A Quantitative Inoculator Capable of Inoculating Cereal Plants at All Growth Stages with Rust Urediniospores

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ABSTRACT

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An inoculator, designed to quantitatively apply a spore/oil mixture on single stems or leaves of cereal plants, was constructed and tested. The tests consisted of counting urediniospores of *Puccinia graminis* f. sp. *tritici* applied to a glass slide, or counting uredinia that formed on seedling leaves and adult plant stems of susceptible or several different resistant genotypes of *Hordeum vulgare,* or susceptible *Triticum aestivum.* Using 2-mg spores suspended in 1 mL of light mineral oil, urediniospores were applied to glass slides at the rate of 125–224 spores/cm^2, with coefficients of variation between 7 and 22. In plant tests, distinctions between resistant and susceptible genotypes could be made using quantitative inoculations, when qualitative differences were not detectable.

Additional keywords: barley, inoculation technique, stem rust, wheat

Resistance in barley (*Hordeum vulgare* L.) to stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn. (*P. g. tritici*), is usually expressed as a range of intermediate infection types, and is difficult to evaluate visually (5). Growth stage of barley plants also may affect stem rust development (5). The evaluation of resistance components or the effects of plant growth stage on disease development require that uniform amounts of inoculum be applied per unit area of all test material, at either seedling or adult plant stages. Two basic types of inoculators, sprayer (1,2,11) and settling tower (4,7), have been designed for this purpose. Both types have produced repeatable results using seedlings of barley (1) or wheat (7). Quantitative inoculation of adult cereal plants has been more difficult. Inoculation of adult plant stems using a settling tower results in inaccessibility of inoculum to tillers, caused by their mutual sheltering and removal of inoculum from the tower atmosphere as it becomes deposited onto the upper plant surfaces. Also, a settling tower must be reloaded with the same number of plants each time a test is conducted. Repeatability between runs using settling towers has been indirectly reported (12). Spray inoculators can be designed to inoculate only one stem of a plant at a time, and different stems can be inoculated at different times with different pathogen pathotypes. However, to make valid quantitative comparisons, the equipment needs to perform reproducibly.

This paper presents the design, construction, and testing of a spray inoculator based on that of Andres and Wilcoxson (1). Modifications in the design included: providing the facility to spray the entire length of an adult plant stem; enhancing the control of spore deposition; and, improving the means to position and hold plants.

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MATERIALS AND METHODS

Design and construction. The quantitative inoculator was constructed in the workshop facilities located in the Winnipeg Research Centre. The details of the inoculator are shown in the line drawing in Fig. 1, and the assembled inoculator is shown photographically in Fig. 2. The inoculator consists of five main parts: *(1)* a base (A), made of plywood and covered with a waterproof hard plastic laminate and with a 25-cm × 41-cm section cut out underneath the shield (Q) to enable the placement of different sized pots; *(2)* an angle iron spine (B) anchored to the base, which has a sprocket (D) at each end; *(3)* a truck (F), consisting of four 5-cm pulley wheels and an atomizer (L), driven from a pin (I) that is incorporated into the chain (E) and rides 123.5 cm along the metal rods (C); *(4)* an acrylic shield (Q) used to separate one tiller from the rest of a cereal plant; *(5)* a variable speed motor (G).

The atomizer (L) sprays a urediniospore/oil mixture that is held in a size 00 gelatin capsule (M). The atomizer, designed to apply micro amounts of inoculum in a very fine spray, was obtained from Scientific Apparatus Services (University of Minnesota, St. Paul, MN).

In preparation for plant inoculation, a weighed amount of spores was mixed with a known volume of oil. This mixture was handled with a glass syringe for distribution into the gelatin capsules. The mixture was agitated after filling halfway and capping each capsule. Prior to inoculation, the atomizer was aimed at the vertical centerline of the shield and a truck speed selected. Spraying an oil blank with the atomizer in a stationary position aided in visualizing the location of the spray pattern. A plant was positioned using the shield to separate one tiller of the plant; the rest of the plant remained behind the shield. A capsule containing the spore/oil mixture was attached to the atomizer and the truck was positioned at the beginning of its path. To inoculate a plant, air was gently backflowed through the atomizer to agitate the mixture in the capsule. Then the motor and air pressure were turned on. The inoculator was designed so that the atomizer began spraying below the lip of the plant pot, allowing the truck to attain the selected speed before spray hit the plant stem. Following inoculation of a plant, excess oil on the shield was wiped off with an absorbent cloth. Using these steps, about 25 plants per hour could be inoculated.

