Techniques

A Test for Randomness of Infection by Soilborne Pathogens

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ABSTRACT

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A simple method is described to test for randomness of infection in inoculum density experiments involving soilborne pathogens. The method tests the goodness-of-fit over a range of inoculum densities of the observed numbers of infected and uninfected roots with those predicted by the Poisson distribution. The method requires scores only of the presence-or-absence of infection on roots and not of the numbers of infections on a root. Data from inoculum density experiments involving the take-all fungus, *Gaeumannomyces graminis*, and seedlings of wheat and barley are used to illustrate the application of the test.

The process of infection of subterranean plant organs may be said to be random if each of a large number of infection sites has an equal and independent chance of being infected. The frequency of infection then approximates to a Poisson distribution. Knowledge of whether infection occurs at random is important to an understanding of the epidemiology of infection by soilborne pathogens. For example, deviation from randomness may indicate unequal distribution of susceptibility of roots within the host population or pathogenicity of the parasite. Prior testing for deviation from randomness would also reduce the risk of misapplication of the often-used multiple infection transformation. This transformation (9), which is widely used by plant pathologists to estimate the mean number of infections per unit from the proportion of infected roots, hypocotyls, or plants (see the reviews of Baker [1,2]), is based upon an assumption of random infection.

The standard mathematical procedure for testing for randomness of infection is to test the goodness-of-fit of the frequency distribution of the observed numbers of infections per unit (usually a root) for agreement with a Poisson distribution (3,8,11), at separate inoculum densities. Perhaps the principal limiting factor in this procedure is the need to count the actual numbers of infections. This is time-consuming and is complicated for many soilborne fungi by the difficulty of identifying primary infections. The term primary infection is taken to describe the symptoms arising from inoculation of a single root by a single

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propagule from the soil inoculum. Primary infections by the take-all fungus, *Gaeumannomyces graminis* Sacc. Arx and Olivier var. *tritici* Walker, are difficult to identify. This widespread pathogen of wheat and barley survives in soil on previously infected host debris of indeterminate size. Upon contacting a host root the fungus grows ectotrophically over the root surface penetrating, at intervals, the cortex and ultimately the stele (see the review by Nilsson [10]). Invasion of the stele results in blockage of the vessels. The symptoms of disease, which result from a single inoculation, are variable and appear as discontinuous, dark lesions within the stele (see Fig. 2 in Gilligan [7]). Counts of primary infections are therefore difficult to make and may be subject to bias due to overlapping of infections at high densities of inoculum.

In contrast to enumerating numbers of infections, scoring for presence or absence of infection on individual roots is easy and unequivocal. This paper describes a test for randomness requiring data only on the numbers of uninfected and infected roots at each of several inoculum densities. Data from experiments involving *G. graminis* and wheat and barley seedlings are used to illustrate the application of the test. Differences between treatments in the experiments described below are discussed only in relation to the tests for randomness.

MATERIALS AND METHODS

Presence-or-absence test for randomness of infection. If infection occurs at random, and hence the frequency of infection is approximated by a Poisson distribution, the proportion of uninfected roots is estimated by e^{-m} in which m is the mean number of infections per root. This is the first term of the Poisson expansion. If the number of susceptible sites is not limiting and there is neither synergistic nor antagonistic interaction between units of inoculum, the mean number of infections per root will vary in direct proportion to the inoculum density.

If the preceding assumptions hold, the expected number of uninfected roots, T_i, at the ith inoculum density, is given by

$$T_i = M_i \exp(-m_{\min} N_i / N_1). \quad (1)$$

M_i is the number of roots available for infection at the ith inoculum density (N_i) and $m_{\min}$ is the mean number of infections per root at the lowest, non-zero density of inoculum, N_1. Hence for an experiment with a single treatment and j densities of inoculum there will be j equations like equation 1, all with the same parameter $m_{\min}$. The expected number of infected roots at the ith inoculum density is simply the difference, $M_i - T_i$. Observed and expected numbers of uninfected and infected roots may be compared by a $2 \times j$ chi-square contingency test. A significant value for the chi-square statistic leads to rejection of the null hypothesis that infection occurred at random.

When roots are scored only for the presence or absence of infection, the value of the parameter $m_{\min}$ is unknown. Therefore, it must be estimated in order to apply the test for randomness based on the presence or absence of infection. The estimate of $m_{\min}$ is obtained by iteration, subject to the constraint of minimum chi-square; the value for $m_{\min}$ is selected, which gives the lowest possible value for the chi-square contingency test.

Although the computations can be done on a pocket calculator, a computer program (obtainable from the author) was written for ease and convenience in the iterative estimation of $m_{\min}$. The program employs a preliminary criterion of minimizing the difference between the observed and expected numbers of uninfected roots, summed over all non-zero inoculum densities. This crude estimation procedure reduced the range of values over which $m_{\min}$ was tested for minimum chi-square.

