Studies on the Use of High- and Low-Nutrient Inoculum for Infection of Wheat by *Gaeumannomyces graminis* var. *tritici*

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ABSTRACT

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A series of pot experiments demonstrated that wheat seedlings can be infected by *Gaeumannomyces graminis* var. *tritici* using a low-nutrient source of inoculum (i.e., fungal hyphae adhering to sand grains). It is shown that there is a proportional relationship between disease incidence and inoculum density for levels of inoculum ranging from 23 to 225 units per 100 cm^3 of soil and seedlings grown for up to 48 days at 10 C, 45 days at 15 C, or 31 days at 20 C. However, in another experiment where wheat was grown for 28 days at 15 C with inoculum densities ranging from 50 to 1,300 units per 100 cm^3 of soil, the relationship between disease incidence and inoculum density became nonproportional, and the disease incidence/inoculum density curve became quadratic. The curve reversed direction at about 900 units per 100 cm^3 of soil. In other experiments using colonized millet seed inoculum, disease incidence and inoculum density was proportional up to about eight units (seeds) per 100 cm^3, but thereafter the relationship was no longer proportional.

Pot experiments involving the use of *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier var. *tritici* Walker, the cause of take-all of cereals, are complicated by the lack of a quantitatively distinct unit of inoculum. Unlike many other soilborne pathogens, *G. g.* var. *tritici* does not possess distinct survival units (e.g., chlamydospores, sclerotia) and consequently any experiment requiring inoculum must involve the use of naturally infected host tissue or an artificially colonized medium. Naturally infected material may range in size from large crown pieces to very small (<0.5 mm) pieces of host residue (5,8,11). The use of such diverse material has many problems, including determining what constitutes a unit of inoculum. This problem may be partially solved by breaking the material into more distinct units by sieving (11), but the problem remains of determining how evenly the *G. g.* var. *tritici* has colonized the natural field material and what level of contamination of the tissue there is by other microorganisms.

The use of artificially colonized media as sources of inoculum has been discussed by Cunningham (2) and Asher (1). Asher divided this form of inoculum into two groups, namely colonized agar and colonized cereal grains or straw. The latter form has the disadvantage of giving the pathogen an unnaturally rich energy source and the introduction to soil of a medium likely to be rapidly attacked by other soil microflora, although this problem would apply less to straw than cereal grains. To overcome this problem Simon and Rovira (10) propose the use of colonized annual ryegrass (*Lolium rigidum* Gaud.) seed, whereas Powelson (9) suggest *G. g.* var. *tritici* could be encouraged to colonize or adhere to the surface of sand grains to form a low-nutrient source of inoculum.

In this paper we report a study on the effectiveness of colonized sand as a form of inoculum for use in studies on the infection of wheat (*Triticum aestivum* L.) roots by *G. g.* var. *tritici*. We contrast this low-nutrient form of inoculum with the nutrient-rich substrate of colonized millet (*Panicum miliaceum* L.) seed inoculum.

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MATERIALS AND METHODS

Soils. The soil used in experiments one to six was a Willamette Valley alluvial sandy loam from Corvallis, OR. This soil had a pH of 6.4 (1:2, v:v, soil:water) and an organic matter content of 1.1%. The gravimetric water content at matric potentials of −0.030, −0.060, −0.080, −0.200, and −1.500 MPa was 27.3, 17.7, 17.1, 12.4, and 11.9%, respectively. The soil used in experiment seven was yellow sand from Lancelin, Western Australia. The pH of this soil was 5 (1:2, v:v, soil:0.01 M $CaCl_2$) and the organic content 0.2%. The gravimetric water content at matric potentials of −0.003, −0.010, −0.067, −0.300, and −1.500 MPa was 30.0, 10.7, 4.6, 3.9, and 3.2%, respectively. Both soils were sieved to pass a screen with 2-mm openings and stored air dry. Neither soil contained indigenous inoculum of *G. g.* var. *tritici*.

