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LAWRENCE ODELL COPELAND for the M.S. in Farm Crops
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Title CONFIDENCE LIMITS FOR FLUORESCENCE TESTS OF RYEGRASS (LOLIUM SP.)

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Studies were conducted to determine the variability existing in repeated fluorescence tests of annual and perennial ryegrass (Lolium multiflorum and L. perenne, respectively). Sources of variation considered were within-sample error, among-sample error, among-germinator error, and among-laboratory error. These were studied independently and the variation in each was compared to the theoretical variability due to random sampling error alone.

Variability indices were calculated which showed the amount of experimental error observed in fluorescence test results due to each source of variation. These indices showed that the present fluorescence tolerances, based on random sampling from a binomial population and used by the Association of Official Seed Analysts, are not adequate to account for the variability actually existing in repeated fluorescence test results conducted on the same sample in

the same laboratory. When other sources of variation are introduced, the inadequacy of the present tolerances becomes even more pronounced.

The differences between fluorescence tolerances used by the Association of Official Seed Analysts and the International Seed Testing Association are discussed and a new method of calculating fluorescence tolerances is presented.

CONFIDENCE LIMITS FOR FLUORESCENCE TESTS OF RYEGRASS (*LOLIUM* SP.)

by

LAWRENCE ODELL COPELAND

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CONFIDENCE LIMITS FOR FLUORESCENCE TESTS OF RYEGRASS (*LOLIUM* SP.)

INTRODUCTION

The fluorescence test is widely used in seed testing to aid in distinguishing between seedlings of annual ryegrass, (*Lolium multiflorum*, L.), and perennial ryegrass, (*Lolium perenne*, L.). Its usefulness for this purpose is based on the premise that roots of germinating annual ryegrass seedlings exude a substance which emits a bright fluorescent glow when subjected to near-ultraviolet light (300-400 mμ), whereas the roots of perennial ryegrass seedlings do not. Because of the similarity of annual and perennial ryegrass seed, this test has proven very useful to the seed analyst in detecting the percentage composition of mixtures of the two species as well as determining the degree of contamination of one species by another. Seed samples representing all levels of fluorescence between 0% and 100% are commonly received by the Oregon State University Seed Testing Laboratory.

The present tolerances used to account for variability in repeated fluorescence tests are theoretical in nature and are not supported by published experimental evidence. The purpose of this study has been to provide the experimental data needed to set realistic tolerances. The amount of variation, the type of distribution

followed, and the sources of error which contribute to fluorescence test variation were investigated. To ascertain the validity of the present accepted tolerances, the theoretical variation upon which the tolerances are based was compared with the variability determined experimentally.

REVIEW OF LITERATURE

Introduction

In 1929, Gentner (4) discovered that seedlings of annual and perennial ryegrass could be distinguished by their different abilities to fluoresce under ultraviolet light. This process soon became widely used, because of its simplicity and because the distinction of the two species on the basis of seed morphology is difficult.

The fluorescence test has also been applied to other crop identifications with varying degrees of success. Gentner (3) earlier had observed that certain species of cereals as well as beans fluoresced when irradiated. These responses were not adequate to distinguish between two otherwise similar varieties or species. Hellbo (5) also found fluorescence responses in various kinds of germinating seedlings. In general, though such responses have been noted, the fluorescence test has not been widely applied for crop identification except for annual and perennial ryegrasses.

The usefulness of the fluorescence test has long been recognized, but only recently has the chemical nature of the fluorescent substance been determined. In 1958, Axelrod and Belzile (2) reported that after growing large quantities of annual ryegrass and removing their tops, they were able to extract and isolate a substance from the remaining parts. The substance had a formula of $C_{20}H_{19}NO_4$, and

was named annuloline. Their results were later confirmed by Ching[1] by using a chromatographic technique.

Fluorescence Tolerances

The present Association of Official Seed Analysts (AOSA) fluorescence tolerances are based on work published by Leggatt (9) in 1939, who distributed samples of red clover and timothy to seven Canadian laboratories for germination tests. By the use of the Chi-square test, the results were found to follow the binomial distribution within individual laboratories; but when results of all stations were analyzed, the Chi-square value was in excess of that expected at the 1% level of significance.

