# Refined Procedures for Inoculating Wheat Seedlings with *Pyrenophora tritici-repentis* and Rating Their Reactions

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**ABSTRACT**

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Systems for rating reaction of wheat to infection by *Pyrenophora tritici-repentis*, the tan spot pathogen, should be informative and reliable so that resistant cultivars can be efficiently developed. Therefore, the effects of propagule type, inoculum dosage, and spatial interaction among lesions on infection phenotype, infections per unit area, and percent disease severity were studied. Conidiophore inoculation of seedlings of wheat line ND 495, which is susceptible to tan spot, produced a lower infection phenotype, fewer infection sites per square centimeter, and less severe damage than conidia at equivalent dosages. Hyphal fragments were rarely infectious under a 24-hr postinoculation wet period. The relative reaction of the resistant wheat cultivar Eric to the three types of inoculum was similar to that of ND 495. Inoculum concentration and type particularly influenced categories of the infection phenotype scale that included coalescence as a criterion. The distal leaf half was more severely damaged and usually had more infections per unit area than the proximal half, but there was little or no evidence of interaction among lesions between leaf halves. Uniform inoculum dosage, exclusion of conidiophores from inoculum, and collection of infection type data from the middle of the uppermost fully expanded leaf at the time of inoculation should be employed to discriminate resistant and susceptible genotypes effectively.

Additional keywords: *Triticum aestivum, T. turgidum* var. *durum*, yellow leaf spot

Reaction of bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* var. *durum* Desf.) to infection by *Pyrenophora tritici-repentis* (Died.) Drechs., the cause of tan spot, has been assessed by several different systems. Various point systems based on lesion size or leaf coverage were formerly used extensively (5). These largely have been replaced either by the qualitative scale of Lamari and Bernier (10), due to their interpretation of reaction phenotypes, or by quantitative measures of percent leaf area diseased (17). Standardization of methodology is crucial for effective discrimination of resistance and susceptibility as well as for other goals such as comparing results among experiments. It is important to continue using and refining methods and rating systems that are both informative and reliable so that progress can be made toward development of resistant cultivars.

Infection phenotypes often can be mixed on a leaf, leading to difficulty in categorization of potentially useful genotypes (4). Also, susceptible or partially resistant wheat genotypes tend to show a more severe reaction to tan spot in the distal portion of the leaf than in the proximal portion (*personal observations*). The causes of these phenomena are unknown. However, *P. tritici-repentis* produces a phytotoxin that causes cellular disruption some distance from the invading fungal mycelia (11,13,19), and this may play a role if toxin movement occurs preferentially in the distal direction. Other hypotheses include a limited pathogen invasion in the proximal end of the leaf due to an abundance of structural fibers and greater sensitivity to disease by cells that are oldest in the distal leaf portion.

*P. tritici-repentis* produces conidia singly on conidiophores in a diurnal manner wherein conidiophores are formed in the light and conidia in the dark (9,16). Abundant conidia can be produced in culture more quickly and uniformly than ascospores. Current methods of inoculum production therefore involve manipulation of the light-dark regime to harvest conidia (2,16).

The effect of propagule type, inoculum dosage, and spatial interaction among lesions on infection type, lesions per unit area, and percent disease severity was investigated to improve our tan spot screening procedure. A preliminary report has been published (8).

## MATERIALS AND METHODS

Erik, a hard red spring wheat cultivar moderately resistant to tan spot (10,11), and ND 495, a hard red spring wheat line susceptible to tan spot (5,6), were planted in Fison sunshine blend No. 1 soilless medium (Fison Horticulture, Vancouver, BC) in Cone-Tainers (Steuwe and Sons, Corvallis, OR). Seedlings (one per container) were grown for 2–3 wk to growth stage 12 or 13 (20) without additional fertilizer in a growth chamber with a 16-hr/8-hr light/dark cycle at 21 C day and 18 C night temperatures.

