Variation and Error in Estimates of Ascospore Maturity and Discharge Derived from Examination of Crushed Pseudothecia of *Venturia inaequalis*

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ABSTRACT

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Crushed pseudothecia from overwintered, infected leaves are routinely used to assess the maturity and release of ascospores of *Venturia inaequalis* for the purpose of advising growers on timing fungicide sprays for the control of apple scab. A recent study has shown a disparity between the morphological maturity of ascospores and the physiological maturity of asci. Furthermore, the accuracy and reproducibility of assessments by different observers, using different methods of sample selection, preparation, and interpretation, have not been documented. We investigated variability in the ratings of a standard set of crushed pseudothecia by 17 observers and also quantified leaf-to-leaf, within-leaf, and temporal variation in ascospore maturity and discharge in populations of *V. inaequalis*. Ratings of the same pseudothecia by different observers were approximately twice as variable as repeated ratings by the same observer. Substantially more variation in ascospore maturity and discharge was encountered among populations from different leaves than within populations on a single leaf. The variance of assessments would, therefore, be minimized by sampling as many leaves as possible. A sampling plan was developed to specify sample sizes required to estimate the mean number of asci within a certain class based on the probable number of asci in that class (based on historical data) and the allowable error in the estimate. The sampling plan was validated with an independent set of observations of 1,800 pseudothecia. The statistical considerations of sampling and the delay between morphological maturity of ascospores and physiological maturity of asci are incorporated into guidelines for using squash mount assessments in disease management programs.

In commercial apple production, fungicide sprays for apple scab usually are initiated when the first susceptible tissue emerges or when mature ascospores of *Venturia inaequalis* (Cooke) G. Wint. are first detected. These two events are normally, but not always, coincident (9). Microscopic examinations of crushed pseudothecia have been used for more than 40 yr by regional specialists in the northeastern United States to advise growers on scheduling fungicides to control apple scab. Prebloom fungicide sprays are recommended when mature ascospores are detected in crushed pseudothecia. When squash mount assessments show the supply of ascospores is exhausted or nearly so, growers usually increase the interval between fungicide sprays and may also decrease the rate of fungicide (1,9).

A detailed description of a method to prepare squash mounts was published in 1969 by Szkolnik (9). Gadoury and MacHardy (1) later revised Szkolnik's method to increase accuracy in early- and late-season assessments. Today, extension plant pathologists, fruit specialists, and private consultants collect and distribute the results of squash mount assessments obtained by a variety of published and unpublished methods throughout the northeastern United States. Computer-linked information services, recorded telephone messages, and newsletters have made possible the rapid dissemination of this information. However, the statistical reliability of squash mount assessments has never been adequately addressed, and sampling methods to minimize variance in estimates have not been developed. Furthermore, a recent study has shown a disparity between the morphological maturity of ascospores and the physiological maturity of asci; i.e., ascospores that are morphologically mature are not always released even under the most favorable conditions (6). This disparity was not considered in previously described methods.

There is no documented basis for excluding the possibility that large differences in ascospore maturity or discharge between adjacent areas are attributable to variability among observers in collecting samples or in preparing or interpreting the mounts. The magnitude of variation in ascospore development or release among individual pseudothecia within a population has not been considered in earlier studies. A critical analysis of the sources of variation inherent in the preparation and interpretation of squash mounts is needed to determine the reliability of this pest management tool. Leaf-to-leaf and within-leaf variation in maturity also must be investigated to devise appropriate sampling plans. Ideally, methods should be standardized to allow a comparison of results obtained by different observers in different areas, and sampling schemes should be developed to minimize variance in estimated maturity of ascospores.

Our first objective was to measure observer-to-observer variation, i.e., the variation in estimates of ascospore maturity introduced by individual observers who viewed the same mounts. The second objective was to determine temporal variation of estimates by individual observers who repeatedly viewed the same set of squash mounts. Our third objective was to determine the magnitude of variation in ascospore development among individual pseudothecia on the same leaf and among populations of pseudothecia from different leaves in early, mid-, and late spring. From this analysis of variation, we have devised a standardized method and sampling scheme that maximizes the precision of estimates based on fixed sample sizes, and we describe the probable error of such estimates. Finally, we offer some suggestions on the application of squash mount assessments in management of apple scab.