Preparation of inoculum. An isolate of *G. g.* var. *tritici* from infected wheat from Jake's Hill, OR, was used for experiments one to six; an isolate from infected wheat from Esperance, Western Australia, was used for experiment seven. Isolates were stored on glucose-asparagine media before use (7). To produce inoculum, the fungus was grown on a medium composed of equal parts by volume of millet seed (>1.4 mm and <2.00 mm, average weight and volume 5.52 mg and 0.008 cm^3, respectively), silica sand (double sieved, >1.2 mm and <1.4 mm, 2.88 mg, and 0.002 cm^3), and water. The three components were placed in a tray, mixed, covered with foil, and autoclaved for 60 min at 121 C. Twenty-four hours later the components were again thoroughly mixed and autoclaved as above. The next day the mixture was added to 1-L glass jars (half full) and autoclaved for 20 min. The tray method prevented a hard coagulation of the sand and millet forming in the bottom of the jars.

The medium was seeded with agar freshly colonized by *G. g.* var. *tritici*, then incubated for 3 wk at 25 C. Jars were shaken regularly. The contents were air-dried and split into colonized millet seed and

surface colonized sand (Fig. 1) by passing over a 1.4-mm-mesh sieve. To clean the sand of small pieces of millet seed, it was winnowed in front of a household fan.

Both millet and sand inoculum were stored in the dark at 4 C in a screw top jar containing a saccule of silica gel. Survival of the Jake's Hill isolate on sand was tested after 10 mo by plating 50 sand grains on a selective medium (6). After six days incubation at 25 C, 49 grains had produced colonies, and by 10 days all had produced colonies.

Experimental design, assessment, and analysis. For all experiments, one wheat seedling (experiments one–six, cultivar Hill; experiment seven, cultivar Gamenya) was grown in 100 (experiment one, two, and three) or 133 cm^3 of infested soil in plastic tubes (supercells, Ray Leach Cone-Tainer Nursery, Canby, OR), which taper in diameter from 4 to 3 cm and are 20 cm deep. Loss of soil from the drainage holes was prevented by applying paper tissue on the outside. Soil was brought to near field capacity by placing the base of the tube in water and allowing the water to percolate to the soil surface. The soil was drained for 24 hr before sowing. At sowing, the Willamette and Lancelin soils had gravimetric water contents of 25.2 (approximately −0.036 MPa) and 7.5% (−0.030 MPa), respectively. Seed was sterilized for 2 min in 1% sodium hypochlorite before incubation for 24 hr on moist filter paper. Only plump seeds with the first sign of the radical were used.

Seed was covered with 15 cm^3 of uninfested soil plus a shallow layer of vermiculite. Tubes were watered to constant weight every 2 or 3 days. All experiments were randomized complete block design with three or more replications.

The cone of soil containing the plant was removed from the tube by applying a gentle stream of water to the drainage holes. Roots were then washed free of soil and the length of vascular discoloration within the proximal 10 cm of all seminal main axes was recorded on root maps. Vascular discoloration within the secondary axes and the main axis distal to the first 10 cm was not recorded. Assessments were made in water over an orange background using a ×2 magnification lamp. This background eliminated the effects of dark runner hyphae on the visual symptoms. In a preliminary test on the same 10 roots, the average length of diseased root over orange and white was 22.1% ± 3.7 and 30.2% ± 5.5, respectively.

Results were analyzed using analysis of variance. In experiments four, five, and six, the percentage of root length was transformed to a logit $[\log_n (x/100 - x)]$, and the disease progress curves analyzed using regression analysis.

Rates of inoculum and growth conditions. In experiment one, seven rates of millet seed inoculum (0, 1, 2, 4, 8, 16, and 32 seeds per 100 cm^3 of soil) were employed. The required number of millet seeds was added to 100 cm^3 of dry soil in a flask, tumbled by hand for 2 min, and then added to a supercell tube. The experiment was grown for 28 days in a greenhouse with a daily temperature range of 12–22 C and daylight supplemented with 12 hr of artificial light from fluorescent bulbs.

