Estimating the Efficiency of Partial-Vacuum Inoculation of Barley with Ustilago hordei

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ABSTRACT

Barley seeds (cultivar Hannchen) were inoculated with various ratios of sporidia of the *A* and *a* mating-types of *Ustilago hordei*. Treatment ratios of *A:a* ranged from 10,000:1 to 1:10,000. With increasing ratios a significant reduction in percentage of diseased plants was seen at 100:1 and at 1:500. A simple method was devised which, based on the relationship between the treatment ratios and percentages of diseased plants, provided an estimate of n, defined as the average number of sporidia available, per seed, for dikaryon formation and infection resulting in smutting. The value of n was calculated as 135, 201, and 195 for the 100:1, 500:1, and 1000:1 ratios, respectively. The conclusion is that n is sufficiently large, so that with a normal 1:1 mating-type ratio in the inoculum, the probability of disease escape is small. Hence, when less than 100% of the inoculated plants are smutted, which is nearly always the case, the nonsmutted plants must be the result of disease resistance.

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Additional key words: disease escape, disease resistance, infection court, barley covered smut, partial-vacuum inoculation.

In any genetic study of disease reactions, it is important that the contributory role of environment be understood. An important aspect of environment in a host-parasite system is the inoculation regime, including the method of inoculation and conditions to which the host and parasite are subject during and shortly following inoculation. It is with part of this aspect that the present work is concerned. It is known that, using the partial-vacuum inoculation technique (7), a high percentage of plants remain nonsmutted, even when those genotypic combinations of barley (*Hordeum vulgare* L.) and covered smut [caused by *Ustilago hordei* (Pers.) Lagerh.] are employed which yield the highest known percentage of plants with one or more smutted spikes. This percentage of apparently healthy plants has varied from 15-20% (6, 7) to 30-40% (3).

To discover if the inoculation technique was allowing these nonsmutted plants to escape contact with the fungus, an approach was chosen which was based on a probabilistic inoculation model. Infective smut dikaryons must be composed of two haploids of opposite (usually called *A* and *a*) mating-type (2). In the past, approximately equal amounts of sporidia of each of the two mating-types were mixed and shaken together for at least 24 hours in liquid broth (8). More recently, it was found (T. Ebba, *personal communication*) that inoculum composed of *A* and *a* sporidia mixed just prior to seed inoculation was no less effective in smutting plants than inoculum composed of sporidia mixed a day or more prior to inoculation. From this it may be inferred that the dikaryons which ultimately infect and cause smutting can be formed after the seeds have been inoculated; they need not exist prior to the time of inoculation.

Because of the above considerations, using inocula composed of increasingly disparate mating-type ratios, a point should be reached at which percentages of smutted plants will begin to decline. Moreover, if certain assumptions are made, then by using the binomial relationship, an estimate should be possible of the efficiency of the inoculation procedure. A useful expression of this efficiency is the "effective number" of sporidia, defined simply as the average number of sporidia that, for each inoculated seed, would be potentially capable of taking part in a "successful" infection (i.e., in an infection which actually results in disease expression). The "effective number" would thus relate to the expression of the disease by individual plants, and would be a measure of the number of sporidia potentially capable of taking part in the infection process. It would of course give no indication of the actual physical location of these sporidia. Two necessary assumptions upon which the reality of this model depend are: that sporidia are randomly mixed in the inoculum, and that the size of the "effective number" does not vary greatly from seed to seed. These assumptions will be dealt with later.

A short study was also made to determine whether known sporidial mixtures remain constant over time, in vitro, or whether the initial ratios are brought closer to unity by faster growth (via vegetative budding) of the minority component of the mixture.

The purpose of these studies was to determine the approximate effective number for a representative susceptible combination of barley and *U. hordei* and, in so doing, to assess the efficiency of the inoculation procedure in establishing host-pathogen contact. Such information is important for detailed studies of disease reactions caused by *U. hordei*, where it is often difficult to distinguish between disease resistance and disease escape. Some of the work was reported previously (4).