When considering the appropriate tolerances to be established for fluorescence test results, the AOSA Statistics Committee was advised by Leggatt that fluorescence test results should follow the binomial distribution similar to that of germination.[2] Consequently, tolerances based on the binomial distribution were accepted to account for variability in fluorescence test results without experimental evidence as a basis for this action. The evidence is still lacking today. Although the AOSA Statistics Committee considered

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[1] Personal correspondence from Dr. Te May Ching to Dr. O. L. Justice, dated March 26, 1958.

[2] Personal correspondence from Dr. O. L. Justice to L. O. Copeland, dated February 1, 1965.

that germination and fluorescence tests followed the same type of distribution (binomial), and established fluorescence tolerances based on this statistical model (binomial distribution), the present AOSA Rules prescribe germination tolerances that are considerably wider than the present fluorescence tolerances. The basis for this difference in fluorescence and germination tolerances is not apparent.

The present International Seed Testing Association (ISTA) fluorescence tolerances appear to be more realistic. The ISTA Rules (6) prescribe that the germination tolerances shall be applicable to cover variation among fluorescence tests. These ISTA tolerances for both germination and fluorescence are considerably wider than can be accounted for by the binomial distribution and more closely approximate the present AOSA germination tolerances.

Germination Tolerances

Experimental evidence that variation in germination test results within a particular laboratory is represented by the binomial distribution is abundant. As early as 1889, Rodewald (15) showed that for properly mixed seed, results of germination trials closely corresponded to the expected variation of a binomial distribution on a theoretical basis. Rodewald realized that for germination, the errors diminished in proportion to the square root of the number of seeds in a test, and calculated tolerance ranges for germination.

Tolerance ranges of 3% for germinations above 90% and 5% for germinations below 90% were considered suitable. Yakusherskaya (21) reported that distribution of germination results followed the binomial distribution and that the influence of personal error and lack of symmetry in the distribution may be disregarded when 400 or more seeds are tested. Stahl (18) compared the observed and expected standard deviations of a large number of germination trials over mean germination ranges of 50% to 100%. Although the theoretical standard deviations on the whole were slightly lower than the observed values, good agreement existed between the two sets of values. Stahl considered that latitudes adopted for tolerances should be wider than are theoretically required, and suggested the wider tolerance to cover those few results which are expected to fall outside the latitudes calculated on a theoretical basis. The existing tolerances covered only the variations expected to occur by repeated tests of the same sample within the same seed testing laboratory, but Stahl felt that the adopted tolerance should cover variation arising from tests made on different carefully drawn samples from the same lot.

In 1935, Leggatt (11) proposed a study of germination trials to determine the average extent of experimental error separate from the sampling error in different kinds of seeds. Leggatt noted the possibility of greater variation in certain kinds of seeds than others, thus necessitating a wider tolerance. Rather than basing the wider

tolerance on the actual variation, Leggatt suggested that the level of significance be lowered while retaining the statistical model, and the use of the Chi-square test to determine if germination tests were out of tolerance. Later, in 1939, after noting the distribution of germination test results within and among laboratories, Leggatt (9) stated that a number of laboratories over a wide geographical area may show differences in the level of interpretation. Wider tolerances were suggested for comparison of results obtained by two different laboratories to account for the extra sources of variation involved. Leggatt again suggested that the level of significance should be lowered in order to increase the tolerance, while still retaining the statistical model as a basis for the tolerance, but stressed the need to standardize the methods of seed analysis throughout the world in order to eliminate the need for the additional tolerance.

Stevens (19), working with alfalfa and sweet clover, found that agreement between observed and expected variation was very close between 100% and 80% germination levels. He compared the theoretical and actual standard deviations of repeated test results. There was considerable disagreement between expected and observed variation between 80% and 25%. The agreement between 25% and 0% was close, but not as close as that between 100% and 80%. This was attributed to the difficulty of evaluating weak sprouts which are common in many samples of low germination. Stevens considered it

reasonable to expect greater variations in germination results of sweet clover and alfalfa, because of the hard seed coat characteristic of many legume seeds. His hypothesis could not be verified by Leggatt (9). Leggatt observed that variation in red clover and alfalfa germination results closely corresponded to the binomial distribution.

