Shu Geng, R. N. Campbell, M. Carter, and F. J. Hills
University of California, Davis

Quality-Control Programs for Seedborr

Seed transmission of fungal, bacterial, or viral plant pathogens is universally recognized by plant pathologists as the most effective method of randomly distributing primary inoculum in crop production fields. The seeds of many crops are produced in the arid western United States to avoid seed-transmitted fungal and bacterial pathogens. These seed production patterns, however, often are not accompanied by a formal testing program, and occasional epidemics in western seed fields have led to major epidemics in the eastern crop production fields (6,11).

Virtually every issue of PLANT DISEASE or *Phytopathology* contains papers reporting methods for detecting seedborne pathogens, for determining the rates of seed transmission from infected mother plants, or for determining distribution of the pathogen within seeds. There are, however, few reports of programs attempting to certify that seed lots are pathogen-free and few discussions of associated problems. Such programs must determine the lowest detectable rate of seed transmission, the highest rate of seed transmission that can be tolerated in the field without suffering economic loss, and the physical limits on the number of seeds that can be processed in a seed health assay.

The programs for the control of lettuce mosaic virus and bean bacterial diseases (*Pseudomonas phaseolicola*, *Xanthomonas phaseoli*, and *X. phaseoli* var. *fuscans*) provide models for solving these problems and for statistical evaluation of the probability of detecting infected seeds. There are two programs for control of lettuce mosaic virus. In France, a tolerance limit of one infected seed per 1,000 seeds has controlled the disease satisfactorily (8). In California, several factors have mandated a lower level of infection, which was arbitrarily set at 0 infected seeds in 30,000 and has given excellent control of the disease (5,7); 0/30,000 implies a 99.9% probability of seed lots having 0–0.022% infection (5). Bacterial blights of bean have caused total crop destruction at infection rates of 1/16,000 (6), but the current program in Michigan certifies seed lots if no bacteria are detected in an assay from one sample unit of about 10,000 seeds (3).

Taylor (10) investigated the sensitivity of detection of *P. phaseolicola* and used the most probable number method to estimate the level of infection in a seed lot by assaying a series of samples of decreasing sizes. The immunofluorescence technique was used by Coleno et al (2); they presented graphs to find critical values for accepting or rejecting a seed lot based on Poisson and binomial probabilities.

These selected examples show some commonly employed techniques for detecting seedborne pathogens. These methods can be divided into two approaches: direct inspection of seeds or seedlings and indirect assay for the pathogen in a number of seed sample units. Not many workers have used statistical procedures for estimating the true incidence rate in the population or for deciding on acceptance or rejection of a seed lot.

Few guidelines are available to help plant pathologists develop statistically reliable assay programs. This paper originated from discussions concerning seed testing for black rot in the NCR-100 committee (12). Its objective is to present two quality-control methods, one each for the direct and indirect assays, for determining the necessary sample sizes that will allow detection of low levels of disease contamination with a guaranteed probability of success.

Elements of the Procedures

A certain percentage of seeds (I) in a lot may be contaminated by the pathogen. This contamination may range from a superficial infestation of the seed surface, to infection of the seed coat, to infection of the embryo. All of these will be designated infected seeds because they have viable propagules of the pathogen, even though embryos and seedlings may not have been infected.

Programs for detecting infection can never certify that there is absolutely no contamination, even if no diseased seedlings are observed in a large sample. The researcher must decide on a tolerable level of contamination (I_t), no matter how small. This level must be set lower than the minimum nontolerable level (I_{nt}) that has the potential to cause economic losses in crop production. Both the direct inspection and the indirect assay will be discussed, and a quality-control program will be suggested for each approach.