**Preparation of inoculum.** Inoculum in all experiments was derived from a single-ascospore isolate, Pti2 (ATCC accession No. 44143), collected from wheat straw in 1978 (1). Pti2 has been maintained in culture and routinely used as inoculum without loss of aggressiveness. Conidia were produced according to the method of Lamari and Bernier (10) with minor modifications. Two dried mycelial plugs were placed 3 cm apart on petri plates containing V8-PDA medium. Plates were incubated for 5–6 days at 20 C under continuous darkness. After incubation, 15–20 ml of sterile distilled water was poured on the plate and hyphae were flattened with a flame-sterilized glass slide. The water was decanted and plates were incubated under fluorescent light for 18–24 hr at

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20 C, followed by 18–24 hr of dark at 16 C. Conidia were harvested by flooding the plates with 15 ml of sterile distilled water, then dislodging spores by gently scraping the colony with a flame-sterilized glass slide. The spore suspension was blended for 2 min to separate aggregates, after which Tween 20 was added at 0.04 ml/100 ml of spore suspension. The spore suspension was stirred and the number of conidia in six 5-$\mu$l samples was counted under a compound microscope to estimate spore concentration. Conidial concentration was adjusted to 3,000/ml unless noted otherwise. Some culture plates were not exposed to light to collect mycelial fragments and others were not returned to the dark after light exposure to collect conidiophores.

**Method of inoculation.** A spore settling tower, previously used to inoculate plants with rust uredinia in a dry talc carrier (3), was modified to inoculate wheat seedlings quantitatively with 25 ml of a spore suspension (7). The youngest fully expanded leaf was taped to a horizontal plastic block to minimize variability due to leaf orientation. For a study of spatial interaction among lesions, leaf half-lengths were covered with glassine bags during inoculation to prevent infection. Treatments were proximal leaf half covered, distal leaf half covered, and uncovered leaves, with data collected from each half separately.

After inoculation, plants were allowed to dry for 15 min and then were placed in mist chambers at 20 ± 2 C. An ultrasonic humidifier was used to mist each chamber for 1 min of each 5 min. After a 24-hr wet period, plants were placed in a growth chamber at 21 C day/18 C night with a 16-hr/8-hr light/dark photoperiod and were subirrigated to avoid further leaf wetting.

**Rating and analysis.** Percent disease severity and lesion phenotype of the newest fully expanded leaf at the time of inoculation were recorded 1 wk after inoculation. The lesion type rating used was based on the rating system developed by Lamari and Bernier (10): 1 = small (approximately 1 mm in diameter) dark brown to black spots without any surrounding chlorosis or tan necrosis (resistant); 2 = small dark brown to black spots with very little chlorosis or tan necrosis (moderately resistant); 3 = small dark brown to black spots surrounded by a distinct chlorotic or tan necrotic ring, lesions generally not coalescing (moderately resistant to moderately susceptible); 4 = small dark brown or black spots surrounded by chlorotic or tan necrotic zones, some lesions coalescing (moderately susceptible); and 5 = dark brown or black centers that may or may not be distinguishable, most lesions consisting of coalescing chlorotic or tan necrotic zones (susceptible). We added a 0 rating to the scale to indicate an apparent immune response or an escape.

In some studies, the number of infection sites was counted under magnification using a colony counter (Model 3325, AO Reichart Scientific Instruments, Buffalo, NY). The area of the leaf at the time of disease rating was measured with an LI-3100 area meter (LI-COR, Lincoln, NE) to derive the number of infections per square centimeter. Leaf tissue shrinkage due to disease-induced senescence leads to inflated values of infection sites per unit area when leaf area is measured after infection rather than at inoculation. However, a destructive leaf harvest is necessary when using the LI-3100.