In the preliminary stage, the mean number of infected roots at the lowest non-zero inoculum density is taken as the first approximation for $m_{\min}$. At the end of the first iterative cycle, the initial value of $m_{\min}$ is reduced or increased, respectively, by 0.2, depending upon whether the difference between the observed and expected values of T_i is positive or negative. In subsequent iterative cycles, sequential values for the estimate of $m_{\min}$, say m_{k+1}, are obtained by the following empirically derived expression:

$$m_{k+1} = m_i + (S - (\Sigma T_i)_k)(m_k - m_{k-1}) / ((\Sigma T_i)_k - (\Sigma T_i)_{k-1}) \; k \geq 2 \quad (2)$$

in which S is the observed value of uninfected roots summed over all inoculum densities and $(\Sigma T_i)_k$ is the corresponding estimated value. For the data discussed in this paper, seven iterations were usually sufficient to obtain a value for $m_{\min}$ that reduced the difference $(S - \Sigma T_i)$ to 0.001. Next, chi-square statistics for the contingency table (infected and uninfected roots by inoculum density) were calculated for values of m incremented by 0.001 within the range $m_i \pm 0.02$. The value of m corresponding to the minimum chi-square was then designated as $m_{\min}$ and the test for randomness was based upon that chi-square value. For an experiment with j inoculum densities there are $j - 1$ degrees of freedom. That is, suppose there were six densities of inoculum. Six of the original 12 degrees of freedom are lost by using the six values of M_i to estimate the expected numbers of uninfected roots from equation 1. One further degree of freedom is lost for the estimation of the parameter $m_{\min}$ from the data, thus giving five degrees of freedom for the chi-square test.

Inoculum density experiments. *Inoculum.* Foxtail millet grains, *Setaria italica* (L.) Beauv., colonized with a strongly pathogenic isolate of *G. graminis*, were used to provide small, uniform inoculum units (7). Twelve grams of millet, 12 g of sand, and 10 ml of distilled water were added to 100-ml Erlenmeyer flasks and autoclaved twice at 120 C for 1 hr with a 3-day interval between autoclavings. The flasks were then incubated for 28 days at 19 C with six disks, 4 mm in diameter, cut from the margin of a colony of *G. graminis* growing on potato-dextrose agar (7). The percent colonization of grains in individual flasks and the ability of the grains to cause infection were both assessed as follows. A sample of 40–50 grains was removed from each flask. Wheat seedlings were grown in narrow, sand-filled polyethylene tubes arranged on a slope so that the roots grew down the surface of the sand in contact with the transparent polyethylene (7). The millet grains were placed individually in contact with the roots by cutting a small window in the polyethylene and then resealing it with adhesive tape. The inoculum units were separated by a minimum distance of 40 mm along roots with a maximum of three inoculations per root. The roots were examined after 15 days of incubation at 19 C. The proportion of inoculum units that had caused dark discoloration of the stele close to the site of inoculation was recorded. Flasks of inoculum used in the experiments described below all had scores for proportion of successful inoculations ≥ 0.95.

Soils. Three types of soil were used:

1) Conducive soil—a heavy clay soil, pH 6.2, from the Oxford University Field Station at Wytham. It had not been sown to crops susceptible to *G. graminis* for 4 yr prior to collection and the site had been fallowed in alternate years. The soil did not contain any detectable inoculum of *G. graminis*.

2) Suppressive soil A—a heavy clay soil, pH 6.5, also from the Oxford University Field Station. Winter wheat had been grown in it continually for 12 yr and it was showing take-all decline. It contained only low levels of inoculum of *G. graminis* as revealed by baiting samples of the soil with wheat seedlings.

3) Suppressive soil B—a stony clay loam soil, pH 6.5, from a site on Little Knot Field, Rothamsted Experimental Station, in which susceptible cereals had been grown continually for at least 15 yr. It was also showing take-all decline and contained only low levels of *G. graminis* inoculum. After collections from the field, each soil was bulked and sieved through a 12-mm mesh screen to remove stones and large pieces of debris and to break up aggregates. Long pieces of straw that passed through the sieve were removed from the suppressive soils to reduce the level of naturally occurring inoculum. It was appreciated that this action might also have reduced the level of microbiological antagonists (4) associated with the phenomenon of take-all decline. It was, however, of greater importance in the present investigation to ensure that the amount of infection arising from naturally occurring inoculum was

minimized. Subsequently the soils were mixed with washed quartz sand (2:1, v/v) to facilitate easier washing of roots from the soils. The term soil is used below to describe these soil and sand mixtures.