MATERIALS AND METHODS.—A single smut dikaryon and a single barley cultivar were used. The two

smut haploids which made up the dikaryon were originally isolated by Thomas (8) from separate smutted barley plants found near Winnipeg, Manitoba. They were designated E3a and 14A. The two-rowed barley cultivar Hannchen was chosen for two reasons: it is moderately to highly susceptible to the dikaryon employed, and in the greenhouse, where this study was carried out, more plants of this cultivar can be brought to maturity in a given amount of space than can plants of other cultivars.

Seeds were treated with a dilute solution of formalin (one part formalin to 320 parts water) prior to inoculation in order to kill any contaminant smuts and to loosen the seed hulls. They were soaked in the solution for 1 hour, rinsed for 30 minutes in running tap water, and thoroughly dried before inoculation. Seeds were inoculated using the partial-vacuum technique described by Tapke and Bever (7). Haploid smut cultures were maintained for up to three weeks on modified Vogel's (9) complete agar medium in petri plates stored at 4 C. Sporidia from each culture were transferred, separately, to 125-ml Erlenmeyer flasks containing 50 ml of complete broth. A drop or two of aqueous achromycin suspension (10 mg/ml) was added to inhibit possible bacterial growth, and the flasks were shaken in a 22 C incubator for 3-4 days, by which time sporidial suspensions had reached maximum density. About 100 seeds were put into a 1-dram vial. For standard inoculation, equal quantities of the two haploids (of opposite mating-type) were mixed and 8-10 ml of the mixture pipetted into each vial of seeds. The vials were then placed in a vacuum desiccator, without dessicant, and subjected to a partial vacuum (which resulted in boiling) for 30 minutes. Upon rapid release of the vacuum, sporidia are drawn under the seed hulls. The excess liquid was then poured off and the seed put into small coin envelopes. Seeds were allowed to dry for at least 24 hours in the open envelopes, and were planted within three days after inoculation.

To manipulate the sporidial ratios of the two smut haploids, the absolute concentrations of the 3- to 4-day-old sporidial suspensions were first determined by diluting each culture 1:100 with sterile water and determining the sporidial concentrations using a hemocytometer. Where necessary, the more concentrated culture was adjusted by adding sterile water to obtain equal concentrations of sporidia for the two cultures. The sporidial ratios were then realized in the inoculum by mixing unequal amounts of the standardized culture in the ratios desired. *A* and *a* sporidial ratios of 1:1, 100:1, 500:1, 1000:1, and 10,000:1, as well as reciprocals of these, were used. The extreme ratios (10,000:1 and 1:10,000) were necessarily obtained by using a 1:100 dilution of the minority culture initially. This resulted in a very slight decrease in the overall sporidial density of the final inoculum for these treatments.

Seeds were planted in soil benches in the greenhouse, about 50 seeds in each 130-cm row. Rows were randomized. The plants were given supplemental fluorescent lighting 16 hours per day. Disease levels, assessed as the percentage of plants showing at least one smutted head, were recorded about three months after seeds were sown, when the plants had reached maturity.

Standard dilution plating techniques were used to obtain samples, as single sporidial colonies, from 1:100 and 100:1 ratio mixtures of the two haploids in complete broth shake culture. From the dilution plates, colonies five days old were transferred to fresh petri plates of complete medium to form 25-colony master plates. These were replica-plated according to the method of Dinoor and Person (2) on complete agar plates containing sporidial lawns of E3a and 14A (separately). Samples were taken from the mixtures at 0, 7, 24, and 72 hours after mixing, and at 24 hours an equivalent volume of fresh medium was added to the cultures to initiate another burst of growth. Colony mating-types, based on the presence or absence of infection hyphae or "suchfaden" (2), were recorded two days after transfer to the tester lawns.

Statistical testing involved a confidence interval about the difference between two proportions (5). The 1:1 control was the standard against which all other treatments were compared.