Seven rates of sand inoculum (0, 4, 8, 16, 32, 64, and 128 grains per 100 cm^3 of soil) were used in experiment two. The weight of grains equivalent to the required number of grains was added to 400 cm^3 of dry soil, tumbled as above, split into four aliquots and added to four tubes. Growth conditions were the same as experiment one. Experiments one and two had four replications.

For experiment three, eight rates of millet seed inoculum (0, 2, 4, 6, 8, 10, 12, and 16 seeds per 100 cm^3 of soil) were employed and the wheat seedlings were assessed 14, 21, 28, 34, or 47 days after sowing. Other details were as experiment one.

Seven rates of sand inoculum (0, 22.5, 45, 90, 135, 180, and 225 grains per 100 cm^3 of soil) were used in experiment four. Plants were grown in a controlled environment (12 hr of fluorescent light per day and 10 C constant temperature) and assessed after 21, 28, 34, 42, or 48 days. Results from experiment two and other preliminary experiments indicated that the tumbling method of mixing may not have been achieving a completely even distribution of sand inoculum in the soil. In this and following experiments, the soil for each treatment was spread (1 cm deep) over a large bench, and the sand inoculum sprinkled evenly over the soil surface before mixing the soil in such a way that most of the mixing was in a vertical plane with minimal lateral movement. The soil was added to the containers without further mixing. Experiments five and six were the same as experiment four except that five was grown at 15 C constant temperature for 17, 24, 32, 38, or 45 days, whereas six was grown at 20 C constant temperature for 13, 19, 25, or 31 days. Experiments three to six had three replications.

Experiment seven was conducted in Australia because there was insufficient time to complete this experiment in Oregon. Quarantine restrictions prevented the use of Jake's Hill isolate of *G. g.* var. *tritici* or Willamette soil. Thirteen rates of sand inoculum (0, 50, 100, 150, 200, 250, 400, 550, 700, 850, 1,000, 1,150, and 1,300 grains per 100 cm^3 of soil) colonized by the isolate of *G. g.* var. *tritici* from Esperance, Western Australia, were used in this experiment. Wheat seedlings were grown in a growth room for 28 days at 15 ± 2 C and 12 hr of fluorescent light per day. There were five replications.

Growth in soil. The ability of *G. g.* var. *tritici* to grow into soil from millet seed or colonized sand inoculum was determined using the soil sandwich technique of Grose et al (4). A single propagule of millet seed or sand colonized by the Esperance isolate of *G. g.* var. *tritici* was placed in the center of a 45-mm-diameter filter (0.45-μm pore size, Millipore Corporation, Bedford, MA) placed over natural or irradiated (285.7 min at 4 Mrds in a gamma cell 220) Lancelin soil. A second filter was placed over the propagule before

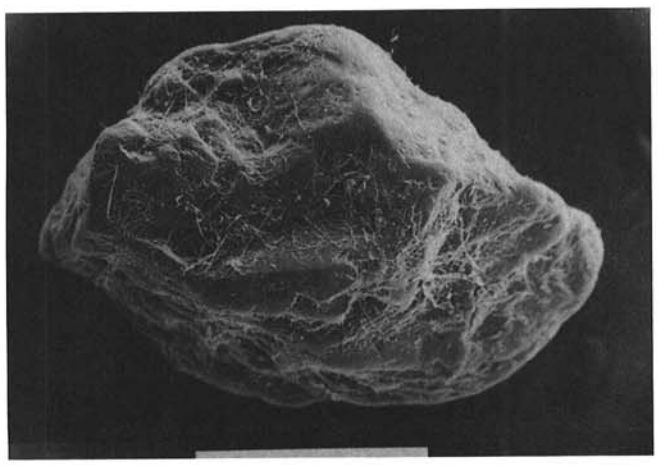

Fig. 1. Micrograph of a sand grain showing surface colonized by *Gaeumannomyces graminis* var. *tritici* (×40, bar = 1 mm).

