Those with a carmine red radicle were considered viable, although no evaluation of the condition of the plumule could be made.


1
2
3
a
b
c
d
4
5
6
7
8
d
e

Bahia grass

Determination of viability in seeds of Bahia grass is one of the most difficult problems in seed technology. Recommended procedures are to dehull the fresh fruit seed, to scrape lightly with a scalpel, and moisten with 0.1% potassium nitrate solution. These procedures are not entirely satisfactory as dehulling is a very tedious process and nitrate solution usually results in an abundance of fungous growth around each seed, making evaluation of sprouting very difficult.

TABLE II

Comparative determination of seed viability by laboratory germination and by staining reaction with 1% solution of tetrazoliumchloride

Sample		Staining test				Laboratory germination	
		No. seeds	Colored	Staining time		No. of seeds	Normal sprouts
			%	*hrs.*	*min.*		%
Barley	1A*	200	90.0	5		200	93.0
”	1A†	200	63.0	5		200	80.5
”	27C*	200	75.0	5		200	74.0
”	27C†	200	61.0	5		200	60.5
Buckwheat	1	200	98.5	4		200	95.0
Oats	36	179	83.2	2		200	94.5
”	38	200	75.0	2		200	89.5
”	Marion	200	94.0	1		400	98.0
”	Tama	200	47.0	1		400	50.0
Peas	55101	194	90.2		20	200	81.5
”	55105	192	91.6		20	200	78.5
”	55106	197	90.3		20	200	85.0
Popcorn	43610	200	32.0	2		200	27.5
Wheat	61	200	84.0	5		200	85.0
”	120	190	73.6	5		200	71.5
Soybean	1	200	100.0		30	200	96.0
Sorgo	9466	200	93.5	2		200	76.0 (80)‡
”	9467	200	75.5	2		200	49.0 (68)‡
”	49545	200	69.5	2		200	45.0 (51)‡
Vetch	1	200	58.0		15	200	75.5

* New crop.
† Old seed.
‡ Seed treated with Arasan.

Two types of tests using tetrazoliumchloride solution were undertaken. The first consisted of soaking the seeds from a pure seed fraction in water at 20° to 35° C. for 1 to 7 days. At the end of each day 3 lots of 50 seeds each were removed, sectioned, and soaked in ½%, 1%, and 2% solutions of tetrazoliumchloride. The results showed that a 2% solution was too strong, that there was little difference between the results obtained with a ½% or a 1% solution, and that after 3 days of soaking the percentage of colored embryos declined. The declining of the colored embryos suggests that seeds of Bahia grass, if soaked several days, begin to lose vitality. Such a response is not unexpected, because many seeds will not germinate in water.

The seeds absorb water and germination is initiated, but, if kept too long, decomposition occurs.

The second type of test with Bahia grass consisted of soaking the seeds 16 hours in distilled water at 20° C. The seeds were then placed in a Petri dish on moist filter paper and kept in a germinator with an alternating temperature of 20° C. for 16 hours and 35° C. for 8 hours. Immediately after the initial period of soaking and at subsequent intervals of 24 hours, 50 seeds were removed, sectioned, and placed in a 1% solution of tetrazoliumchloride. At the end of 8 and 24 hours the sections were examined and classified as light-stained, dark-stained, viable, and non-viable. Only those with the entire embryo and scutellum stained light or dark were considered viable (table III).

TABLE III

RESULTS OF SOAKING 50 CUT SEED SECTIONS OF BAHIA GRASS IN 1% SOLUTION OF TETRAZOLIUMCHLORIDE FOR 8 AND 24 HOURS*

No. of days after soaking	No. of dark-stained seeds	No. of light-stained seeds	Viable	Non-viable	Time in solution
			%	%	*hrs.*
0	2	48	100	0	8
	35	15	100	0	24
1	8	42	96	4	8
	37	13	96	4	24
2	29	20	96	4	8
	44	5	94	6	24
3	35	15	100	0	8
	47	3	98	2	24
4	22	24	88	12	8
	42	3	90	10	24
5	25	25	100	0	8
	40	10	100	0	24
6	31	19	96	4	8
	38	9	94	6	24

* Seeds were soaked 16 hrs. in water at 20° C., kept at temperatures alternating between 20° and 35° C. for period of 1 to 6 days, and sectioned.