Direct Test of a Seed Sample

A large number (N) of seeds is planted or plated individually on media, and the observed number of diseased plants or seeds is designated X. The observed percentage of diseased plants $\hat{I}$, or 100 (X/N), provides an estimate of the true I in the seed lot. If I_t and I_{nt} are established, then a critical decision point, C, which is a compromise value between I_t and I_{nt}, can be defined. The seed lot will be accepted if I is less than C and rejected if it is greater. This is a classical statistical problem of hypothesis testing. Due to sampling errors, two types of mistakes may occur: Either a seed lot with an actual I greater than I_{nt} is accepted or a seed lot with an actual I smaller than I_t is rejected. The probabilities of these errors are designated β and α, respectively. A quality-control program can be developed by examining enough plants so that the probability of either mistake will be confined to a small and predescribed range. Therefore, by specifying these probabilities, the idea of hypothesis testing can be applied reversely to determine a sample size for quality control. When the percentage of diseased seed is very low, the probability of the occurrence of diseased seeds in a sample follows the Poisson distribution. Examples of the application of Poisson probabilities for the purpose of quality control were given at least a half-century ago (9). For easy application, we have extended Thorndike's chart of the cumulative Poisson probabilities (4) to cover a wider range of possible mean values up to 100 (Fig. 1). The sample size

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

0191-2917/83/02023607/$03.00/0

athogens

can be determined from Figure 1 using five steps:

1. The researcher defines the quality standard. Suppose information available about a disease indicates that 0.1% or more seed contamination in a seed lot can cause an economic crop loss but that 0.05% or less contamination will not be important. The researcher would like to have a high probability (eg, $1-\alpha = 95\%$) that the true I in an accepted seed lot is less than 0.05%. At the same time, the probability (β) that the true I in an accepted seed lot is equal to or greater than 0.1% could be set at 1%. One is usually more concerned about accepting an unacceptable lot than rejecting an acceptable lot, so β is set smaller than α. In this case, the researcher has defined and required a quality standard for the seed lot with the following specifications: The tolerable disease rate (I_t) is 0.05%, and the probability of accepting a tolerable seed lot ($1-\alpha$) is 95%, the nontolerable disease rate (I_{nt}) is 0.1%, and the probability of accepting a nontolerable seed lot (β) is 1% (or the confidence of rejecting an unacceptable seed lot is 99%).

2. The expected number of diseased seedlings in the sample is calculated for an initial guess as to sample size, N. If N = 10,000, then $NI_t = 10{,}000\ (0.0005) = 5$ (the expected number of diseased plants from an acceptable lot), $NI_{nt} = 10{,}000\ (0.0001) = 10$ (the expected number of diseased plants from an unacceptable seed lot).

3. Based on $NI_t = 5$ for the acceptable seed lot, the vertical line from 5 on the abscissa is traced to the intersection with the horizontal line of the 95% probability. The critical value (C) is obtained from the next sloping line above this point (9 in this example).