Infestations. Inoculum units were restricted to a layer of infested soil sandwiched between layers of uninfested soil in the middle of plastic pots, with maximum diameters of 152 mm. This arrangement prevented infection spreading from one root to another via the crown of infected plants and avoided repeated infection of proliferating roots at the bottom of the pot. The inoculum layer was of thickness equivalent to that of the inoculum units so that it was effectively two dimensional. The thickness of the layer is not, however, pertinent to the present investigation other than to note that multiple infection of roots by inoculum in soil would only occur over a short length of each root. The diameter of the inoculum layer was 120 mm. Pots were filled with uninfested soil to a depth of 65 mm; aliquots of inoculum units numbering 20, 60, or 100 were shaken onto a soil to form an infested plane. This was covered with more uninfested soil to a total depth of 140 mm. The pots were sown on the same day with three germinated wheat or barley grains placed 60 mm above the inoculum. The experiment consisted of four treatments carried out at two temperatures, 19 and 10C, respectively, giving eight treatment combinations in all. The treatments were wheat growing in conducive soil, wheat growing in suppressive soil A, wheat growing in suppressive soil B, and barley growing in conducive soil. All treatment combinations were replicated three times. The experiment was carried out in two phytotrons running at 19 C, 16 hr of light, and 10 C, 8 hr of light, respectively. The moisture status of the soils was maintained at approximately field capacity by watering to constant weight.

Examination of roots. After 28 days, roots were washed free of soil. The numbers of seminal roots long enough to have passed through the inoculum layer were identified and summed over the replicates for each inoculum density to give the values for M_i. These roots were examined against a white background under a dissecting microscope for symptoms of take-all infection.

Roots from seedlings grown at 19 C were recorded as infected if they showed any dark discoloration of the stele. Because of the slower development of symptoms at lower temperatures (7) the criterion for infection of roots of seedlings grown at 10 C was extended to include roots showing any cortical discoloration. *G. graminis* was routinely reisolated from samples of root taken from within discolored regions.

The number of roots not showing any signs of infection were summed over all inoculum densities to give the value of S used in equation (2) for each treatment within each experiment. There was no sign of root infection by *G. graminis* in any of the controls to which killed inoculum had been added. The level of natural infection was therefore assumed to be of negligible importance.

RESULTS AND DISCUSSION

Application of the test for randomness based upon the presence or absence of infection yielded evidence of significant ($P = 0.05$) deviation from randomness in two of the eight treatment combinations tested. The two treatments were wheat growing in suppressive soil A and barley growing in conducive soil, both at 19 C. The chi-square values for two other treatments, wheat growing in conducive soil at 10 and 19 C, respectively, were close to statistical significance (Table 1). The observed and expected numbers of infected roots are given in Table 2 to illustrate the

TABLE 1. Results of chi-square contingency tests to compare the observed numbers of infected and uninfected roots with those estimated[a] on the null hypothesis of random infection

Temp (C)	Treatment: Host	Treatment: Soil	Estimated[b] value of m_{min}	Minimum chi-square value (2 d.f.)	Probability of exceeding minimum chi-square	Evidence for deviation from randomness ($P = 0.05$)
19	Wheat	Conducive	0.322	5.23	0.073	No
	Wheat	Suppressive A	0.216	2.22	0.330	No
	Wheat	Suppressive B	0.204	7.47	0.024	Yes
	Barley	Conducive	0.287	12.28	0.002	Yes
10	Wheat	Conducive	0.083	5.68	0.059	No
	Wheat	Suppressive A	0.072	1.60	0.450	No
	Wheat	Suppressive B	0.050	2.98	0.225	No
	Barley	Conducive	0.091	4.06	0.131	No

[a] Estimated numbers of uninfected roots (T_i) were calculated from the equations $T_i = M_i \exp(-m_{min} N_i / N_1)$ for $i = 1, \ldots, 3$. The number of roots available for infection at the ith inoculum density (N_i) is M_i: m_{min} is the mean number of infections per root at the lowest, non-zero, inoculum density. Estimated numbers of infected roots were obtained by the difference, $M_i - T_i$.

[b] The estimate of the parameter m_{min} was obtained iteratively such that the value of the chi-square statistic for the contingency test was minimized.