Experiments comparing inoculation with conidia, conidiophores, or mycelia were repeated five times with ND 495 and three times with Erik. Three experiments compared inoculum concentrations of conidia (0–4,000/ml) and conidiophores (0–18,000/ml). Finally, experiments concerned with an interaction among lesions between leaf halves were repeated four times. The number of observations per treatment within an experiment ranged from four to 12. Experiments were separately analyzed by randomized complete block designs, completely randomized designs, or regression analysis, as appropriate. Analyses of variance were conducted with SAS PROC GLM (18). Planned contrasts were used to detect differences among half-leaf responses.

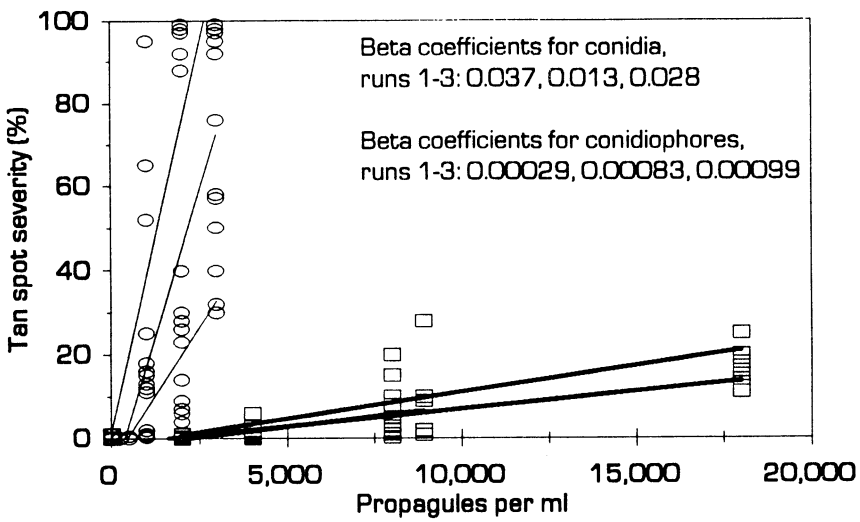


**Fig. 1.** Severity of tan spot on seedlings of the susceptible wheat line ND 495 after inoculation with conidiophores (□) or conidia (○). Slope ($\beta$) coefficients are from simple linear regression models, and slope comparisons based on $t$ tests all indicate significant differences between conidia and conidiophores (all $P < 0.01$).

**Table 1.** Tan spot severity, infection sites per square centimeter, and infection phenotype following inoculation with propagules of *Pyrenophora tritici-repentis* on the susceptible wheat line ND 495 and the resistant wheat cultivar Erik

| | Disease severity (%) | | Infection sites/cm$^2$ | | Infection phenotype[w] | |
|---|---|---|---|---|---|---|
| Inoculum | ND 495 | Erik | ND 495 | Erik | ND 495 | Erik |
| Water check | 0.0 c[x] | 0.0 c | 0.1 c | 0.0 c | 0.7 d | 0.0 d |
| Mycelia[y] | 0.2 c | 0.0 c | 0.2 c | 0.1 c | 1.3 c | 0.5 c |
| Conidiophores[z] | 10.3 b | 0.3 b | 5.3 b | 1.5 b | 2.8 b | 1.0 b |
| Conidia[z] | 22.6 a | 6.8 a | 6.9 a | 11.1 a | 4.1 a | 1.7 a |

[w] Rated on a 0–5 scale after Lamari and Bernier (10).
[x] Values in a column followed by the same letter are not significantly different at $P < 0.05$ based on a protected LSD test on least squared means.
[y] From 45,000 to 155,000 mycelial fragments per milliliter were used as inoculum in eight experiments.
[z] From 1,700 to 3,400 conidia or conidiophores per milliliter were used as inoculum in eight experiments at equal rates within each experiment.