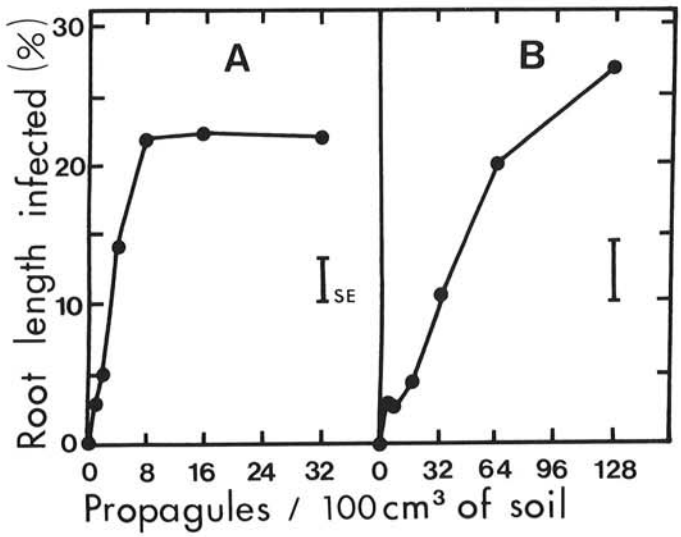

Fig. 2. Inoculum density/disease incidence curves for wheat seedlings grown for 28 days in soil plus millet seed **A** or sand **B** colonized by *Gaeumannomyces graminis* var. *tritici*.

adding more soil. The soil was maintained at −0.01 MPa for 14 days at 17 C. These conditions were chosen to maximize the possibility of growth (4). Hyphal growth was determined by measuring the diameter of the colony. There were three replications for millet seed and six for sand.

RESULTS

Within each of the seven experiments there was no significant effect of inoculum or time of sampling on the length of seminal root assessed (i.e., most seminal roots were established and had reached a length greater than 10 cm before the first time of sampling). Consequently, the effects of inoculum densities and times of sampling on length of root discolored can be compared.

Results from experiments one and two showed there was a highly significant correlation between the percentage of vascular length discolored (y) and average number of distinct lesions per root (x) (experiment one, $y = -0.26 + 5.07x$, $r^2 = 0.702$; experiment two, $y = 2.54 + 4.36\,x$, $r^2 = 0.685$). Following results are presented only in terms of the percentage of length discolored.

The inoculum density/disease incidence (ID/DI) curve for experiment one showed that increasing rates of millet seed inoculum caused a linear ($y = 0.44 + 2.81\,x$, $r^2 = 0.972$) increase in root length infected up to eight seeds per 100 cm^3 of soil after which length of discoloration ceased to increase (Fig. 2A). The analysis of variance (ANOVA) showed a significant inoculum effect ($P = 0.001$) composed of both a linear ($P = 0.001$) and quadratic ($P = 0.001$) component. In contrast, the sand inoculum in experiment two had a significant inoculum effect ($P = 0.001$) accounted for only by a significant linear component ($P = 0.001$) (Fig. 2B).

Experiment three was designed to give closer intervals of increasing rates of inoculum, about eight seeds per 100 cm^3, and to allow the study to be assessed after a range of growth periods. The slope of the ID/DI curve for 28 and 34 days reversed at eight units and declined, whereas the slope for 47 days reversed at four units (Fig. 3). The ANOVA showed significant inoculum ($P = 0.001$), time ($P = 0.001$), and inoculum × time interaction ($P = 0.05$). This interaction is accounted for by a significant quadratic (inoculum) ×linear (time) interaction ($P = 0.001$) reflecting the reversing of the slope of the ID/DI curve after about eight units of inoculum and a significant ($P = 0.05$) deviations (inoculum) × linear (time) interaction probably reflecting the changed shape of the curve of 47 days compared with the shapes of the curves at the previous times of sampling.