Thompson (20) recognized two kinds of seed present in any germination test--germinable seed and non-germinable seed. He stated that a third category of seed was sometimes also present. This type was sensitive to small changes in environmental conditions and its germination response depends on the physical conditions of the test. This was considered to influence the judgment of the analyst and thus become an additional factor in test variability. Besides causing greater actual variability of germination, it was also considered to cause different degrees of germination which imposed a burden of greater choice on the analyst.

Using a slightly different technique to determine if the sampling variation in analyses of red clover, alfalfa, sweet clover, and bluegrass followed the binomial distribution, Leggatt (9) prepared bulk lots of these seed containing 1%, 7%, 10%, 15%, and 50% stained seed. Germination tests on repeated replicates of 100 seeds showed close agreement between the expected and observed standard deviations. This seemed to indicate that sampling variation within one laboratory from a particular sample could be accounted for

adequately by tolerances based on random sampling variation alone. Any variation beyond that expected from a binomial distribution was attributed to experimental error or to true differences in sample means. At this time Leggatt did not consider experimental error important in establishing germination tolerances.

Shoorel (17) emphasized that germination tolerances based strictly on a theoretical statistical model would cause a disproportionately large number of retests. Johnston and Miller (7) working with ryegrass found that the actual variation among replicated germination tests from a particular working sample corresponded to theoretical variation from a binomial distribution. All replicates were tested in the same laboratory where all conditions were constant. Miranda (14) analyzed germination data from many different kinds of seeds and found that results closely followed the binomial distribution, and strongly urged that all tolerances be based strictly on a statistical model as in quality control, rather than actual variation existing among all possible test results. It was noted that within a particular laboratory, the agreement between the statistical model and reality, or the observed variation, was very close. Miranda realized that the statistical model gave the inevitable sampling variation which cannot be eliminated, or decreased without introduction of errors of bias, and like Leggatt, suggested that for tests conducted in different laboratories, the tolerances should be widened. It was

suggested that this be done by decreasing the level of significance rather than rejecting the statistical model in favor of a tolerance based on actual variation. Miranda realized that the actual variation includes all sources of variation in addition to that due to sampling error.

Miles (12) compared the observed with the expected standard deviation of the AOSA referee samples from 1953 to 1955, and found that the actual variation was 2.55 times greater than that expected. But within a single laboratory, the actual variation was smaller than that theoretically expected in 65% of all cases. This small actual variation was attributed to analyst bias. When counting the seedlings, the analyst, by remembering the count from previous replicates, tended to make later replicate counts similar. Miles considered this phenomenon to be an unconscious act by the analyst. Within a particular laboratory Miles found no relationship between the difficulty of differentiating between normal and abnormal seedlings either at high and low germination percentages. However, among laboratories such a relationship existed. When among-laboratory tests were considered, the ratio of the actual to the expected variation was greater at lower than at higher germination percentages. This was attributed to the different concepts of abnormal and normal seedlings by analysts in different laboratories. Miles proposed tolerances to be used for inter-laboratory results which were based on the actual

variation existing in the inter-laboratory results from the AOSA referee tests of 1953 to 1955.

Miles, Shenberger, and Carter (13) reported that germination test results may vary because of six reasons: (1) random sampling variation which cannot be avoided; (2) differences in methods, techniques, or conditions; (3) differences in equipment; (4) differences in interpretation of normal seedlings and hard seed; (5) bag to bag variation; and (6) changes or actual differences in seed being tested. All these factors were considered important in the establishment of tolerances. Germination tolerances were presented showing different degrees of certainty of 5%, 2.5%, 1%, and 0.5% to be used in comparison of results conducted in the same, or different laboratories. These were considered to be tentative only, and were expected to be modified when information became available as to a reasonable amount of variation due to other than random causes.

By conducting a sweet clover seed germination referee test among analysts within a single laboratory, Shenberger (16) found a tendency for the 4-replicate tests to be a little too much alike. This causes the observed variance to be less than the expected variance. This was similar to results found by Miles (12), who found that when replicates were unidentified by the analysts there was closer agreement between the observed and expected variance. Shenberger found that the analysts were fairly consistent in the classification of normal

and abnormal seedlings when the germination was high as well as when germination was low.