RESULTS.—Figure 1 shows the relationship between differing *A:a* sporidial ratios and level of disease. In the figure, the level of disease obtained with the 1:1 ratio of

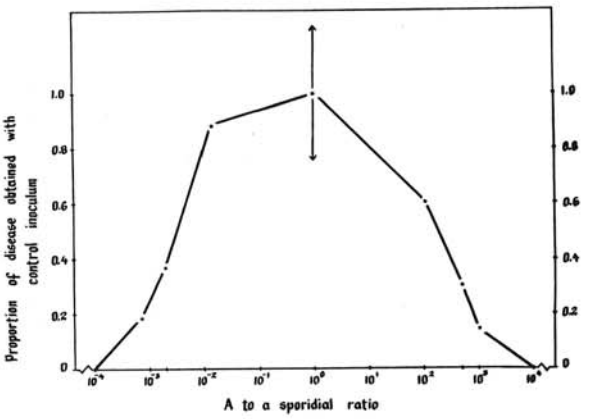

Fig. 1. Levels of disease observed for each *Ustilago hordei* sporidial ratio of mating-types *A* and *a* in inoculum applied to barley seeds (cultivar Hannchen) with the partial-vacuum method. Disease level is presented as proportion of that observed using 1:1 ratio control inoculum. Arrows indicate $P = 0.05$ confidence interval (shown only for control for reasons explained in text).

TABLE 1. Mating reactions of *Ustilago hordei* sporidia (isolates 14A and E3a) sampled at four different times after mixing *A* and *a* mating-type sporidial suspensions in 100:1 and 1:100 ratios and shaking in flasks

Time of sampling (hours)	Sporidial ratio of initial mixture[a]	
	100 14A: 1 E3a	1 14A: 100 E3a
0	1/54	1/55
7	0/54	0/54
24	2/54	0/55
72[b]	0/55	0/54

[a]Number of sporidia, over total examined, showing the mating-reaction of the minority mating component of the inoculum. All other sporidia in the sample showed the other mating-reaction.

[b]Sampled 48 hours after 50 ml of fresh medium was added to each culture.

TABLE 2. Three estimates of the average number (n) of *Ustilago hordei* sporidia available per barley seed (cultivar Hannchen) which can form dikaryons and cause smutting of the plant. Each estimate is based on smutting observed after partial-vacuum inoculation using a different ratio of *A* and *a* sporidial mating types

Sporidial ratio used[a]	Infection level (proportion of control) (S)	Calculated effective Number (n)[b]
100:1	0.747	135
500:1	0.331	201
1,000:1	0.175	195

[a]A weighted mean of disease levels for the reciprocal ratios of *A* and *a* sporidia.

[b]Based on the relationship $S = 1 - P^n$ in which P = the proportion of sporidia of the majority mating-type.

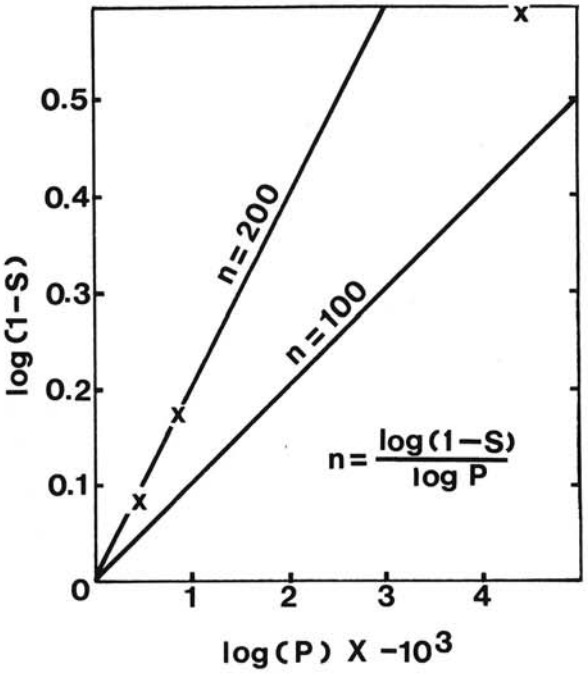

Fig. 2. Linear (log-log) plot of three separate estimates of n, the average number of *Ustilago hordei* sporidia available per barley seed (cultivar Hannchen) which form dikaryons and cause smutting of the plant, expressed as the slope of a regression line of log (1−S), where S is the level of infection observed, over log (P), where P is the proportion of sporidia which are of the majority mating-type in the inoculum applied by the partial-vacuum method. Calculated experimental n values are indicated by X, and reference regression lines for n = 100 and 200 are shown.