4. C = 9 is traced to its intercept with the unacceptable mean, 10, and the value

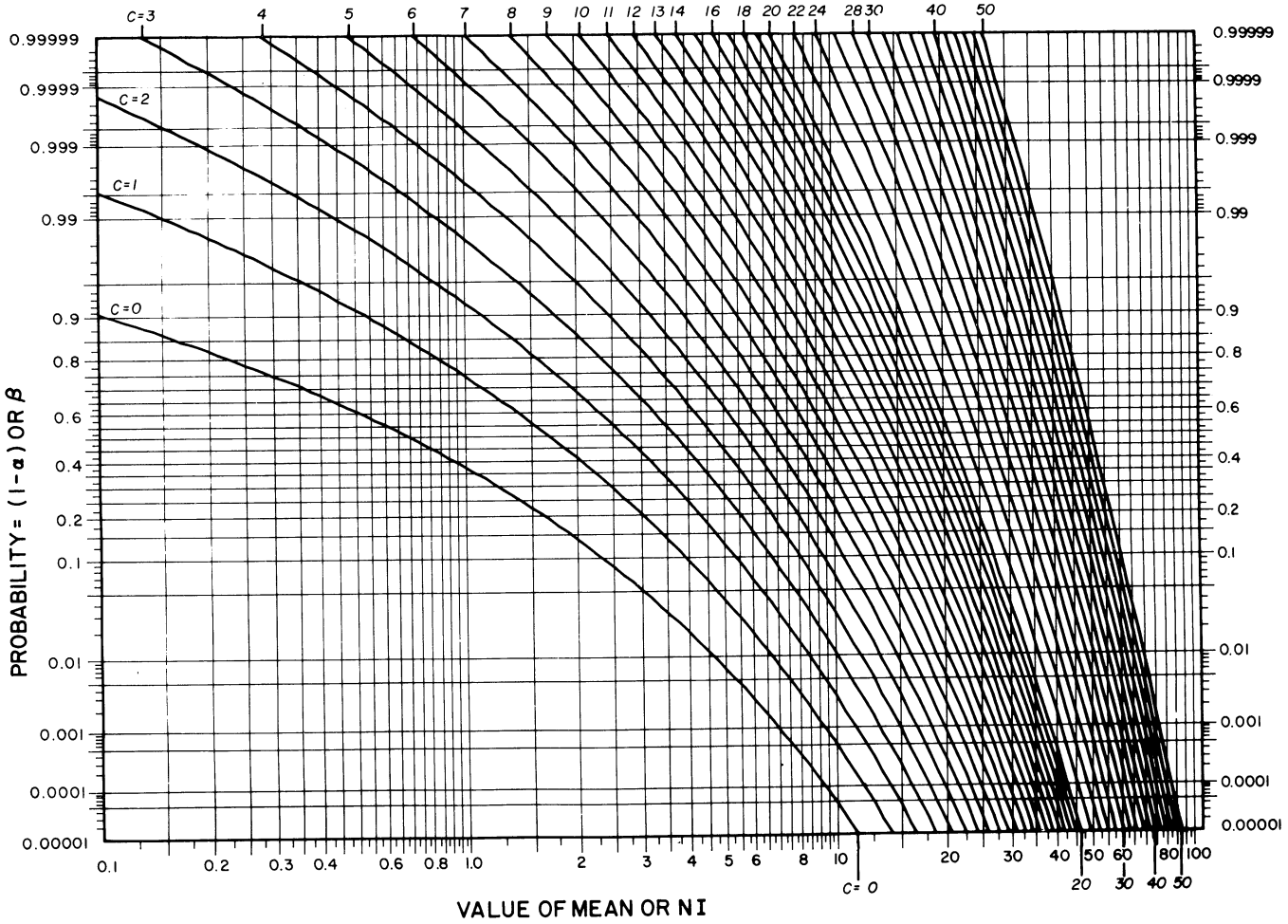


Fig. 1. Cumulative probability curves for the Poisson distribution.

on the ordinate is read. This gives β = 45%. Because this probability of accepting an unacceptable seed lot is much higher than the desired level of 1%, the sample size of 10,000 is too small.

5. This process is iterated starting with step 2 for larger sample sizes until β is less than 1%. In this example, we found that a sample size of 48,000 seeds reduces β to about 1%, so the recommended sample size N is about 48,000. With this size, we will have 95% confidence that a seed lot will not be rejected if its true disease rate is less than 0.05% and be 99% sure that a seed lot will be rejected if its disease rate is greater than 0.1%.

Table 1 illustrates how sample size changes with different requirements in the probabilities for the acceptance of a tolerable seed lot and against the acceptance of a nontolerable seed lot. Obviously, a more stringent quality standard requires a larger sample size of seeds.

Table 1. Sample sizes for various levels of probability for accepting a seed lot in which a tolerable disease rate (I_t) is 0.05% and a nontolerable disease rate (I_{nt}) is 0.1%

	$(1-\alpha)^b$			
β^a	0.80	0.90	0.95	0.99
0.005	35,947	45,976	53,324	71,267
0.01	31,846	39,308	47,813	63,231
0.05	19,443	25,500	31,415	45,266
0.10	14,206	20,129	24,757	37,199

[a] Probability of accepting a seed lot with a true incidence of 0.1% or more diseased seeds.
[b] Probability of accepting a seed lot with a true incidence of 0.05% diseased seeds or less.