Testing the inoculator. The effectiveness of the inoculator in uniformly depositing urediniospores of *P. g. tritici* onto a surface was tested by spraying glass slides with a urediniospore/oil mixture and then counting the number of spores on the slide. Based on preliminary work, the inoculator was set to operate with a truck speed of 9 cm/sec, and an air pressure of 55 kPa. These inoculator settings were used for all experiments and represented an atomizer output of approximately 200 μL for a full 123.5-cm run of the inoculator truck. Dustrol light mineral oil (Ciba-Giegy Canada Ltd.) was used to suspend the urediniospores. In all tests, urediniospores were used that had been stored at −80 C in glass Pyrex or plastic microcentrofuge tubes immediately after collection. Different spore collections were used for each experiment.

A completely randomized design experiment was conducted three times using six replicates of five urediniospore concentrations (0.25, 0.5, 1.0, 2.0, and 4.0 mg/mL). A clean glass slide was exposed to the spore/oil spray for each replicate, and each replicate represented a separate gelatin capsule containing the desired spore suspension. Three additional experiments were done in which 18–32 slides were sprayed using a single concentration urediniospore/oil mix of 2 mg/mL. These latter slides were obtained from tests in which plants were inoculated for reasons not related to this paper. Three other tests were done to determine the evenness of spore application within a run. Five slides were placed at roughly equal distances along the shield's vertical center line and then sprayed.

Prior to spraying a slide, a line was drawn across it so that this line could be matched to the vertical center line on the sprayer shield. Urediniospores were counted by selecting a band alongside the line drawn on the slide. The vertical spray segment dimensions were 25 mm (width of the slide) × 1.8 mm (diameter of field of view of the microscope at 100×) which equaled an area of 0.45 cm^2. Urediniospore counts are presented on a per square centimeter basis.

In plant tests, seedling or adult plants of barley or wheat were inoculated with an isolate of race QCC (9) of *P. g. tritici*. The purity of the isolate was determined by evaluating reactions on the *P. g. tritici* differential set (9). Seedlings were grown in a greenhouse using a 3:1:1 soil/sand/peat moss mix, a temperature of 20 ± 5 C, and 16 hr of supplemental fluorescent lighting (276 μmol m^{-2}s^{-1}). Using the same soil mix, adult plants were grown in a growth cabinet set to provide 16 hr/18 C day and 8 hr/15 C night periods. The experimental designs created for all plant experiments were