A:a is set at 1.0, and other levels are presented as proportions of this maximal level. Except for the 10,000:1 and 1:10,000 ratios, which were based on samples of about 100 plants, each point in the figure was based on about 150 plants (250 in the 1:1 control). In preliminary studies, no detectable reduction in smutting was seen for 10:1 and 1:10 ratios and these were not included. Data shown were from four separate experiments.

The curve shown in Fig. 1 is not symmetrical. Its skewness is evident when the 100:1 and 1:100 ratios of *A:a* sporidia are compared: in the former the disease level was significantly lower than that of the control, but in the latter, the difference in disease level was not significant. The confidence interval placed about the 1:1 point illustrates this. This confidence interval is a graphic illustration, based on sample sizes of 150 plants, of the confidence interval about the difference between two proportions. It is placed about the control which is always involved in comparisons. The test accounts for variation in both points, hence more than one set of arrows is not shown. The skewness is also evident when the reciprocal 500:1 and 1:500 ratios and the 1000:1 and 1:1000 ratios are compared. In all cases, it is the ratio which favors mating-type *a* that associates with the higher disease level.

The point at which no smut was found was, in both directions, about 10,000:1. Only one smutted plant was found at these ratios–where *a* was the more numerous component.

The number of colonies (over the total examined) which showed the mating reaction of the minority culture when 100:1 and 1:100 mating-type mixtures were made and sampled at various times is presented in Table 1. The conclusion is that when sporidia are mixed together unequally and maintained in vitro, the ratios do not change appreciably toward less disparity over a three day period.

The effective number was estimated by using the following relationship:

$$S = 1 - P^n$$

where S = the disease level measured as the proportion of that level observed in the control population (those plants inoculated with a 1:1 mating-type mixture of sporidia); P = the proportion of majority sporidia in the inoculum mixture; and n = the effective number of sporidia.

In logarithmic form the relationship becomes:

$$\log (1 - s) = n \log (P)$$

$$\text{or} \quad n = \frac{\log(1 - s)}{\log(P)}$$

which is more easily solved for n.

Table 2 presents the proportion of disease shown by three different sporidial ratios as compared with the controls, along with three separate effective number calculations, one for each ratio.

DISCUSSION.–The haploid smut lines used were genetically different, hence the observed skewness of the decrease in disease could be due to several factors: difference in survival between the two lines under planting conditions, nonequality of role of the mating types (including unilateral production of substances which stimulate mating), or differences in production of, or sensitivity to, mating substances whether they are unilaterally or bilaterally produced. Some of the above possibilities could be tested by using near-isogenic *A* and *a* lines in similar studies. If mating substances do play a role, it is possible that all three calculated effective numbers underestimate the true number. This would occur if the minority sporidial type, because of its rarity,

fails to produce sufficient substance(s) to induce maximum possible dikaryon formation. Opposing this idea is the fact that the effective number estimate, which might be expected to decrease as the ratio becomes more disparate (and the effect becomes more important), does not do so in reality.

Qualitatively, one would expect that if the effective number of sporidia is large, so that relatively many sporidia have the potential of forming dikaryons and of taking part in a successful infection which results in smutting, then a reduction in the percentage of diseased plants should not be realized until rather disparate ratios of the two mating-types are used. Likewise, if the effective number is small, the number of plants showing smut should drop off relatively quickly as the ratios increase. This is because some of the seeds, which would otherwise produce smutted plants if inoculated with the usual 1:1 mating-type mixture, will, at high ratios, fail to receive sporidia of the minority mating type. Since a significant decrease in the percentage of smutted plants did not occur until the ratio reached 100:1 on the one hand, and 1:500 on the other, it would seem likely that the effective number must be rather large.