Indirect Bioassays for Pathogens in Sample Units

The second approach utilizes sample units. Several samples (k), each having a number (N) of seeds, are extracted, and the extract of each sample is tested for the pathogen. The test result will show the presence or absence of the pathogen. Two elements dictate the result: whether the sample contains any infected seed and whether the assay technique will detect the contamination. Let P_c represent the probability of having contaminated seeds

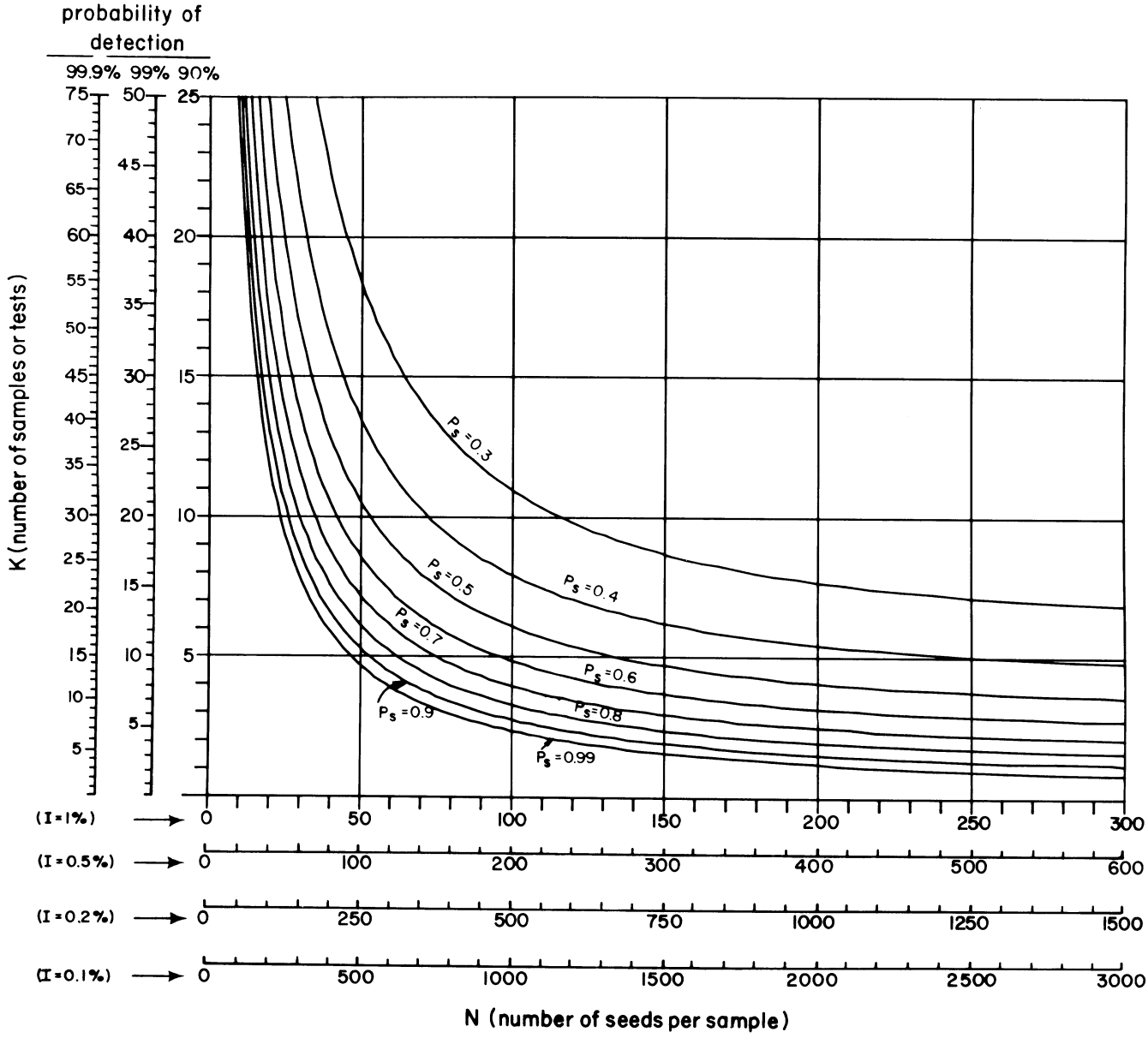


Fig. 2. Number of seeds per sample (N) and number of samples (k) for an indirect assay at various levels of assay sensitivity (P_s) and tolerable disease levels (I from 0.1 to 1%).