MATERIALS AND METHODS

Preparation of the standard slide set. On 23 April 1986, leaves bearing numerous apple scab lesions were collected in Geneva, NY, from beneath McIntosh trees that had not received fungicide sprays during the previous growing season. The leaves were fixed in FAA (formalin, acetic acid, and 50% ethanol, 10:10:100, v/v) for 2 hr, rinsed in several changes of tap water, and stored between wet paper towels. Pycnidia and ascocarps of many fungi are found on overwintered apple leaves and are difficult to distinguish from pseudothecia of *V. inaequalis* when illuminated with reflected light. Selective removal of pseudothecia of *V. inaequalis* was accomplished by viewing moist leaves through a stereomicroscope

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at ×50 magnification with transmitted light from a 150W halogen source. Pseudothecia appeared as darkened, subepidermal structures, approximately 100 μm in diameter, with visible connections to the subcuticular stromata of *V. inaequalis*. Setae often were visible around the ostioles of pseudothecia.

Twenty pseudothecia were removed at random from leaf tissue, individually mounted in lactophenol on glass slides, covered with No. 2 coverslips, and placed on the stage of a compound microscope. While a pseudothecium was observed at a magnification of ×126, fine forceps were used to apply pressure to the coverglass to rupture the ascocarp wall. More pressure then was applied, and the coverglass was rotated and moved laterally to spread the asci in a thin layer. The coverslip then was sealed with Permount (Fisher Scientific, Pittsburg, PA). The slides were examined five times, and the number of intact (immature and morphologically mature) and discharged asci on each slide was recorded. Intact asci were examined under brightfield illumination, and discharged asci were examined under phase-contrast illumination. The slides then were repeatedly examined until the mean of the number of intact and discharged asci in two consecutive examinations differed by less than 5% from the mean of the previous five assessments. The resultant grand mean was assumed to represent the actual number of intact and discharged asci on each slide.

Seventeen observers from a variety of professional backgrounds viewed the standard slide set and counted the asci in each pseudothecium that were immature, mature, or discharged, as described by Gadoury and MacHardy (1). Each observer was provided with captioned micrographs that depicted the various ascus maturity categories and listed the distinguishing characters of each category. Immature asci contained only cytoplasm or delimited spores in which the apical cell was not noticeably wider than the basal cell. Epiplasm was absent in mature asci, and the apical cells of the ascospores were broader than the basal cells. Discharged asci viewed under phase-contrast illumination were approximately twice the length of other asci because of the extrusion of the endoascus, and the outline of the apical portion often was indistinct. Each observer was given the same brief (approximately 15 min) verbal instructions on the objectives of the research project, ascocarp morphology and anatomy, data to be recorded, and operation of the microscope used.

Six observers examined the standard slide set from two to five times as a means of determining the variation in individual observer's assessments of ascocarp maturity. Repeated examinations were made no less than 48 hr after the previous examination, and, in most cases, a period of at least 3 wk elapsed between repeated counts. Maximum likelihood estimates of variance components were calculated using the SAS VARCOMP procedure (8) for unbalanced samples.

Leaf-to-leaf variability and temporal variation in ascocarp maturity. Overwintered leaves were collected from beneath unsprayed McIntosh trees at the Mast Road Research Orchard in Durham, NH, and from unsprayed McIntosh trees in a research orchard at the Hudson Valley Fruit Research Laboratory in Highland, NY. Leaves were collected at three times that coincided with the onset, peak, and final stage of maturation of pseudothecia of *V. inaequalis* and to the green tip, pink, and petal fall phenophases, respectively, of apple fruit buds at each location. The actual leaf collection dates were 6 April, 16 May, and 2 June 1987 in New York and 13 April, 19 May, and 1 June in New Hampshire. The leaves were fixed in FAA and were examined by a single observer in each state with a stereomicroscope and transmitted light as before. Twenty pseudothecia were selected from each leaf, and a total of 30 leaves were examined per location and collection date. Random selection of pseudothecia was achieved by placing a leaf on the microscope stage and then removing the ascocarp closest to a reference point in the ocular. The pseudothecia were mounted and examined as before, and the numbers of immature, morphologically mature, and discharged asci were recorded.