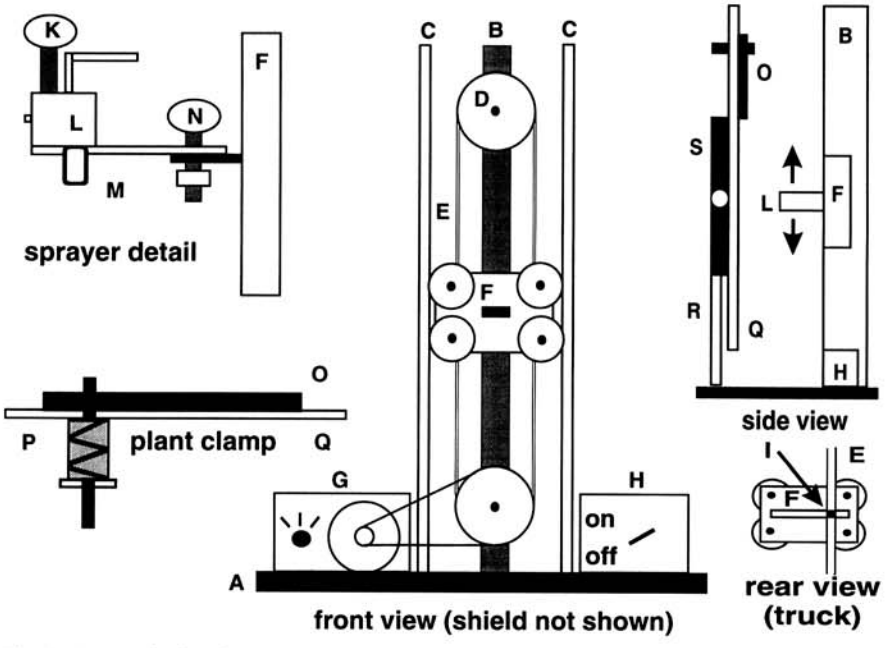

Fig. 1. A quantitative inoculator to inoculate seedling leaves or adult plant stems of cereals. The inoculator consists of: a base (**A**) covered with a waterproof hard plastic laminate; an angle iron spine (**B**); two metal rods (**C**); two 60-tooth sprockets (**D**); a chain (**E**); a truck with four 5-cm pulleys (**F**); an alternating current motor and a variable resistor (**G**) that provides a truck speed of 6 to 14 cm/sec; a power switch (**H**); a pin (**I**) integrated into the chain; a pressure regulated air line (**J**) providing 55 kPa air pressure; an atomizer clamp (**K**); an atomizer (**L**); a size 00 gelatin capsule containing urediniospore/oil mixture (**M**); a positioning bolt (**N**) that allows the atomizer to be aimed; four plastic plant clamps (**O**, only one shown) used to hold a plant stem or leaf in position during inoculation; a spring (**P**); an acrylic shield (**Q**) with a vertical center line used to position plants in the center of the spray target; and a metal rod (**R**) anchored on the motor side of the base that supports the shield by fitting through a hole in each of two aluminum shield stiffeners (**S**). Measurements are in centimeters.

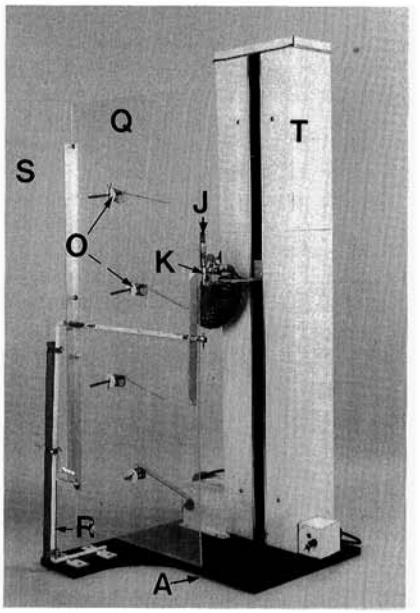

Fig. 2. Photograph of the inoculator illustrated in Fig. 1. The components indicated are the same as in Fig. 1: (**A**) base; (**J**) regulated air pressure line; (**K**) atomizer assembly; (**O**) plant clamp; (**Q**) the acrylic shield supported by (**R**) metal rod and (**S**) aluminum stiffener; (**T**) metal housing to enclose the mechanical components.

maintained where the plants were grown. The seedling test involved a six replicate completely randomized design experiment using 8-day-old seedlings of four barley lines and the susceptible wheat line Little Club. The barley cvs. Bonanza, Heitpas-5, and PI 382313 carry the independently inherited stem rust resistance genes *Rpg1* (5), *Rpg2* (8), and *Rpg3*, respectively (6). Hiproly barley was included as a susceptible cultivar. The plants were inoculated with a 2 mg/mL urediniospore/oil mixture. The adult plant test involved a randomized complete block design experiment with eight replicates of Husky-9, Hiproly-15, and four BC3F4 lines developed from Husky-9 and Hiproly-15. All lines were derived from a genetic study conducted by the second author (5). The backcross lines were selected for the presence of the stem rust resistance gene *Rpg1*. Each treatment consisted of two plants per 15-cm pot. Any uredinia on the entire stem that were sporulating 12 days after inoculation were counted as successful infections. The adult plants were inoculated at growth stages ranging from awn emergence to completion of spike emergence. All replicates of the line SF338 headed 1 wk earlier than the rest of the lines, necessitating their inoculation prior to the other lines.