in a given sample unit and P_s represent the probability of detecting the pathogen in the sample unit (the sensitivity of the assay method). The probability of obtaining a positive result from a sample unit assay, P_d, is the product of the two probabilities:

$$P_d = (P_c)\,(P_s). \quad (1)$$

The probability of contamination in a sample unit (P_c) depends on the true percentage of infection (I) in the seed lot and the sample size (N) according to the Poisson distribution:

$$P_c = 1\text{-}e^{-NI}. \quad (2)$$

If a seed lot contains 0.015% contaminated seeds, the chance of having one or more diseased seeds in a sample of 500 seeds is:

$$P_c = 1\text{-}e^{-500(0.00015)} = 0.0723.$$

The sensitivity (P_s) characterizes the reliability of the assay method and must be determined before the general use of the assay method for quality control programs. For example, Kimble et al (7) evaluated the *Chenopodium* test for detecting lettuce mosaic virus and found that the probabilities of detecting contamination rates of 1 in 500, 1 in 1,000, and 1 in 2,000 seeds per sample were 80, 60, and 50%, respectively. Therefore, the probability of obtaining a positive assay from a 500-seed sample taken from a population with 0.015% diseased seeds is calculated from formula 1 as:

$$P_d = (0.072)\,(0.8) = 0.058.$$

If k is the number of sample units assayed, the probability of finding at least one positive result, P_+, is:

$$P_+ = 1\text{-}(1\text{-}P_d)^k. \quad (3)$$

Thus in this example, if 10 sample units are tested, the probability of detecting at least one infected seed in 10 tests is calculated using formula 3 as:

$$P_+ = 1 - (1\text{-}0.058)^{10} = 0.45.$$

For the purpose of quality control, the problem is to determine the appropriate number of seeds in a sample unit and the appropriate number of tests so that any specified level of contamination in the seed lot can be detected with a high probability of success. From the relationships of sample sizes and probabilities described above, N and k can be calculated:

$$N = -\ln (1\text{-}P_c)/I_t \quad (4)$$

and

$$k = \ln (1\text{-}P_+)/\ln (1\text{-}P_d), \quad (5)$$

in which I_t is defined as the maximal

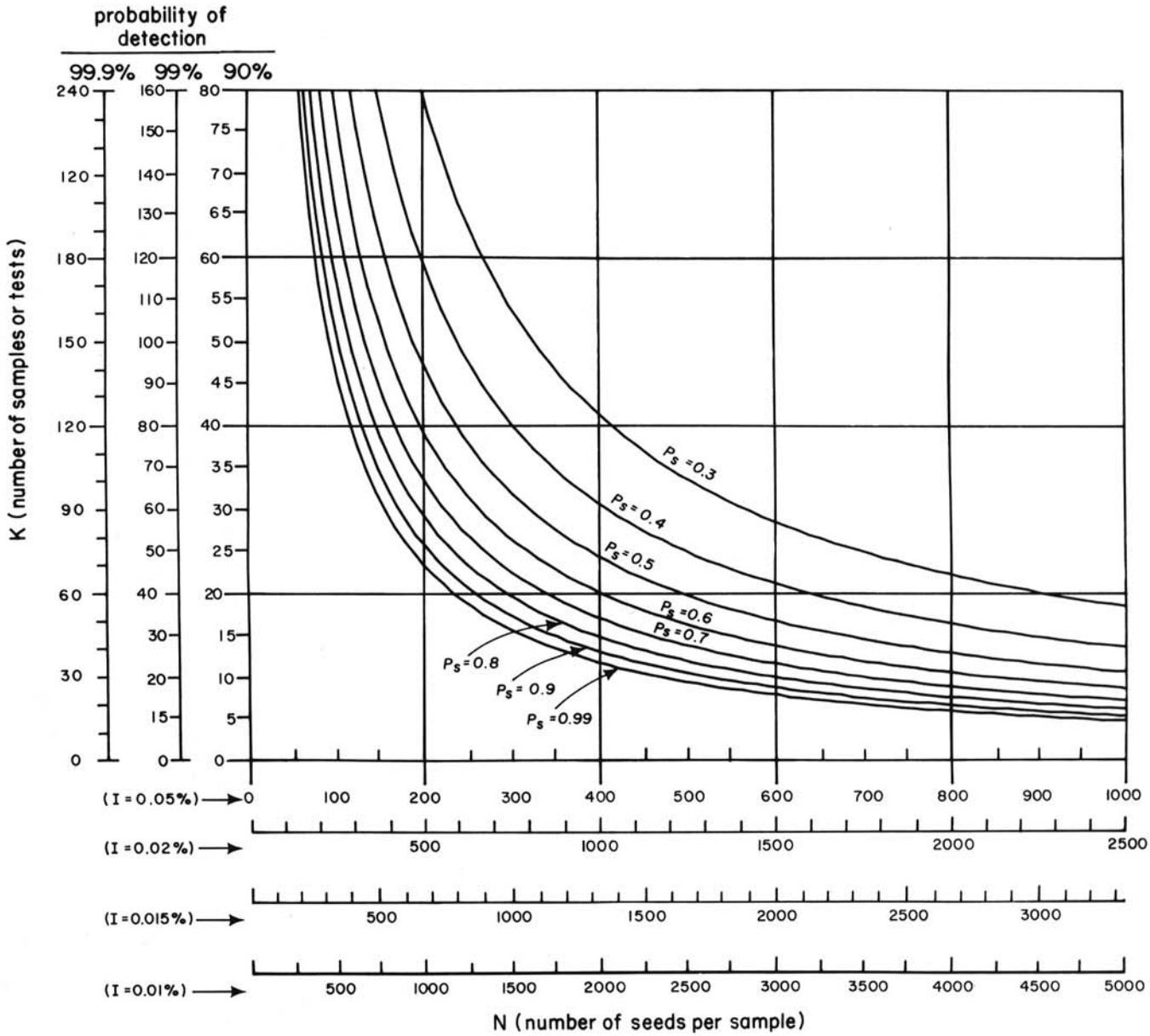


Fig. 3. Number of seeds per sample (N) and number of samples (k) for an indirect assay at various levels of assay sensitivity (P_s) and tolerable disease levels (I from 0.01 to 0.05%).

tolerable level of contamination in the seed lot.

The size of sample unit, N, often is fixed by the researcher for convenience and reflects practical limitations on handling the samples. In this situation, P_d needs to be calculated first from formula 1 based on the validated sensitivity of the method and the maximum tolerable disease level in the seed lot. The number of tests, k, can then be determined for any required probability of detection. Using the earlier example to detect an incidence level of 0.015% seedborne lettuce mosaic, Kimble et al (7) worked with samples of 500 seeds with a sensitivity of 80%. The number of tests required to have at least a 99% confidence of detecting a disease level equal to or above 0.015% is:

$$k = \ln (1-0.99)/\ln (1-0.058) \cong 77.$$

If a larger sample size (eg, N = 1,000) is used, the number of tests required to maintain the 99% confidence of detection must be determined. In this case the probability of contamination per sample is:

$$P_c = 1-e^{-1,000\,(0.00015)} = 0.139$$

and the sensitivity for a 1,000-seed sample is 60%. Thus,

$$P_d = (0.139)\,(0.6) = 0.084$$

and

$$k = \ln (1-0.99)/\ln (1-0.084) \cong 52.$$

Therefore, 52 tests of sample units of 1,000 seeds are equivalent to 77 tests of sample units of 500 seeds in that both give a 99% confidence of detecting a contamination level of 0.015% or greater. For easy application, we plotted these relationships for some commonly used cases in Figures 2 and 3. These figures can be used to solve for N and k in several situations given assumed values for I and P_s: 1) given N, find k, or 2) given k, find N, or 3) given P_c, find N and then solve for k, or 4) given P_+, find k and then solve for N.