Experiments four to six were designed to study the effectiveness of sand inoculum over a wider range of inoculum densities than used in experiment two. One ID/DI curve for each temperature regime is shown in Figure 4, whereas the equations for all curves and their r^2 values are shown in Table 1. With several exceptions, the ID/DI curves for all times of sampling in all three temperature regimes are described by a linear equation with a high r^2 value. This, plus the fact the curves all pass close to zero, shows a proportional relationship between inoculum density and disease incidence over the range of inoculum densities used. This is further

TABLE 1. Linear equations and r^2 values for three inoculum experiments comparing rates of sand inoculum of *Gaeumannomyces graminis* var. *tritici* (x) and percentage of root length discolored (y) on wheat seedlings grown for varying periods under three temperature regimes

Days of growth	Linear equation	r^2 value
Sand 10 C (Expt. 4)		
21	$y = 0.06 + 0.004\,x$	0.558
28	$y = -0.07 + 0.017\,x$	0.820
34	$y = 0.04 + 0.033\,x$	0.929
42	$y = 0.23 + 0.050\,x$	0.691
48	$y = 1.03 + 0.066\,x$	0.881
Sand 15 C (Expt. 5)		
17	$y = -0.31 + 0.014\,x$	0.920
24	$y = 1.60 + 0.021\,x$	0.465
32	$y = 2.51 + 0.088\,x$	0.641
38	$y = 2.00 + 0.176\,x$	0.926
45	$y = -0.08 + 0.245\,x$	0.978
Sand 20 C (Expt. 6)		
13	$y = -0.06 + 0.012\,x$	0.938
19	$y = 2.38 + 0.017\,x$	0.262
25	$y = 2.10 + 0.089\,x$	0.838
31	$y = 1.43 + 0.181\,x$	0.988

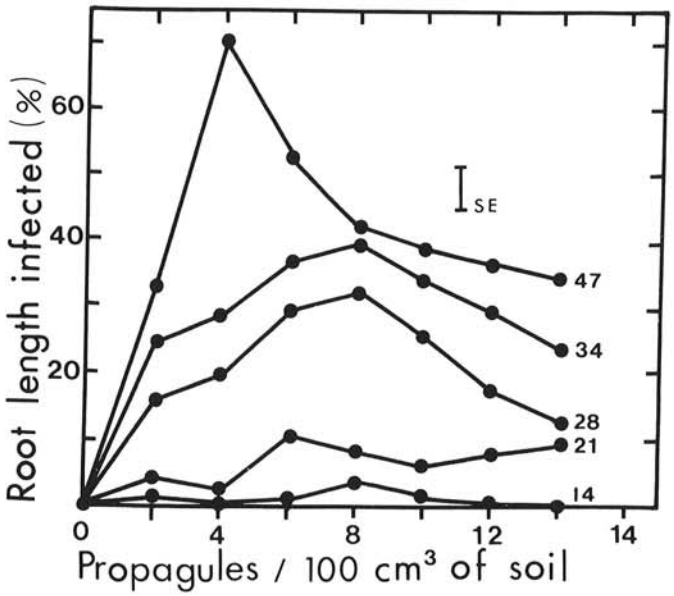

Fig. 3. Series of inoculum density/disease incidence curves for wheat seedlings grown for 14, 21, 28, 34, or 47 days in soil plus millet seed colonized by *Gaeumannomyces graminis* var. *tritici*.