In all cases the percentage of dark-stained embryos increased markedly as the period of immersion was increased from 8 to 24 hours. Since the embryos not deeply stained were colored throughout the scutellar region as well as in the embryo proper, they were considered viable. Among the lots kept in the germinator for 1 to 6 days and soaked 24 hours in the chloride solution, the differences in viability, on the basis of 50 seeds, were not significant. On the other hand, the large percentage of dark-stained seeds retained in the germinator for 2 to 4 days after soaking, indicated that such a procedure may be preferable. The viability by this method of this particular lot of Bahia grass seed appeared to be between 90% and 100%.

A germination test of this lot was made as follows: 100 seeds were dehulled, soaked 5 minutes in a 25% solution of chlorox, placed on moist filter papers in a Petri dish, and kept in a germinator with alternating tempera-

tures of 15° C. for 16 hours and 30° C. for 8 hours. At the end of 9 days all the seeds had produced strong, normal sprouts. Another sample of 200 seeds, treated in the same way except that the temperature alternated from 20° to 35° C., gave 95% germination in 5 days. The results indicated that the viability of this lot of seed was nearly 100% which agrees with the index of viability as measured by the tetrazoliumchloride method. They also suggested that alternating temperatures of either 15° to 30° C. or 20° to 35° C. were favorable for the germination of Bahia grass seed.

Rice seed

Two hundred seeds from each of four varieties of rice were soaked in water for 16 hours at room temperature. Each seed was sectioned longitudinally through the embryo, and one half of each placed in a 1% solution of tetrazoliumchloride for 4 hours. At the end of the soaking period the

TABLE IV

Comparative determination of the viability of rice seed by staining with 1% solution of tetrazoliumchloride and laboratory germination based on 200 seeds tested

Variety	Viability by staining	Laboratory germination		
		Between blotters	Between blotters 20°–30° C.	Sand
	%	%	%	%
Nira	66.0	65.5	55.0	70.0
Fortuna	87.0	84.0	77.5	81.0
Prelude	84.5	75.5	72.0	71.5
Zenith	94.5	90.0	78.0	86.5

sections were examined under a binocular microscope and all those with the embryo and scutellum stained light or dark red were classed as viable. Three lots of 200 seeds each from the 4 varieties were prepared and tested for germination by three methods: (1) between blotters, (2) presoaked 16 hours and planted between blotters, and (3) presoaked 16 hours and planted in sand. All tests for germination were made at 20° C. for 16 hours and 30° C. for 8 hours each day for 10 days (table IV). With the exception of the Prelude variety soaking before planting resulted in a higher percentage of germination, and germination in blotters or sand of presoaked seed agreed with the embryo color reaction.

Cotton seed

A few attempts were made to apply the tetrazolium color test to cotton seed. One difficulty was that some seeds after soaking overnight were hard and could not be sectioned. Such seeds presumably would not have absorbed water, and germination processes could not have been initiated. Many seeds that did absorb water when sectioned and soaked in a 1%

tetrazoliumchloride solution stained readily in the embryo region; but the seed coats separated easily from the embryos and cotyledons, and the latter further separated from each other. An evaluation of the color test, therefore, was difficult to make.

Discussion and summary

Determination of living embryos of seeds by means of the color indicator 2,3,5-triphenyl-tetrazoliumchloride as recommended by LAKON (**12**) was investigated, using seeds of several members of the grass family, three leguminous species, buckwheat, and cotton. A comparison of the percentage of stained embryos in a given lot with the percentage of normal sprouts obtained in a standard germination test showed close agreement in 13 samples of corn, two samples each of wheat and oats, three samples each of barley and rice, and one sample each of buckwheat, popcorn, soybean, peas, and Bahia grass. The comparisons for vetch, sorgo, two samples of oats and peas, and one sample of barley were not in close agreement, yet the differences in results shown by the two methods were not great.

With the exception of corn the criterion used for classifying grass seeds as viable was the almost complete staining of the embryo proper and scutellum. Absence of color in the radicle below the mesocotyl region or in the lower half of the scutellum, however, was not considered sufficient grounds for classification of a corn seed as non-viable.

In seeds of pea, soybean, and vetch it is impossible to section through the epicotyl or plumule region regularly. The only cut part, therefore, with which the solution can readily make contact, is the radicle. Development of a radicle in leguminous seeds without consideration of the plumule or epicotyl is insufficient evidence for classification as a normal seedling. For that reason the method investigated may not be applicable to seeds producing baldheads when they germinate.