In the above lettuce mosaic example, if I = 0.2% and the sensitivity P_s = 80% for sample units of 250 seeds each, we must determine the number of tests required to be 99.9% confident that at least one sample will show a positive result. In Figure 2, using the scale for I = 0.2% and N = 250, we move vertically to the interception of the sensitivity curve P_s = 80%. The corresponding k for P_+ = 99.9% is about 19, the correct answer.

If, however, the researchers can do only 10 tests (k), how many seeds must be included in each sample unit to maintain a 99.9% confidence for detecting at least one positive result? Reversing the steps with Figure 2, the N that corresponds to the intersection of the 80% sensitivity curve and k = 10 is found to be 500 for I = 0.2%. If the sensitivity of larger sample units is only 60%, the intersection of k = 10 with P_s = 60% shows that the sample size should be 850 seeds.

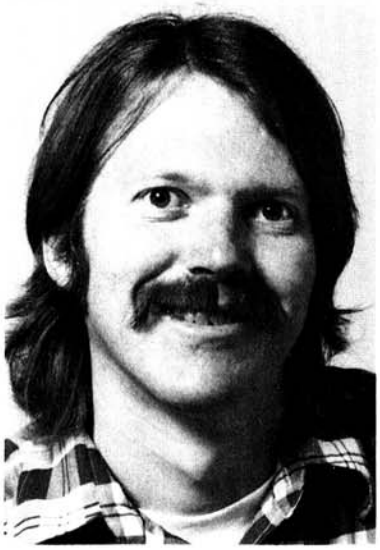
Another approach to the problem may be stated as follows: Suppose the tolerable disease rate is 0.2% and the sensitivity of the method is determined as 80%. The investigator can calculate how large a sample to take in order to be 50%

Shu Geng

Dr. Geng is an associate professor in the Department of Agronomy and Range Science and an associate biometrician at the Experimental Station of the University of California, Davis. He teaches two applied statistics courses for the College of Agricultural and Environmental Sciences and does research in experimental designs and multivariate analysis.

R. N. Campbell

Dr. Campbell is a professor in the Department of Plant Pathology at the University of California, Davis. He teaches an introductory plant pathology class and does research on diseases of vegetable crops.



M. Carter

Mr. Carter is a research programmer in the Department of Agronomy and Range Science at the University of California, Davis. He is responsible for the operation and management of the computer laboratory in the department and for the development of research computer programs.

F. J. Hills

Dr. Hills is an extension agronomist and biostatistician with the Cooperative Extension Service of the University of California. He does research in sugar beet production and is responsible for instructing extension personnel in the design and analysis of field experiments.

sure that each sample contains at least one diseased seed and how many such samples are needed to be 99% confident that at least one test will be positive. The procedure is to first solve for N from formula 4, ie,

$$N = -1n\,(1-0.5)/0.002 \cong 347,$$

and then use Figure 2 to find k. From the figure, k is 9 for $P_+ = 99\%$. Thus, nine assays of sample units containing 347 seeds are required to meet the specified conditions.

Similarly, if P_d is given, k can be calculated from formula 5. Figure 2 or 3 can then be used to find N from k.

Discussion

Two techniques are described in this paper to determine the sample size and the number of tests to be used for having a high confidence of detecting contaminated seed lots if the disease incidence in the seed lot is above a specified tolerable level. An advantage of the first procedure, used for direct planting or plating tests, is that an estimate of the true incidence rate in the population can be obtained from the number of diseased seedlings observed. Disadvantages are the time, labor, and costs of the experiment. However, although preferable in many aspects, the indirect assay procedure will not provide an accurate estimate of the actual incidence rate. Both procedures will satisfy any required assurance for detecting an unacceptable level of disease if the number of seeds in a sample and the number of tests are large enough.

One important consideration in determining an adequate number of tests in the indirect assay technique is the sensitivity of the assay technique. The probability of detecting the pathogen, ie, the sensitivity of the assay, must be established before the technique is adopted for general use. Furthermore, the sensitivity of a technique is a function of the level of contamination in the sample unit: the higher the percent contamination, the greater the chance for a positive result by the technique and, hence, the greater the sensitivity.