It is perhaps easiest to picture the model by using the simplistic concept of an "infection court" which is associated with each seed. This court is a space or volume which, in accordance with the two assumptions mentioned above, does not vary a great deal from seed to seed and is filled at inoculation with a random sample, equal to the effective number, of sporidia from the inoculum mixture. Genetic uniformity of the seeds and the sporidia supports the assumption that the size of the infection court, while certainly subject to some variation, does not vary to the degree that some seeds would have an infection court capable of holding only a few sporidia. The in vitro study of the inoculum supports the assumption that each seed will receive a random sampling of the sporidia present, since minority sporidia remain free in the inoculum for at least the first 24 hours, and probably longer. If these assumptions are true, the reduction in smutting due to inequality of mating types should largely follow a simple binomial expectation. The proportion of smut observed should equal the total possible smut, defined as 1.0, minus the probability of not including in this infection court at least one sporidium of the minority-type, or 1.0 minus the probability, P, that a sporidium is of the majority type multiplied by itself the effective number of times (hence P^n). The only unknown in the equation is n.

When the above equation was solved three separate times for different dilutions, three distinct but fairly similar estimates of n were obtained. Figure 2 shows this graphically as a log-log plot of 1−S over P. For each of the three points, the value of n was obtained as the slope of the line from the origin (which represents inoculum made up entirely of one mating type) to each point. If the model is valid, the points should occur in a straight line. Since only three points exist, however, regression analysis would be of little value. According to the model, the origin must be included in any regression. A visual idea of the confidence with which n is determined is obtained by including in the figure two regression lines which have n values (slopes) of 100 and 200, respectively. Although this does not establish linearity, nor does it allow a quantitative confidence statement to be made about n, it seems safe to say from the graphic appearance that n is at least 100.

Also contributing to the model's validity is the finding that in vitro the ratios of mating-type in the inoculum do not tend to become less disparate over a three day period. There is no reason to think that what occurs in vitro would not also occur in the seeds. Appel and Gassner (1) determined that the first three days of seed germination is the critical period for infection of barley by covered smut.

In a preliminary study (Groth, *unpublished*), further support for the validity of the model was obtained. The study was similar to these above, except that instead of diluting only one mating-type, whole 1:1 sporidial suspensions were diluted. According to the model, one would expect this to result in infection courts which are not being filled, but rather are receiving only a fraction of the sporidia they would receive if the inoculum were not diluted. For example, if inoculum is diluted 1:100 with growth medium, only 1/100 the number of sporidia would occupy each infection window, on the average. The rest of the volume would presumably be filled with medium. The drop in smutting should be greater in this case than for corresponding single mating-type dilutions. For reasonably large n values, the smutting decline should behave according to the relationship:

$$S = 1 - 2(P^{n/2})$$

where P is, in this case, the proportion of sporidia replaced by medium.

The equation is the same, except that either mating-type can be eliminated from the infection court due to replacement of all n/2 sporidia by medium. The results fit this expectation for an n of about 170 sporidia (calculated from 100:1 values, where S was 0.15 based on 60 plants). As one would expect, no infection was seen when 1:300 and 1:500 dilutions were used, since these are both beyond the vanishing point for the likely n values. These studies were not continued because it was felt that mating-type dilution was the simpler, and hence better, approach. They do, however, support the conclusion that n is a large number.

The extended conclusion to be drawn from these experiments is that virtually all seeds are being effectively contacted by the fungus when the inoculum is composed of the normal 1:1 mating-type ratio. If it is conservatively estimated that 100 sporidia are potentially capable of taking part in dikaryon formation and successful infection of a single barley seed, and allowing for variation due to sampling error (accidental deviation from the 1:1 ratio in the inoculum), the probability of one of the two mating types being totally absent is expected to be extremely small [about $(1/2)^{100}$]. In practice the numbers of plants which fail to show the disease are always much greater than this. This must mean that the potential for disease expression is not entirely determined by the capacity of the inoculum to produce infective dikaryons, and points to the conclusion that host resistance is also an important factor. How and when resistance might operate is not indicated by the results of this study. Such resistance appears to be universal in barley; however, and presumably not having been purposely bred either into or out of the crop, one can

speculate that it may represent a remnant of the "natural" resistance of wild barley ancestors to *U. hordei*. Examining whether the level of such resistance could be affected by selection would provide further information about its nature.

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