MATERIALS AND METHODS

All seed used in the studies described herein were taken from samples representing varying levels of fluorescence between 0% and 100% as indicated by results on file at the Oregon State University Seed Laboratory. Twenty-seven samples, representing twenty-seven different fluorescence levels, were included in the first study on within-sample, within-laboratory variation. Seven samples were selected from the original twenty-seven to test for two additional sources of variation. These were within-sample, among-germinator error and within-sample, among-laboratory error. Another study involved testing for variability in fluorescence test results due to among-sample, within-laboratory error using fourteen different seed lots having different fluorescence levels.

The results obtained from each different study were analyzed similarly. F values were calculated for each level of fluorescence under all sources of variability studied. This method, described earlier by Miles (12) and Shenberger (16), was applied using the following formula: $F = s^2/\sigma^2$, where s^2 = the observed variance for all sources of error for each fluorescence level, and σ^2 = the expected variance under the same conditions. From a binomial population the mean F value for each fluorescence level should equal 1.00 over a large number of tests, with one-half the F values above 1.00

and one-half below. A variability constant was calculated for each source of variability by the following formula: $K = \frac{\Sigma s^2}{\Sigma(P^2-100P)}$, where Σs^2 = the sum of all variances at each fluorescence level, and P = each fluorescence level. This constant represents the overall variability value above that theoretically expected at each fluorescence level. Theoretically a K value of 1.00 would be expected if random sampling alone was contributing to the observed variability.

The variability observed among laboratories was compared by a t-test as described by Miles (12). This t-value was calculated for each fluorescence level for each cooperating laboratory by the formula: $t = \frac{(\bar{x} - \bar{\bar{x}})}{\sigma}$, where $\bar{\bar{x}}$ = the grand mean for all laboratories at each fluorescence level, $\bar{x}$ = the level obtained by a particular laboratory, and σ = the theoretical standard deviation at each fluorescence level, assuming a binomial variation only.

Within-Sample, Within-Laboratory Variation

Twenty-six samples representing twenty-six fluorescence levels were subdivided by means of the Gamet Precision Divider. Ten 100-seed replications of each of the twenty-six samples were subjected to germination and fluorescence tests by routing testing procedures used at the Oregon State University Seed Laboratory. The procedure consisted of counting and positioning the seeds on the

seedlings were removed at this time and recorded. The fluorescence determinations were made in a darkroom by use of a near-ultraviolet-emitting lamp (300-400 mμ) of the type described by Justice (8). The tests were then replaced in the germinators for seven additional days at which time a final count was made. A preliminary count was necessary because early germinating and fluorescing seedlings tend to grow together and become difficult to count accurately.

Among-Germinator, Within-Sample Variation

Seven samples representing different fluorescence levels were selected as described earlier. Ten replications were subdivided by means of the Gamet Precision Divider. Each of the ten replicates for all seven samples was placed in ten different germinators providing a representative cross section of germinators commonly used for conducting the fluorescence test of ryegrass. Conditions of light, temperature, and humidity differed to varying degrees, and no attempt was made to standardize germinator conditions. Although the tests were conducted in different germinators the fluorescence interpretations were made by the same analyst, well-trained in performing such routine fluorescence tests.

A supplementary among-germinator study was conducted by repeating the tests in a similar manner, but in this case, all interpretations were made by another analyst familiar with the

fluorescence technique, but unaccustomed to making routine fluorescence counts. Comparisons were made between the variability observed among tests interpreted by the two unequally trained analysts.

Among-Laboratory, Within-Sample Variation

The seven samples used above were divided by the same procedure into ten subsamples, each of which was distributed to ten cooperating laboratories located in various parts of the United States for 100-seed fluorescence tests. These laboratories included only those experienced in conducting fluorescence tests.

Among-Sample, Within-Laboratory Variation

Fourteen lots representing appropriate levels of fluorescence were selected as described earlier. From each of these lots, ten officially drawn samples were taken by a representative of the Oregon State Department of Agriculture. These samples were subsequently subdivided into small working samples, from which a 100-seed fluorescence test was conducted by the Oregon State University Seed Laboratory. Samples received identical treatment as described for within-sample, within-laboratory tests. The results were analyzed and compared as described previously.

RESULTS AND DISCUSSION

Within-Sample, Within-Laboratory Variation

Comparison of observed to expected variances at different fluorescence levels is shown in Table 1. The actual variability is only slightly above that expected from sampling error alone. The mean F value obtained in comparing observed with expected variances was 1.11, compared to the value of 1.00 expected from random sampling error alone. Fourteen of the 27 fluorescence levels had F values above 1.00 and 13 had F values below 1.00.