After inoculation, oil was allowed to evaporate from the plant surfaces for 1–2 hr. Seedlings were incubated in a dew chamber, (Model 1-60D, Percival Manufacturing Co., Boone, IA), and similarly adult plants were incubated in a dark cabinet with an ultrasonic cool mist humidifier (Solaray, Div. of Sunbeam Corp. [Canada] Ltd., Brantford ON, Model 456) to provide 100% relative humidity. Plants were incubated for 16 hr at 20 C, then removed from the chambers and covered with translucent plastic to maintain moisture on them while they were exposed to light (276 μmol $m^{-2}s^{-1}$) for 4 hr. The plastic was then removed and the plants were allowed to dry slowly. Seedlings were returned to the greenhouse and were provided with a temperature of about 20 C and supplemental fluorescent lighting. Adult plants were returned to a growth cabinet set to provide 16 hr/18 C day and 8 hr/15 C night periods. The pustules on seedling leaves and adult stems were counted 12 days after inoculation, before pustules began to coalesce. Leaf area of the first seedling leaf was determined by measuring the leaf width and length, and adult plant heights were measured. This allowed for expression of disease per unit of plant tissue.

Analysis of variance (ANOVA) was performed for pustule count data. We conducted two analyses for adult plants: one for each of the two plants per pot individually (two subsamples per pot) and one for the mean pustule count for the two plants in each pot.

RESULTS AND DISCUSSION

Performance testing. Under the conditions of a truck speed of 9 cm/sec, 55 kPa atomizer air pressure, and a urediniospore/oil mixture of 2 mg/mL, 125–224 spores/cm^2 were applied to plant surfaces (Table 1 and Fig. 3). Variability in urediniospore numbers for a given mass of spores may occur due to differences in relative humidity during spore collection and in spore size (10). It is important to use one source of urediniospores for an experiment so that the concentration and viability of urediniospores will be consistent. Also, the spores settle in the oil quickly, and it is important to agitate immediately before a run. The standard errors observed from these tests are similar to those obtained by Mortensen et al (7). Variability in spore counts was similar between and within spray runs. No vertical gradient in spore density was observed. Within an experiment that represents the use of a single collection of spores, spore density was easily controlled as indicated by the strong linear relationship between spore concentration and spore density (Fig. 3). These results suggest that this spray-type inoculator can provide a level of uniform spore deposition similar to that of a settling tower (7), but the spray-type inoculator has the adaptability to inoculate plants at all growth stages.

The operating conditions were selected based on a number of compromises. A low air pressure was desired that would provide even application of oil but would not force oil into the plant tissues. The urediniospore/oil mixture of 2 mg/ml was used so that the amount of oil that was sprayed was minimized. Higher concentrations of spores were not used because they tended to settle out of the oil too rapidly. Minimizing the amount

Table 1. Mean number and standard error of *Puccinia graminis* f. sp. *tritici* urediniospores per square centimeter applied on surface of glass slides

Experiment	N	Spore number	CV
SC #1[a]	18	224 ± 11	21
SC #2	32	144 ± 5	20
SC #3	29	158 ± 5	17
WR #1[b]	5	222 ± 14	14
WR #2	5	207 ± 7	7
WR #3	5	187 ± 15	18

[a]Single concentration experiment.
[b]Within run experiment.

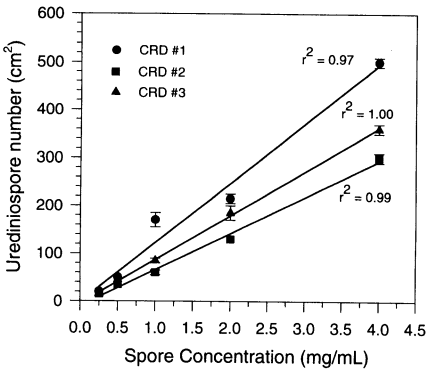

Fig. 3. Number of urediniospores of *Puccinia graminis* f. sp. *tritici* deposited per square centimeter on the surface of glass slides in three completely randomized design experiments.

Table 2. Mean pustules per square centimeter on first seedling leaves of four barley cultivars and Little Club wheat inoculated with *Puccinia graminis* f. sp. *tritici* race QCC

Cultivar	Known resistance genotype	Mean[a] pustules/cm^2	Waller-Duncan *k*-ratio *t* test[b]
Hiproly	...	6.4 ± 0.91	A
Little Club	...	6.1 ± 0.91	A
Heitpas-5	*Rpg2*	3.4 ± 0.80	B
Bonanza	*Rpg1*	1.4 ± 0.33	C
PI 382313	*Rpg3*	0.2 ± 0.05	D

[a]Mean of six replicates.
[b]Groupings are based on square root transformed data.