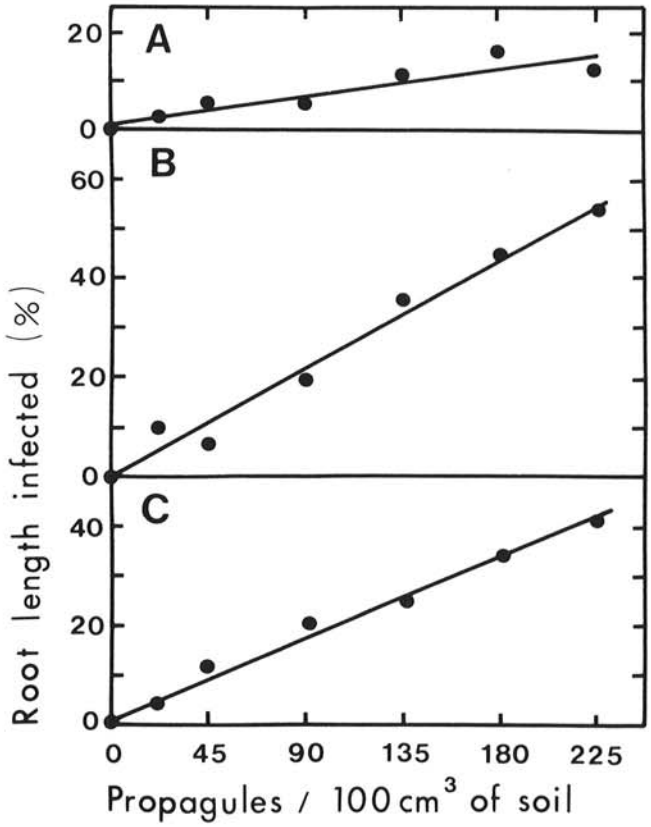

Fig. 4. Inoculum density/disease incidence curves for wheat seedlings grown for 48 days at 10 C **A**, 45 days at 15 C **B**, and 31 days at 20 C **C**, in soil plus sand colonized by *Gaeumannomyces graminis* var. *tritici*.

supported by the ANOVA where the significant inoculum × time interaction ($P = 0.01$ or 0.001) is composed mostly of a highly significant linear (inoculum) × linear (time) interaction ($P = 0.001$) for all three temperature regimes. For both 15 and 20 C there was also a small component of the inoculum × time interaction accounted for by a linear × quadratic interaction ($P = 0.01$ and 0.05, respectively). This would be due to the change of the disease progress curves from lag phase to exponential phase.

An examination of the disease progress curves suggested data from experiments four, five, and six could be combined on the basis of thermal time (days of growth × temperature). The data were transformed to logit and a series of disease progress curves prepared for each inoculum density. These curves were analyzed by regression analysis. The first model fitted included thermal time, six levels of inoculum, and the thermal time × levels of inoculum interaction. This model accounted for 85.4% of the variance. In a modified model, a common slope was fitted, and the model still accounted for 85.4% of the variance. The disease progress curves are shown in Figure 5. The results can be expressed as a series of parallel curves with significant differences between some inoculum levels (Fig. 6). The interval between curves reduced with increasing inoculum density so that by 135 units per 100 cm^3 of soil, increases were small and not significantly different (Fig. 6).

Before the commencement of experiment seven, two preliminary experiments similar to experiments one and two above, but using the Esperance isolate, gave results showing the same patterns as those experiments (millet seed inoculum rates of 0, 2, 4, 8, 16, and 32 seeds per 100 cm^3, $y = 7.13 + 4.341x - 0.1004x^2$, $r^2 = 0.900$; sand inoculum rates 0, 16, 32, 64, 128, and 256 grains per 100 cm^3, $y = -0.39 + 0.085x$, $r^2 = 0.973$; where y is the percentage of root length discolored and x is inoculum density). The results from experiment seven indicate that levels of sand inoculum equivalent to approximately 900 grains per 100 cm^3 caused the ID/DI curve to reverse and above that to decline (Fig. 7). The fitted quadratic curve had an r^2 value of 0.821. The ANOVA showed a significant ($P = 0.001$) inoculum effect composed of both significant ($P = 0.001$) linear and quadratic components.

The study on the hyphal growth in soil gave the following results. Mycelium covered the entire filter paper (>45 mm) for millet seed in irradiated soil and averaged 20.8 mm diameter in natural soil. For sand in irradiated soil four colonies covered the entire filter paper and the remaining two had diameters of 14 and 40 mm. In natural soil colonies failed to grow on three filter papers but averaged 9.3 mm of very sparse growth on the remaining filter papers.