RESULTS AND DISCUSSION

The experience of an observer was expressed as the product of the number of years an observer had performed squash mounts and the number of days on which squash mounts were prepared each year. The value of this product ranged from three to 120 among the 17 observers and was not significantly correlated ($P = 0.10$) with the accuracy of counts of total asci or discharged asci by the various observers. Most observers underestimated the actual number of empty asci present on slides. In many cases, this may have been attributable to misalignment of phase annuli during counts of empty asci. We found that misalignment of less than 10% can result in a loss of phase-contrast and, thus, a loss of the resolution of the hyaline empty asci.

The mean/variance relationships were examined. Means and variances appeared to be linearly related and the data were transformed as $(x + 1/2)^{1/2}$ to stabilize the variances. The variances of all classes (total asci, asci with immature ascospores, asci with morphologically mature ascospores, and discharged asci) were of similar magnitude (expressed as a proportion of the mean), and the coefficients of variation did not differ by more than 10% from the earliest to the latest sampling date for the immature ascus class. Thus, the variances associated with asci containing immature ascospores at bud break were used in subsequent calculations.

The data were analyzed to compute the variance in counts that could be attributed to within-observer variation (i.e., the variation in ascus ratings when an observer rates the same pseudothecium on different occasions), between-observer variation (i.e., the variation in ascus ratings when different observers rate the same pseudothecium), within-leaf variation (i.e., the variation in ascus ratings of different pseudothecia on the same leaf), and between-leaf variation (i.e., variation in the mean ratings of populations of pseudothecia from different leaves).

To identify sampling plans that minimized variance in the sample means within a range of practical sample sizes, a model was developed from the above variances. The resultant model for a given ascus count was: $y_{ijkl} = \mu + l_i + p_{ij} + o_{ijk} + r_{ijkl}$, where y is the count on a specific leaf (l) and pseudothecium (p) by a specific observer (o) on a specific occasion (r). Each of the four components can cause deviation of the observation from the overall mean of all such observations, and the variation when all observations are considered can be summarized by the corresponding variance components: ∂^2_l, ∂^2_p, ∂^2_o, and ∂^2_r. The data from leaves collected in New York provided the estimates of ∂^2_l and ∂^2_p, and the data from different observers provided estimates of observer-related variation.

The data on observers included replicated counts of 19 slides by six individuals. As before, a $(x + 1/2)^{1/2}$ transformation was used to stabilize the variance. Estimates of the variance components were $s^2_o = 1.02$ and $s^2_r = 0.27$. Counts of the same pseudothecium from different observers were about four times as variable as counts from a single observer. Therefore, although individual observers differed in the classes to which they assigned asci, once individuals decided on the criteria that determined assignment, they were consistent in the application of those criteria.

A full sampling plan would specify four values: the number of leaves, the number of pseudothecia per leaf, the number of observers per pseudothecium, and the number of readings by each observer. In practice, a single observer would view a given pseudothecium only once. It was left to determine the number of leaves and the number of pseudothecia per leaf.

Estimates of the variance components for asci containing immature ascospores for the New York data were $s^2_l = 2.81$ and $s^2_p = 4.78$. Assuming equal costs of sampling pseudothecia from the same or different leaves, the number of pseudothecia to be sampled from a single leaf is given by: $(s^2_p/s^2_l)^{1/2} = (4.78/2.81)^{1/2} = 1.3$, or approximately one pseudothecium per leaf. The estimated standard error of a sample mean will be given by: $s_y = [(s^2_r + s^2_o + s^2_p + s^2_l)/m]^{1/2} = [(0.27 + 1.02 + 4.78 + 2.81)/m]^{1/2} = (8.88/m)^{1/2}$, where m is the number of leaves. If $\pm L$ describes the 95% limits on the sample mean (L is the allowable error in the sample mean with a 5% probability that the error will exceed L), then approximately: $L = 2s_y$, which can be solved for m, when $m = 4[(s^2_r + s^2_o + s^2_p + s^2_l)/L^2]$. Thus, the approximate 95% limits for two, four, and nine pseudothecia, each taken from a different leaf, would be 5, 2, and 1, respectively. Note that these limits are expressed on a square-root scale. On the arithmetic scale, the limits would be $(z^{1/2} \pm L)^2$, where z is the arithmetic mean. For example, if four leaves were sampled and a single pseudothecium was selected from each leaf, the limits would be $(z^{1/2} \pm 2)^2$. If z were equal to 49 mature asci per pseudothecium, then the 95% limits on z would be $(49^{1/2} \pm 2)^2 = (7 \pm 2)^2 =$ 25–81 mature asci per pseudothecium. Assuming a total of 120 asci per pseudothecium, this is equivalent to an estimate of 21–68% ascospore maturity. Selecting nine pseudothecia from nine leaves would yield $(49^{1/2} \pm 1)^2 = (7 \pm 1)^2 =$ 36–64 mature asci, or 30–53% ascospore maturity.