Table 3. Mean pustules per centimeter of stem on lines of *Hordeum vulgare* inoculated with *Puccinia graminis* f. sp. *tritici* race QCC[a]

Line[b]	Mean pustules/cm stem	Waller-Duncan *k*-ratio *t* test[c]
SF338[d]	3.0 ± 0.21	A
Hiproly-15	1.2 ± 0.07	B
SF292	1.2 ± 0.15	B
SF220	0.6 ± 0.12	C
SF216	0.5 ± 0.07	CD
Husky-9	0.3 ± 0.05	D

[a]Plants ranged in growth stage from appearance of awns to complete spike emergence.
[b]SF lines are BC_3F_4 lines in which SF216 and SF220 have Husky-9 as the recurrent parent, and SF292 and SF338 have Hiproly-15 as the recurrent parent.
[c]Groupings were the same for untransformed and square root transformed data.
[d]This line was inadvertently subjected to additional light and possibly higher temperature during incubation, perhaps accounting for the higher infection levels.

of oil sprayed was necessary so that phytotoxic effects of the oil would be avoided. The number of spores applied per square centimeter was selected based on previous experience by Rowell (10), which showed that only 15–25% of spores that are deposited on a wheat plant will infect. Rowell (10) also noted that the linear relationship between spores applied and uredinia formed is maintained up to 10 uredinia/cm^2. Distinctions between genotypes would most likely occur when this relationship is linear.

Plant tests. Both the dew chamber and cool mist humidifier provided adequate humidity conditions, and gave reproducible infection levels. Providing uniform moisture during incubation is very important for reproducibility, as shown by Browning (3). Quantitative inoculation of seedlings effectively distinguished, as measurements of receptivity, the reactions of barley cultivars with different *Rpg* genes (Table 2). These differences in receptivity to infection can only be made when reproducible amounts of inoculum are applied. Making distinctions between these resistance genes in the seedling stage has been hampered because barley stem rust resistance genes confer mesothetic reactions to most pure races of stem rust (5). Both the susceptible barley line Hiproly and Little Club wheat showed similar levels of receptivity.

The results of the adult plant test showed the precision of this experimental procedure (Table 3) by comparing the reactions of the lines Husky-9 and Hiproly-15. Husky-9, although carrying the *Rpg-1* gene, maintains a moderate level of resistance to *P. g. tritici* race QCC, but this resistance is difficult to evaluate visually, and Hiproly-15 is susceptible (5). The method of inoculation used in this study clearly distinguished the reaction of these two lines based on pustule frequency. The expression of resistance by each BC_3F_4 line was related to its recurrent parent despite the presence of the *Rpg1* gene for which these lines were selected (Table 3).

Since two plants were grown in each pot, an ANOVA was done in which each plant was a subsample, the pot being the sample. It was found that experimental error was not significantly different from sampling error, indicating that variability between plants in a pot was not significantly different from variability between plants in different pots. However, the mean square for treatment, mean square error, and coefficient of variation (CV) was 2 times smaller, 3.5 times larger, and 2 times smaller, respectively, when the ANOVA was done using the means of the plants in each pot. When only one of the two plants per pot was considered in the ANOVA, the experiment was less conclusive. Using the means of 2 plants per pot is statistically more robust than using single plants and makes better use of space than using single plants and increasing the number of replicates.

The CV was 47% for seedling data and 30% for adult plant data; the square root transformation to equilibrate variances was useful in reducing the CV to 26% and 16%, respectively. In the seedling test, the transformation also increased the ANOVA F value from 16 to 27 and allowed the Waller-Duncan k-ratio t test to separate an additional group. The square root transformation was of similar value in analysis of the adult plant test when plants were considered as subsamples.

The high CVs that were observed reflect the nature of the host-pathogen interaction between barley and stem rust. About 2% of deposited urediniospores were able to infect and cause a pustule to develop on a susceptible barley plant. This is much lower than the 10–25% frequency reported by Rowell (10) for wheat. The high rate of abortion occurring during the infection process (J. Liu and D. E. Harder, *unpublished*) may be an important factor contributing to the high CVs.

The inoculator allows for many variations in cereal rust studies: repeatable urediniospore numbers can be applied to stems of adult plants or leaves of seedling and adult plants; single tillers can be inoculated so that the remaining tillers can be left unaffected to be used for other comparative studies or for seed production. With appropriate adaptations, the inoculator could also be used for other plant pathogen/host interaction studies.

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