## RESULTS

**Inoculum type and amount.** Inoculation of tan spot–susceptible ND 495 with 500 or fewer conidia per milliliter did not damage leaves appreciably (Fig. 1). At somewhat higher concentrations, conidiophores were 3–12% as damaging as conidia at the same rate of inoculum. Conidiophores in a similar experiment were 5% as damaging as conidia on Erik and 46% as damaging on ND 495 (Table 1). Hyphal fragments were nearly noninfectious on both Erik and ND 495, with percent disease severity and infections per square centimeter not significantly different ($P < 0.05$) from the water check.

Conidiophore inoculation produced lower infection phenotypes and fewer

lesions per square centimeter than inoculation with conidia at equivalent dosages (Table 1). The infection phenotype on Erik seemed less affected by inoculum type than that of ND 495 because the difference in the former comparison was less than one phenotypic class. However, inoculation with conidia resulted in a significantly ($P < 0.05$) higher phenotypic score than inoculation with conidiophores for both susceptible and resistant wheats. Conidial inoculum produced 30% more infections per square centimeter than conidiophore inoculum on ND 495, but in the dosage experiment, the ratio between infection sites per square centimeter for conidia and conidiophores on ND 495 varied from 8:1 to 35:1 as dosage increased (*data not shown*). The combination of fewer infection sites and smaller lesions resulted in a lower percent disease severity for conidiophores than for conidia. Also, there were significantly more infections per square centimeter on leaves of Erik after inoculation with conidia than after inoculation with conidiophores, and this difference was more disparate than that found for ND 495 (Table 1).

**Spatial interaction.** The distal leaf half was more heavily damaged than the proximal leaf half in four replicate experiments (range, $0.02 < P < 0.0001$). In three of four experiments, covering the basal or distal leaf half had no significant effect on damage in the other half when compared to uncovered leaf halves, suggesting no interaction between halves for tan spot severity.

There were 9.9 and 6.7 infection sites per square centimeter on the distal and basal halves of the leaf, respectively, when averaged across experiments ($n = 96$). The number of infection sites per square centimeter within each experimental replicate was usually greater on the distal leaf half than on the proximal half, but results were more consistent for covered than uncovered leaf halves (Table 2).

Interpretation of interaction effects was difficult with respect to the number of infection sites per unit area (Table 2). Contrasts examining an interaction between leaf halves showed no consistent pattern among experiments. Therefore, no spatial interaction could be inferred regarding the number of lesions per unit area.

## DISCUSSION

Ideally, conidiophores should be excluded from the inoculum, since they contribute little as an infectious propagule and they can produce a lower infection phenotype than can conidia, thereby resulting in a mixture of lesion types. Compared with scraping the surface of the plate, gently washing the surface of a sporulating culture reduced conidiophore concentration in solution (2). Hyphal fragments should not be considered infective propagules under incubation conditions similar to the ones described here. Chopped hyphal suspensions had been used in earlier inoculation methods (5,14), but these have largely been abandoned in favor of conidial suspensions. Hyphae can contribute to clumping of conidia, which creates problems in counting and inoculating procedures (J. G. Jordahl, *personal observation*).

The type and amount of inoculum appeared to influence most strongly infection phenotypes that included coalescence as a criterion, i.e., classes 3, 4, and 5 of Lamari and Bernier (10). Therefore, placement in these phenotype classes must be interpreted carefully. Coalescence depends on fungal isolate, inoculum density, and number of lesions per square centimeter, plus plant factors such as leaf age and orientation and environmental factors such as temperature and wet period duration (6,12,15). Consequently, placement of a wheat line in phenotype category 3, 4, or 5 is probably subject to more variability than placement in category 1 or 2. This is an important consideration for analysis of genetic studies and perhaps for some breeding objectives but is not crucial in a screening program where all susceptible classes are discarded.

The distal half of the susceptible wheat leaves was more severely damaged by *P. tritici-repentis* than the basal half, where lesions were restricted in development. This confirms our observations during screening for tan spot resistance. Infectivity or receptivity per unit area was often significantly more on the distal half than on the basal half. Repeatability of this result was not absolute, perhaps merely because of difficulty in finding all sites of infection on tissue with coalesced lesions.