The proposed sampling plan developed from the New York data was evaluated by simulation. The New Hampshire collection of pseudothecia was used as the test population: the means of each ascus class, at each sampling time, were taken as the population means to be estimated. The sampling plan was evaluated for five, 10, and 20 pseudothecia from as many leaves at $P = 0.05$. Leaves were randomly selected and the pseudothecia were randomly drawn from them. Simulations required the selection of 10,000 samples from the test population for each sample size, for each ascus class, and for each sampling time. The means were calculated for each sample. Between 85 and 100% of the sample means fell within the theoretical 95% limits for sample sizes of five, 10, and 20 pseudothecia (Table 1). Most sample means that fell slightly outside of the theoretical limits were on the first two sampling dates in the class of asci containing morphologically mature ascospores (Table 1).

The sample sizes required to estimate means of five to 100 asci per pseudothecium were computed for two levels of probability ($P = 0.10$ and 0.05) and three levels (10, 20, and 50%) of allowable error (Table 2). This information could be used to select sample sizes that were appropriate for the intended use of the information. For example, if the exhaustion of the ascospore supply was to be detected, it would be necessary to accurately assess the mean number of asci remaining in pseudothecia at the end of the primary infection season. If one assumed that pseudothecia form a total of approximately 100 asci and that 90% or more of the asci must discharge before there was no longer a danger of primary infection (1,9), the mean number of undischarged asci that would remain would be 10 or less. A sample size appropriate to estimate a mean of five asci would therefore provide a conservative estimate of the mean number of asci remaining in pseudothecia at the conclusion of the primary infection season. A sample of 10 pseudothecia would be appropriate to estimate a mean of five asci with a probability of 90% that the error would not exceed 50% (5 ± 2.5) of the mean (Table 2).

Some knowledge of the number of asci that are likely to be found at various times during the primary infection season is required to use Table 2 in planning

Table 1. Percentage of sample means within the 95% confidence limits for immature, mature, and discharged asci of *Venturia inaequalis* for three different sample sizes on three collection dates

		Leaf collection[a]		
Ascus class	**Sample size**	**Bud break**	**Pink**	**Petal fall**
Immature	5	99	99	97
	10	99	99	96
	20	99	98	96
Mature	5	88	89	98
	10	89	85	98
	20	89	87	97
Discharged	5	99	99	99
	10	100	99	100
	20	98	99	99

[a]Ten thousand samples were selected randomly from data on a population of 600 pseudothecia examined on each collection date in Durham, NH. The actual means of asci in each class were 74.8 immature, 32.6 mature, and 1.9 discharged at bud break; 33.9 immature, 18.9 mature, and 30.8 discharged at pink; and 18.6 immature, 9.7 mature, and 31.2 discharged at petal fall. Confidence limits were computed for each sample size from the variances of each ascus class from a similar population of 600 pseudothecia examined in Highland, NY.

Table 2. Number of pseudothecia of *Venturia inaequalis* examined to estimate the mean number of immature, morphologically mature, or discharged asci of based on the projected number of asci present in the ascus class of interest

Mean number		Number of pseudothecia required for allowable error[c]		
of asci in class[a]	**P[b]**	**10% of mean**	**20% of mean**	**50% of mean**
5	0.05	68	34	14
	0.10	48	24	10
10	0.05	34	17	7
	0.10	24	12	5
20	0.05	17	9	3
	0.10	12	6	2
50	0.05	7	3	1
	0.10	5	2	1
100	0.05	3	2	1
	0.10	2	1	1

[a]Number of asci that can be expected in the class of interest (immature, morphologically mature, or discharged) based on historical data.
[b]Probability that the error in measuring the mean number of asci in a class will exceed the given percentage of the mean in column 1.
[c]Maximum allowable error in measuring the mean number of asci in a class (expressed as percentage of the mean in column 1).