Validation trials must be done to establish the sensitivity of an assay technique either at one level or at several levels of contamination. The number of replications (r) required in a validation trial to estimate the sensitivity at one contamination level can be determined by the following formula:

$$r \cong 4\,\frac{\hat{P}\cdot\hat{Q}}{d^2},$$

in which $\hat{P}$ is an estimated probability of detecting one infected seed in N-1 uninfected seeds, $\hat{Q} = (1-\hat{P})$, and d is an acceptable deviation of the estimated sensitivity from the experiment to the true sensitivity of the assay method. The

true sensitivity will fall within the estimated sensitivity plus or minus d using this number of replications with a 95% confidence. If the estimated probability is 90% and the acceptable deviation is 10%, then:

$$r = \frac{4\ (0.9)\cdot(0.1)}{(0.1)^2} = 36.$$

Thus, 36 replications are needed in a validation trial in order to have a 95% confidence that the true sensitivity is within one d distance from the estimated sensitivity.

If sensitivities at several contamination levels have to be determined in a validation study, the number of replications per level can be reduced to obtain a good estimate of the sensitivity curve. The design of this type of experiment can be found in Brown (1).

Sometimes quality regulations exist for certain testing programs. For instance, in the lettuce mosaic virus control program in Monterey County, California, seed lots are acceptable only if no diseased plants are observed when 30,000 plants are grown. A 0/30,000 result can be observed where there is a wide range in the true incidence I: If $I \geqslant 0.015\%$, there is a 1% chance of seeing 0/30,000 in the direct assay, but if $I \geqslant 0.0023\%$, the chance is about 50%. The 0/30,000 requirement can be translated into the determination of a sample unit assay in the following fashion: If the sensitivity of 500 seed units is 100%, the absence of a positive test in 60 sample units will yield the same quality-control standards as the grow-out test specifying 0/30,000; if the sensitivity of 500 seed units is only 80%, then 75 tests are required to match the same quality control.

Although empirical standards can give excellent control of certain diseases, it is advisable to determine the sample size by going through the exercise of defining required standards for the control program as introduced in this paper. A good quality-control program can be developed with an understanding of the levels of the assurance required.

Literature Cited

1. Brown, B. W., Jr. 1966. Planning a quantal assay of potency. Biometrics 53:322-329.
2. Coleno, A., Trigalet, A., and Digat, B. 1976. Detection of seed lots contaminated by a phytopathogenic bacterium. Ann. Phytopathol. 8:355-364.
3. Copeland, L. O., Adams, M. W., and Bell, D. C. 1975. An improved seed program for maintaining disease-free seed of field bean (*Phaseolus vulgaris*). Seed Sci. Technol. 3:719-724.
4. Dodge, H. F., and Romig, H. G. 1944. Sampling Inspection Tables—Single and Double Sampling. John Wiley & Sons, Inc., New York.
5. Grogan, R. G. 1980. Control of lettuce mosaic with virus-free seed. Plant Dis. 64:446-449.
6. Guthrie, J. W., Huber, D. M., and Fenwick, H. S. 1965. Serological detection of halo blight. Plant Dis. Rep. 49:297-299.
7. Kimble, K. A., Grogan, R. G., Greathead, A. S., Paulus, A. O., and House, J. K. 1975. Development, application, and comparison of methods for indexing lettuce seed for mosaic virus in California. Plant Dis. Rep. 59:461-464.
8. Marrou, J., and Messiaen, C. M. 1967. The *Chenopodium quinoa* test: A critical method for detecting seed transmission of lettuce mosaic virus. Proc. Int. Seed Test Assoc. 32:49-57.
9. Pearson, E. S. 1933. A survey of the uses of statistical method in the control and standardization of the quality of manufactured product. J. R. Stat. Soc. 96:21-60.
10. Taylor, J. D. 1970. The quantitative estimation of the infection of bean seed with *Pseudomonas phaseolicola* (Burkh.) Dowson. Ann. Appl. Biol. 66:29-36.
11. Walker, J. C., and Patel, P. N. 1964. Splash dispersal and wind as factors in epidemiology of halo blight of bean. Phytopathology 54:140-141.
12. Williams, P. H. 1980. Black rot: A continuing threat to world crucifers. Plant Dis. 64:736-742.