A variability constant, K, of 1.27 was obtained which describes the amount of variability above the K value of 1.00 expected from normal random sampling. This K value is shown in Table 7 with those K values obtained from other sources of error.

These results indicate that other sources of error are operative in fluorescence testing other than random sampling. Though experimental error could be due to several sources of error, the most likely contributors are errors in technique, procedure, or interpretation of results among replicates. Because of the nature of these tests, which involved only replicates from properly mixed samples that were interpreted and recorded by the same analyst, they represent the minimum variability existing within a single laboratory. In laboratories where several analysts might be involved in interpreting test results, greater variability could be expected.

Table 1. Comparison of observed to expected variances at different fluorescence levels for within-sample, within-laboratory error.

Mean Percent Fluorescence	Observed Variance	Expected Variance	F Value
3.53	5.27	3.34	1.58
4.45	3.14	4.21	0.75
6.73	3.41	6.25	0.55
7.79	7.46	7.19	1.04
9.44	7.20	8.52	0.85
9.74	10.38	8.76	1.18
15.50	8.41	13.10	0.64
18.57	7.51	15.14	0.50
34.22	33.11	22.50	1.47
43.73	15.65	24.60	0.64
45.62	12.70	24.81	0.51
51.67	21.81	24.97	0.87
60.39	37.55	23.92	1.57
69.06	26.37	21.35	1.24
69.42	54.50	21.24	2.57
70.38	37.41	20.84	1.80
84.10	8.88	13.36	0.67
86.05	12.14	11.97	1.01
90.07	6.81	8.44	0.80
91.43	11.48	7.86	1.46
94.20	5.91	5.46	1.08
94.52	4.44	5.20	0.85
95.16	2.51	4.57	0.55
96.60	4.32	3.28	1.32
98.58	0.62	1.38	0.45
99.46	1.51	0.60	2.53
99.66	0.42	0.30	1.42

Mean F value = 1.11

13 less than 1.00 = 48.14%

14 more than 1.00 = 51.86%

Tolerances, based on the statistical model alone, are insufficient to account for all the variation in fluorescence test results, even the minimum fluorescence variation existing within a single laboratory.

Among-Germinator, Within-Sample Variation

Tables 2 and 3 show the comparative and mean F values obtained from inter-germinator fluorescence tests. F values from tests interpreted by the trained analyst showed an average of 1.63, while those interpreted by the untrained analyst average 1.72. Variability constants, K, obtained by the skilled and unskilled analyst were 1.86 and 2.11 respectively. These are given in Table 7.

These results are interesting primarily because of the high variability shown by the mean F and K values. They are indicative of considerable differences in the environmental conditions among the germinators used in fluorescence testing. Although it is generally realized that such variation in environmental conditions exists, the extent of the effect of such variation on test results has not been previously realized. Thus to be accurately interpreted, both the F and K values must be compared to similar values obtained by tests conducted within the same germinator within the same laboratory. These values were reported in the previous section, and represent ever-present variability. This variability must first be compared to the over-all variability observed from each particular source of

Table 2. Comparison of observed to expected variances at different fluorescence levels for among-germinator, within-laboratory, within-sample error (skilled analyst 1).

Mean Percent Fluorescence	Observed Variance	Expected Variance	F Value
6.09	4.77	5.72	0.83
10.59	14.81	9.47	1.56
66.09	32.16	22.41	1.44
87.37	47.81	11.03	4.33
93.70	5.40	5.90	0.92
96.43	3.31	3.44	0.96
98.88	1.34	0.90	1.49

Mean F value = 1.63

3 less than 1.00 = 42.8%

4 more than 1.00 = 57.2%

Table 3. Comparison of observed to expected variances at different fluorescence levels for among-germinator, within-laboratory, within-sample error (unskilled analyst 2).

Mean Percent Fluorescence	Observed Variance	Expected Variance	F Value
5.66	5.33	2.57	0.48
10.48	9.49	29.05	3.06
51.32	24.98	22.08	0.88
81.82	14.87	33.01	2.22
84.53	13.07	64.39	4.93
91.64	7.66	11.52	1.50
97.62	2.32	0.92	0.39

Mean F value = 1.72

3 less than 1.00 = 42.8%

4 more than 1.00 = 57.2%

error, to obtain the net effect of that source of error.