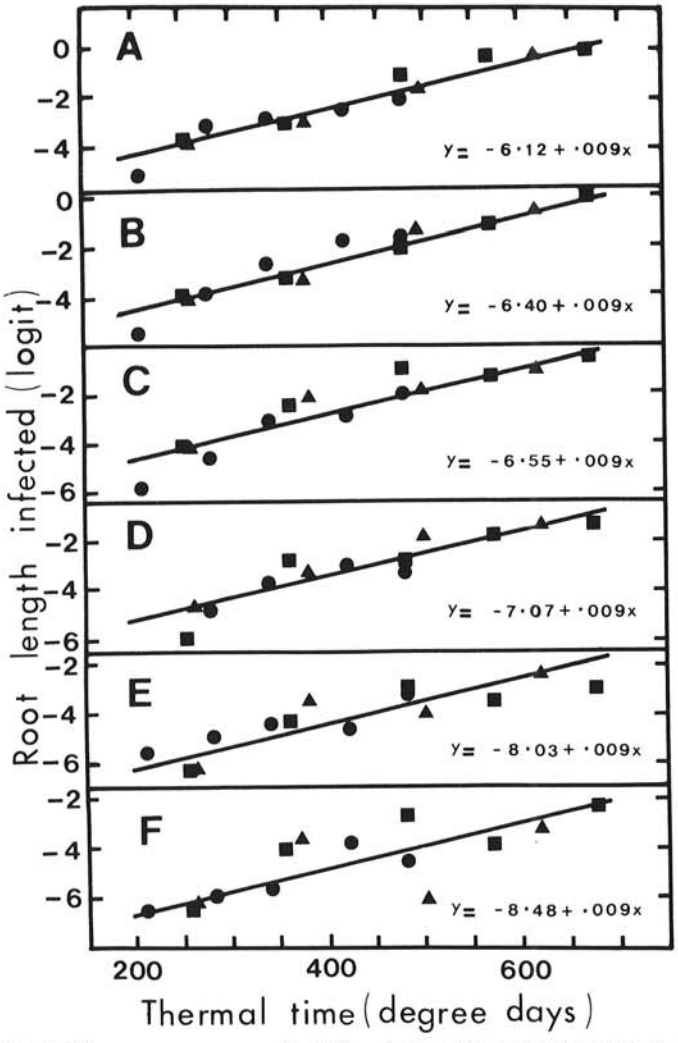

Fig. 5. Disease progress curves (logit) for wheat seedlings grown for various times at 10 C (●), 15 C (■), or 20 C (▲) in soil plus sand colonized by *Gaeumannomyces graminis* var. *tritici*. Inoculum densities of 23.5 **F**, 45 **E**, 90 **D**, 135 **C**, 180 **B**, and 225 **A** grains of sand per 100 cm^3 of soil.

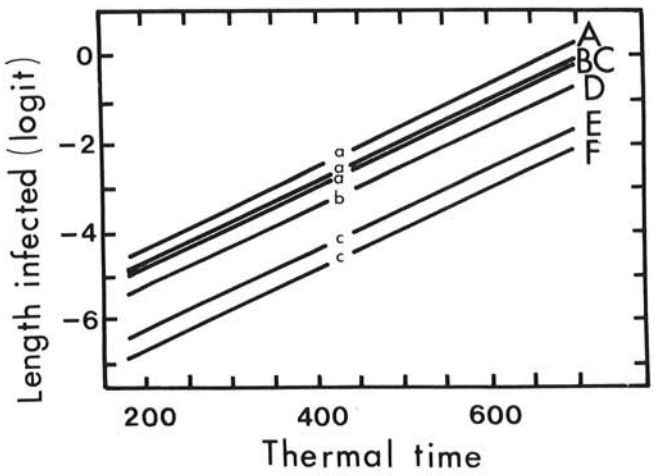

Fig. 6. Comparison of the position of the disease progress curves shown in Fig. 5. Curves denoted by the same lower case letter are not significantly different at $P = 0.05$.