Table 3. Mean number of asci containing immature ascospores, asci containing morphologically mature ascospores, discharged asci, and total asci per pseudothecium of *Venturia inaequalis* at various phenological stages of apple fruit buds in Highland, NY, during 1986–1990

Phenological stage of apple fruit buds	Immature	Mature	Discharged	Total
Green tip	59	12	0	71
1 cm green	67	34	4	105
Tight cluster	56	42	11	109
Pink	45	40	19	104
Bloom	36	39	24	99
Petal fall	25	27	31	83

sample sizes. As a guide in planning sample sizes, we computed the mean and total number of asci in each class at key phenological stages of apple (5). These data were collected in Highland, NY, between 1986 and 1990 (Table 3). Table 3 gives the mean number of immature asci, asci containing morphologically mature ascospores, discharged asci, and total asci per pseudothecium at green tip, 1 cm green, tight cluster, pink, bloom, and petal fall.

Because of the disparity between the morphological maturity of ascospores and the physiological maturity of asci (6), we recommend that squash mounts alone not be used to estimate the maturity of ascospores. However, these assessments can still provide useful information on ascocarp development and release. The physiological maturity of asci can be assessed by counting the number of discharged asci before and after leaf samples are immersed in water (3). Empty asci eventually degenerate and are removed from sampling (1). Nonetheless, their numbers can be deduced from the number of intact asci remaining in pseudothecia at various times in the season (1). Certain rain events may result in the discharge of nearly all of the physiologically mature asci (7). MacHardy and Gadoury reported (7) that in most cases, more than 90% of the ascospores trapped during daytime rain events were trapped within 6 hr of the start of rain. Presumably, under the most favorable conditions for ascospore release, i.e., daytime rains of more than 2.5 mm with temperatures above 10 C (7), the physiologically mature ascospores are released. Assessing the number of discharged asci present immediately after such conditions should then provide the best indication of the actual maturity of the pathogen population.

In management of apple scab, information on the relative maturity of ascospores of *V. inaequalis* is of value at three times during the year: when the first ascospores are available for release, when ascospore maturation is entering its most rapid phase, and when the supply of primary inoculum is exhausted. The first is important because it signals the earliest possible time that fungicidal protection may be required. The second is associated with the maximum numbers of airborne ascospores during rain events and is a time during which growers are likely to adopt low-risk strategies to control apple scab. The last signals the transition to a less intensive fungicide programs or sometimes to the cessation of the fungicide spray program (1,5). The use of squash mounts in Integrated Pest Management programs should be designed to maximize the accuracy of information on ascospore maturity and release at these three key times. We suggest that early assessments focus on the first appearance of empty asci within pseudothecia as a warning of physiological maturity. The results could be provided to growers as a forecast if the water-immersion method mentioned above or the spore release method described by Szkolnik (9) were used. Alternatively, the first actual orchard release could be reported if growers are applying fungicides with extended postinfection activity. Sample sizes could be relatively small because large errors would not affect the application of the results; i.e., it matters little if the number of discharged asci is 5 ± 2.5 or 5 ± 0.5—the recommendation to growers would be the same.

The shift to a rapid phase of ascospore maturation is accompanied by a rapid accumulation of asci containing morphologically mature ascospores. This shift normally occurs approximately 125 degree days (base = 0 C) after bud break (2). Because of the larger mean to be estimated and the lack of precision required, smaller sample sizes can again be used to detect this shift. Once the shift to rapid accumulation of morphologically mature asci has been detected, squash mount assessments provide little useful information until the depletion of the ascospore supply is imminent.

The depletion of the ascospore supply can be most easily be determined by counting the number of intact asci remaining in pseudothecia in late-season assessments. The smaller the number of intact asci remaining, the greater the depletion of the ascospore supply. In general, larger sample sizes are required to accurately estimate smaller quantities. However, this requirement is offset in practical use by a decreasing requirement for precision (increase in allowable error expressed as a proportion of the mean) as total exhaustion of the ascospore supply is approached. Again, relatively small (i.e., <10) sample sizes should be sufficient to assess depletion of the ascospore supply if this depletion is considered to be the discharge of $>90\%$ of the ascospore supply.

In research on the development of pseudothecia or on the effects of treatments on ascospore maturation and release, more precision in estimating ascospore development and release may be required than in the above examples. Also, continued research on the relationship between inoculum dose and the epidemiology of apple scab (4,5) may result in a need for more precise assessments. Sample sizes can then be adjusted as deemed appropriate.

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