The difference in variability obtained by the unskilled analysts presents another source of variation. These differences are not surprising when the procedural problems of performing this test are considered. The difference in the F and K values presented in Tables 2, 3, and 7 represent the actual, additional variability caused by fluorescence interpretations by an unskilled analyst. This information is valuable, only because it represents the type of variation which should not be covered by tolerances, and certainly should not be tolerated in routine seed testing work. Another implication of these tests is that laboratories which perform only a few fluorescence tests each year do not have staff accustomed to conducting and interpreting the results. Thus in such laboratories variability introduced because of the analyst's lack of skill may become more important than difference in germinator conditions.

Among-Laboratory, Within-Sample Variation

Table 4 shows variability observed among the ten different laboratories for each fluorescence level. The mean F value was 1.46, indicating that considerable variability does exist beyond that due to random sampling. Also the comparative F values of 1.10 obtained within a particular laboratory and 1.46 obtained in this study substantiates that more variability exists among laboratories than

Table 4. Comparison of observed to expected variances at different fluorescence levels for among-laboratory, within-sample error.

Mean Percent Fluorescence	Observed Variance	Expected Variance	F Value
3.20	5.19	3.10	1.67
10.90	9.98	9.71	1.03
53.33	22.98	24.89	0.92
84.01	26.10	13.43	1.94
86.07	26.65	11.99	2.22
94.87	9.23	4.87	1.90
98.45	0.69	1.53	0.55

Mean F value = 1.46 M = 1.46

2 less than 1.00 = 28.5%

5 more than 1.00 = 71.5%

within a particular laboratory. The variability constant, K, obtained among inter-laboratory tests was 1.45, compared to the theoretically expected value of 1.00 from random sampling error alone. This value is found in Table 7. The larger K values obtained among laboratories than those obtained within a laboratory also confirms that greater variation exists among laboratories than within laboratories.

Although considerable experimental error evidently occurred among tests conducted by the different laboratories, the mean F value of 1.46 does not appear excessive when compared to possible variability due to other sources of error discussed in this study. It also compares well to inter-laboratory variation in germination reported by other workers. Miles (12) found by comparing inter-laboratory tests involving 68 laboratories and 48 samples, that the observed standard deviation was 2.55 times greater than that due only to sampling. When compared to germination results reported by Miles, the fluorescence test variation found in this study does not appear to be excessive. It should be noted, however, that only laboratories experienced in fluorescence testing were included in the present study. Miles used AOSA referee samples which included many different kinds of seed as well as many different laboratories. If fluorescence samples were sent to laboratories without regard to their experience in fluorescence testing, the observed variability might be expected to parallel the germination variability reported

by Miles.

Table 5 shows t-values computed for each fluorescence level. Simply stated, the t-value tells the number of standard deviations a laboratory fluorescence result deviated from the grand average fluorescence result obtained by all cooperating laboratories. Six of the ten cooperating laboratories obtained positive t-values, indicating a tendency to obtain fluorescence results somewhat above the over-all mean among laboratories. The second and third columns of Table 4 show the number of positive and negative t-values obtained by each laboratory throughout all fluorescence levels. The last column shows the standard deviation of t-values obtained by each laboratory at all levels of fluorescence. Larger values here would indicate that fluorescence results were sometimes considerably above the grand average and sometimes considerably below. Miles (12) calculated such values for inter-laboratory germination tests and found some standard deviations as high as four and five. No theoretical basis was known prescribing a dividing point between good and bad values, but some association existed between the magnitude of the standard deviation of t-values and columns 1, 2, and 3. Compared to the high average value of the standard deviation of t-values noted by Miles in inter-laboratory germination tests, the average standard deviations of the t-values in Table 5 appear similar.

Table 5. Summary of t-values obtained due to among-laboratory error.

Laboratory Number	Mean t-value	Number of Positive t-values	Number of Negative t-values	Standard Deviation of t-values
1	0.37	4	3	1.89
2	0.32	2	5	2.14
3	0.78	5	2	1.95
4	0.12	1	6	4.13
5	0.65	4	3	3.66
6	1.36	1	6	3.69
7	0.51	4	3	4.11
8	0.81	5	2	2.46
9	0.03	4	3	3.12
10	0.31	2	5	1.23

Among-Sample, Within-Laboratory Variation

Results of fluorescence tests conducted on different samples, from the same lots, within a particular laboratory are shown in Tables 6 and 7. A mean F value of 1.46 was obtained among all fluorescence levels. This is approximately the same amount of variability observed among inter-laboratory fluorescence tests conducted on the source samples. The variability constant, K, obtained from tests conducted among samples from the same lot within the sample laboratory was 1.47. Again, this closely approximates the K value obtained from inter-laboratory tests.