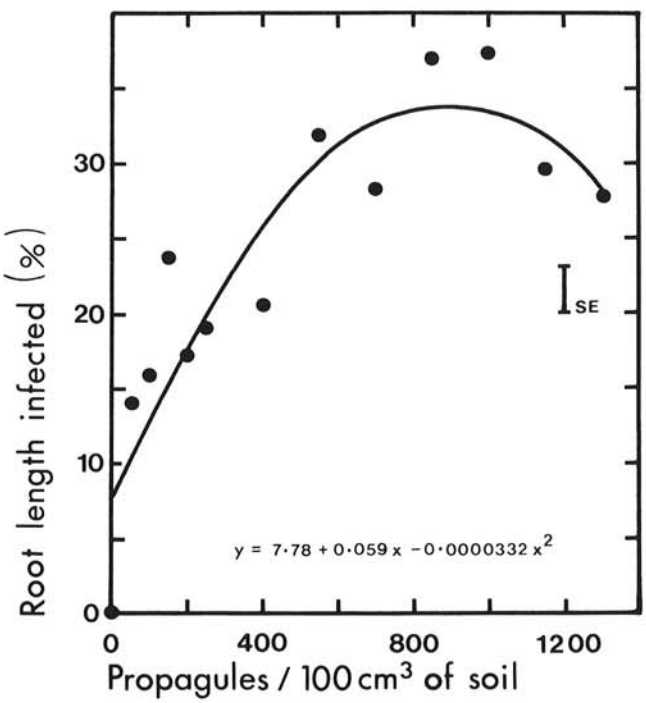

Fig. 7. Inoculum density/disease incidence curve for wheat seedlings grown for 28 days at 15 ± 2 C in soil plus sand colonized by *Gaeumannomyces graminis* var. *tritici*.

DISCUSSION

The results from these experiments show that the ID/DI curve became nonproportional with millet seed and colonized sand as inoculum densities increase above certain levels. This changing from a positive slope to a negative slope with increasing inoculum of *G. g.* var. *tritici* has been observed by Gilligan (3). Similarly, in a study by Wilkinson et al (11), it is shown that 0.75-mm inoculum units of naturally colonized crowns, colonized wheat roots, and axenically colonized oat cause a reversal of the ID/DI curve at about 5 mg of inoculum per gram of soil. The reason for this reversal of the ID/DI curve was not studied in our experiment. However, we believe it is not due to a lack of infection sites, as might be expected with very high levels of inoculum. The effect may be due to antagonism set up by soil microorganisms interacting with introduced organic matter. This could apply to the millet seed where antagonists could colonize the millet seed substrate, providing a biological control of the pathogen. This possibility seems less likely with the low-nutrient sand inoculum, but at high densities of sand sufficient nutrients may be available for antagonists to become established.

The results of the experiments using sand inoculum demonstrate that *G. g.* var. *tritici* can infect wheat roots from a low-nutrient base. Wilkinson et al (11) propose that infection by *G. g.* var. *tritici* is influenced by the total nutrients available from both the colonized crop residue and the soil. Our soils were not fumigated and had low-organic matter contents, suggesting there would be low but not necessarily negligible availability of nutrients from these soils. The results of our experiments show that *G. g.* var. *tritici* can grow into the soil from a low-nutrient base. However, the sparseness of growth and lack of growth in some cases suggests that the proximity of the root to the inoculum propagule becomes more important with a low-nutrient inoculum than with a high-nutrient inoculum. In the irradiated soil, *G. g.* var. *tritici* readily grew into the soil from the sand inoculum. This could be due to the removal of competitive microorganisms but could also be due to the release of nutrients from the soil.

The use of colonized sand provides a source of inoculum that is a uniform size, easily mixed with soil, and produces proportional infection per unit of inoculum, with densities of up to about 250 units per 100 cm^3 of soil (with the isolates used in these experiments). The use of thermal time allows the interchange of a wide choice of temperature and time regimes when linked to a range of inoculum levels. The use of disease progress curves will allow the use of the technique to test the effects of treatments like nitrogen sources, fungicides, and biological control agents on the infection of wheat seedlings by *G. g.* var. *tritici*.

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