The method appears to have some merit for seeds of Bahia grass which are difficult to germinate. It is recommended that the hulls be removed before placing the seeds to germinate. This is a tedious and time-consuming process. By the staining method it was possible to soak the seeds 16 hours, place them on a moist substratum at 20° to 35° C. for 3 or 4 days, cut them into sections, and place in the tetrazoliumchloride solution for 24 hours. More samples must be tested by this method before definite conclusions can be stated. When the hulling method is used it is desirable to soak the seeds in a 25% solution of chlorox to prevent the growth of surface molds.

A standard method of germination may not be the one that measures the true germinability of a particular kind of seed. For example, in the tests with four varieties of rice the standard method failed to measure the maximum germinability of the seed of three varieties. When the seeds were soaked and planted in sand or blotters, the percentage germination compared closely with the percentage of stained embryos that were soaked in the tetrazoliumchloride solution.

As pointed out in the introductory statement, tetrazoliumchloride is colorless but forms carmine red formazans upon reduction. The salt is an oxidation-reduction indicator, and the development of the non-diffusible red color in a specific tissue is presumably an indication of the presence of active respiratory processes in which hydrogen radicals are transferred to the tetrazoliumchloride. If further research should establish this hypothesis, the process should prove to be of general value in physiological experiments as well as an index of germinability of seeds. Because the measurements of "germinability" are indirect, however, and dependent upon all of the factors affecting the oxidation-reduction potential of the cells, careful standardization of procedure will probably be necessary.

DEPARTMENT OF BOTANY
IOWA STATE COLLEGE
AMES, IOWA

LITERATURE CITED

1. BARTON, LELA V. Storage of elm seeds. Boyce Thompson Inst. **10**: 221–233. 1939.
2. CROCKER, WILLIAM, and HARRINGTON, GEORGE T. Catalase and oxidase content of seeds in relation to their dormancy, age, vitality and respiration. Jour. Agr. Res. **15**: 137–174. 1918.
3. DAVIS, WILBUR E. The use of catalase as a means of determining the viability of seeds. Proc. Assoc. Off. Seed Anal. 1925. 1926.
4. EGGEBRECHT, H., und BETHMANN, W. Das Selenfarbeverfahren im Vergleich zu der üblichen Keimprüfung insbesondere bei Wintergerste mit Keimruhe. Angew. Bot. **21**: 448–455. 1939.
5. EIDMANN, FRANZ. Eine neue biochemische Methode zur Erkennung des Augsaatwertes von Samen. Proc. Internat. Seed Test. Assoc. **10**: 203–209. 1938.
6. FLEMION, FLORENCE. Dwarf seedlings from non-after-ripened embryos of *Rhodotypos kerrioides*. Boyce Thompson Inst. **5**: 161–165. 1933.
7. FLEMION, FLORENCE. A rapid method for determining the germinative power of peach seeds. Boyce Thompson Inst. **8**: 289–293. 1936.
8. FLEMION, FLORENCE. A rapid method for determining the viability of dormant seeds. Boyce Thompson Inst. **9**: 339–351. 1938.
9. FLEMION, FLORENCE. Further studies on the rapid determination of the germinative capacity of seeds. Boyce Thompson Inst. **11**: 455–464. 1941.
10. LAKON, GEORG. Das Schwinden der Keimfähigkeit der Samen, insbesondere der Getreidefruchte. Ber. Deutsch. Bot. Ges. **57**: 191–203. 1939.
11. LAKON, GEORG. Topographischer Nachweis der Keimfähigkeit der Getreidefruchte durch Tetrazoliumsalze. Ber. der Deutsch, Bot. Ges. **LX**: 299–305. 1942.

12. LAKON, GEORG. Topographischer Nachweis der Keimfäghigkeit der Mais durch Tetrazoliumsalze. Ber. der Deutsch. Bot. Ges. **LX**: 434–444. 1942.
13. LEGGATT, C. W. A further note on catalase activity as a measure of seed viability. Canadian Jour. Res. **9**: 571–573. 1938.
14. MAR, FRANCIS. Amylase activity as an indicator of seed viability. M.S. Thesis. Iowa State College Library. 1944.
15. MOHS, K., and TORNOW, E. Germinating energy (of grains) and selenium reduction. Ztschr. Ges. Getreidew. **27**: 17–22. (Abstr. in Jour. Soc. Chem. Ind. **59**: 483B. 1940.)
16. PORTER, R. H. Testing the quality of seeds for farm and garden. Iowa Agr. Exp. Sta. Res. Bull. 334. 1944.
17. Report of the Committee on Standardized Tests. Proc. Assoc. Off. Seed Anal. 1943. 70–72. 1945.