As samples studied in this test were drawn in the official manner by an officially designated representative, the observed variability should represent the minimum obtained under actual sampling conditions. When the sampling is properly done, theoretically the sampling variation should be such that observed F values would equal 1.00. In actual practice, however, theoretical sampling efficiency is apparently not obtained. This could be due to lot heterogeneity, inefficient sampling probes, or improper sampling technique. The difference between within-sample variability and among-sample variability observed in these experiments approximates the variability due to sampling beyond that theoretically expected. The numerical value can be approximated by subtracting F values in

Table 6. Comparison of observed to expected variances at different fluorescence levels for among-samples, within-laboratory error.

Mean Percent Fluorescence	Observed Variance	Expected Variance	F Value
2.32	3.76	2.27	1.66
2.39	2.56	2.33	1.99
9.52	2.84	8.61	0.33
10.78	4.84	9.63	0.50
16.33	23.09	13.66	1.69
32.46	27.56	21.92	1.26
41.61	85.27	24.71	3.45
49.60	35.26	24.99	1.41
52.93	25.30	24.91	1.01
80.69	14.23	15.58	0.98
89.88	5.26	9.10	0.58
98.88	3.79	1.11	3.41
99.26	0.66	0.73	0.90
99.80	0.10	0.20	0.50

Mean F value = 1.41

6 less than 1.00 = 42.8%

8 more than 1.00 = 57.2%

Table 7. Variability constants (K) for different sources of variation.

Sources of Variation	Variability Constant
Within-Sample, Within-Laboratory	1.27
Among-Germinators, Within-Laboratories	
Skilled Analyst	1.86
Unskilled Analyst	2.11
Among-Laboratories, Within-Sample	1.45
Among-Samples, Within-Laboratory	1.47
Mean	1.63

Table 1 from the F value in Table 6. The value in Table 1 represents the variability due to within-sample variation and must be subtracted from the F value in Table 6, before the sampling variability can be evaluated properly. This derived value can be called Fs and represents a quantity which can be added to the F value of any source of variability to estimate the actual variability which would be expected to occur when different samples are involved. It is important to realize that the sampling variability observed in these tests may be considerably less than that normally encountered in routine service testing. There are reasons to suspect that many samples are improperly drawn by untrained personnel.

In summary, these considerations stress the importance of proper sampling techniques, as well as reliable well-trained sampling personnel. Even under the most careful sampling procedure, theoretical sampling efficiency is difficult to achieve.

SUMMARY AND CONCLUSIONS

The variability in ryegrass fluorescence test results was investigated. Variation due to within-sample error, among-sample error, among-germinator error, and among-laboratory error was determined. This variability was then compared to theoretical variability due to random sampling alone to estimate the amount of experimental error which exists in fluorescence testing.

Results of the studies indicated that when testing a sample in a given laboratory, fluorescence test variation closely corresponded, but was slightly above that theoretically expected by sampling from a binomial population. However, when other sources of error were introduced, the variability increased considerably beyond that theoretically expected. A review of the literature suggested that a similar situation exists for germination test variability.

The variability constants presented in Table 7 can be used as direct multipliers to compute new, more realistic, confidence limits for each source of variation. The new confidence limits would account not only for variation due to random sampling, but also for experimental error existing through lack of complete standardization. When this is done it is apparent that the present AOSA fluorescence tolerances do not adequately account for fluorescence test variability, even within a particular laboratory. The inadequacy of

the present tolerances becomes even greater when other sources of variation are introduced. It is the conclusion of this study that the present AOSA fluorescence tolerances should be broadened to account for the variation that actually exists. This could be accomplished by calculation of new tolerances based on actual fluorescence test variability similar to that presented in this study; or the AOSA germination tolerances could be adopted to cover fluorescence test variation. This latter alternative would bring the AOSA and ISTA germination and fluorescence tolerances into agreement.

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