There was little or no evidence of interaction among lesions on the distal and proximal halves of the same leaf. Coverage of proximal or distal leaf halves during inoculation had no consistent effect on infections per square centimeter or on tan spot severity on the corresponding leaf half, suggesting that an interaction was not occurring over the distance tested. Despite the lack of an interaction between leaf halves, tan spot was in fact more severe on the distal half than on the proximal half, indicating a differential response had occurred.

Some foliar damage caused by *P. tritici-repentis* is due to a host-selective toxin that is mobile within the leaf (11,13,19). The toxin may be implicated in heavily damaging leaf tips, if preferential movement is assumed to be toward the distal end. Infectivity or receptivity was often, but not always, significantly greater for the distal leaf half than for the basal half (Table 2). Whether the toxin can act quickly enough, however, to affect the number of infections, which presumably occurred entirely within the 24-hr wet period after inoculation, is open to question. Other possible explanations for a greater number of infections on the distal half than on the proximal half include a difference of approximately 7 days in cell age and anatomical disparities.

Our work with propagules of *P. tritici-repentis* affirms that a standardized level and type of inoculum are critical to the repeatability and generalization of results. A de facto standard of 2,500–3,000 conidia per milliliter for seedling inoculation and a postinoculation wet period of 24 hr is to be encouraged. If conidiophores are not excluded, a conservative approach when quantifying inoculum would be to treat 25 conidiophores as equivalent to one conidium.

We suggest that percent disease severity and the predominant infection phenotype of the uppermost fully expanded leaf at the time of inoculation be recorded and any secondary or unusual phenotypes noted. Scoring only the middle portion of the leaf for infection phenotype would avoid extremes at either end.

**Table 2.** Comparison of number of infection sites per square centimeter following inoculation with conidia of *Pyrenophora tritici-repentis* on the susceptible wheat line ND 495 when leaves were partly covered or left uncovered during inoculation

| Measurement | Contrast | Infection sites/cm² in experiment: 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Distal half | Base covered | 8.7 | 7.0 | 12.3 | 11.9 |
| | Base uncovered | 5.3 | 7.9 | 9.4 | 15.7 |
| | $P^z$ | 0.01 | NS | NS | 0.09 |
| Proximal half | Tip covered | 4.2 | 4.0 | 9.9 | 7.4 |
| | Tip uncovered | 7.8 | 2.9 | 8.7 | 7.6 |
| | $P$ | 0.03 | 0.05 | NS | NS |
| Uncovered leaf | Tip | 5.3 | 7.9 | 9.4 | 15.7 |
| | Base | 7.8 | 2.9 | 8.7 | 7.6 |
| | $P$ | 0.03 | 0.009 | NS | 0.005 |
| Covered leaf half | Tip | 8.7 | 7.0 | 12.3 | 11.9 |
| | Base | 4.2 | 4.0 | 9.9 | 7.4 |
| | $P$ | <0.001 | 0.001 | NS | 0.003 |

[z] Probability that two means are identical when contrasted using a general linear model; NS = not significant at $P > 0.10$.

If end extremes exist, they probably could be described adequately by rating percent disease severity for the entire leaf. The meaning of the "middle" of the leaf may be better defined in a future study. For now, the suggestion is left vague with the intent of providing latitude for personal observation and judgment.

In conclusion, standardized environmental conditions, a quantified and uniform dosage of conidia, and exclusion of conidiophores and hyphae to the greatest extent possible should be employed in a inoculation protocol for *P. tritici-repentis*. Of perhaps lesser importance, tan spot rating should include infection type and disease severity. Homogeneity of these conditions is necessary to discriminate resistant and susceptible genotypes effectively and to pursue other goals, such as comparing results from different laboratories.

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