TABLE 2. Observed and expected[a] numbers of infected roots in the inoculum density experiment

Temp (C)	Treatment: Host	Treatment: Soil	Inoculum density (units/cm²): 0.18 Infected roots (no.): Observed	0.18: Expected	0.18: Total[b]	0.53: Observed	0.53: Expected	0.53: Total	0.88: Observed	0.88: Expected	0.88: Total
19	Wheat	Conducive	17	11.6	42	27	26.6	43	31	34.4	43
	Wheat	Suppressive A	11	8.9	46	15	18.6	39	26	24.4	37
	Wheat	Suppressive B	12	6.5	35	16	18.3	40	20	23.0	36
	Barley	Conducive	22	13.5	54	19	26.5	46	44	44.2	58
10	Wheat	Conducive	5	3.4	43	4	9.7	44	17	14.6	43
	Wheat	Suppressive A	5	3.1	44	8	9.0	46	13	14.3	47
	Wheat	Suppressive B	0	2.2	45	8	6.2	45	9	9.9	45
	Barley	Conducive	4	5.2	60	19	14.0	59	18	22.6	62

[a] Expected numbers of infected roots were calculated from the expression $M_i(1 - \exp(m_{min} N_i / N_1)$ in which M_i is the number of roots available for infection at the ith inoculum density (N_i) and m_{min} is the mean number of infections per root at the lowest, non-zero, inoculum density (N_1).

[b] Total refers to number of roots available for infection.

application of the contingency test. Deviation from randomness may have arisen from a number of causes. For example, unequal susceptibility of roots or variation in pathogenicity of inoculum units each might affect the probability of roots remaining uninfected, but the data in the experiment reported here do not permit identification of the cause of any deviation from randomness.

Limitations of the test. The minimum chi-square estimates of m_{min} were close to the preliminary estimates based upon minimum difference between observed and expected total numbers of uninfected roots (*unpublished*). The minimum difference procedure of estimation is, however, a non-standard procedure whose statistical properties are unknown (D. Brown, *personal communication*). Unlike the minimum chi-square procedure, it makes use only of the marginal total and not of individual observations from within the contingency table. Therefore, it is unlikely that it would be precise enough for routine use. The minimum chi-square procedure has been used in bioassay, and Finney (page 521 in reference 5) records that it has the desirable property of being asymptotically efficient for large samples. In other words, minimum chi-square estimators are as precise as any other estimator of a population parameter when samples are large. Unfortunately the statistical properties of minimum chi-square estimates for small samples are not known (5).

However the estimate of m_{min} is made, the presence-or-absence test has two important statistical limitations. The first is that the range of inoculum densities should be such as to minimize the probability of obtaining expected values of uninfected or infected roots of less than one. Such values render the chi-square contingency test unreliable unless classes (in this case inoculum densities) are amalgamated, which requires that degrees of freedom be sacrificed (12). Even with expected values as low as one, Snedecor and Cochran support the use of chi-square test only when most of the expectations are substantially larger (page 241 in reference 12). The second statistical limitation also concerns degrees of freedom. That is, no account is taken of information supplied by replicates within inoculum densities with a consequent loss of degrees of freedom. Only the total numbers of infected and uninfected roots are used. Separate tests could be carried out for blocks or, if the experiment were completely randomized, on randomly selected replicates from each inoculum density. But this would result in as many tests for each treatment as there are replicates. The conclusions of tests within treatments may differ. Such multiplicity would then be of little use in making decisions (eg, as to whether the use of the multiple infection transformation is justified).

Relationship between test and multiple infection transformation. The mathematical bases of the multiple infection transformation and the presence-or-absence test are similar. If the proportion of infected roots at the ith inoculum density is x_i, and the mean number of infections per root is λ_i then:

$$1 - x_i = \exp - \lambda_i.$$

But, if $\lambda_i = m_{min} N_i / N_1$ then:

$$\ln (1 - x_i)^{-1} = m_{min} N_i / N_1$$

The expression $\ln (1 - x_i)^{-1}$ is the multiple infection transformation (9), which estimates the mean number of infections per root. Hence, plotting relative inoculum density against the number of infections per root, estimated by the multiple infection transformation, should give a straight line of slope m_{min}, if infection occurs at random. A crude test for randomness would simply be to fit a line by eye and assess its goodness of fit to the points subjectively. Rigorous statistical testing for deviation from linearity, and hence from randomness, is theoretically feasible but computationally difficult. Fitting of a line by ordinary least squares regression and testing for deviation from linearity by analysis of variance is not valid. The variance of $\ln (1 - x)^{-1}$ is not independent of relative inoculum density, but is a function of sample size and the proportions of infected and uninfected roots. Fulton (6) noted a similar restriction with the logit transformation. Proper fitting of a regression line in these circumstances is by iterative, weighted regression or maximum likelihood and deviation from linearity is properly tested by analysis of deviance (D. Brown, *personal communication*).

The presence-or-absence test proposed in this paper has the advantage of relative computational simplicity. Unlike the standard procedure of using goodness-of-fit to the Poisson distribution to test for randomness (3,8,11), the presence-or-absence test does not require data on the numbers of infections per root. Therefore, it is a simple and rapid means of detecting deviation from randomness in experiments in which several densities of inoculum are employed and in which the sign or symptom of a single infection is unequivocally distinctive.

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