# Enzyme-Linked Immunosorbent Assay Quantification of Initial Infection of Wheat by *Gaeumannomyces graminis* var. *tritici* as Moderated by Biocontrol Agents

H. M. El-Nashaar, L. W. Moore, and R. A. George

Postdoctorate research associate, associate professor, and research assistant, respectively, Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331. Present address of senior author: USDA, ARS, Plant Science Research Laboratory, P.O. Box 1029, Stillwater, OK 74076.

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## ABSTRACT

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An ELISA-double antibody sandwich system was used to measure the quantity of *Gaeumannomyces graminis* var. *tritici* associated with roots of winter wheat. The seedlings were grown at 15 ± 2 C in sand infested with three concentrations of 1-mm particles of oat grains colonized by *G. g.* var. *tritici*. Six days after seeding the pathogen was readily detected in a homogenized suspension ($10^{-7}$ dilution) of roots that were exposed to the lowest concentration. The level of *G. g.* var. *tritici* in the roots was proportional to the amount of inoculum used ($r = 0.998$). When wheat seeds were coated with strains of antagonistic bacteria before sowing, the amount of *G. g.* var. *tritici* detected in the roots was as much as 79% lower than in the absence of the bacteria. The results indicate that the amount of *G. g.* var. *tritici* associated with wheat roots can be measured within 6 days from seeding, and that in this system, potential antagonists to *G. g.* var. *tritici* can be evaluated within 8–10 days from planting.

*Additional key words:* biological control, monoclonal antibodies, soilborne plant pathogens, take-all, wheat.

Take-all, a disease caused by *Gaeumannomyces graminis* (Sacc.) Muller & Von Arx var. *tritici* Olivier, is one of the most important root diseases of wheat worldwide. This soilborne fungal pathogen can exist saprophytically on plant debris in the soil or parasitically on a susceptible cereal crop or on weed hosts (12).

Quantification of inoculum of soilborne plant pathogens can be difficult with some species. Most quantitative methods have relied on direct counts of chlamydospores, colony-forming units, sclerotia, and other spore forms. Other species can be detected only indirectly, by using seedling baiting techniques (12). The latter technique has been commonly used to quantify the inoculum potential of *G. g.* var. *tritici* in soil. Typically, wheat seedlings are grown for a few weeks in a rooting medium infested with debris colonized by the pathogen, after which the root systems are retrieved for visual assessment of take-all symptoms. However, symptoms alone are not entirely specific, and diagnosis must be confirmed by isolation of the causal agent (12). Isolation and identification of *G. g.* var. *tritici* is laborious and requires skill, experience, and a minimum period of a few weeks to complete. Consequently, a better method is needed for detection of the pathogen and/or diagnosis of the take-all. Enzyme-linked immunosorbent assay (ELISA) is now well known for its potential in the identification of plant pathogenic organisms and in some cases can be used as a reliable quantitative technique (4,5).

The present investigation was undertaken to test the feasibility of using ELISA techniques for early detection and quantification of *G. g.* var. *tritici* associated with roots of winter wheat seedlings; and to determine if this method could be used *in-plantae* to identify the effect of potential antagonists against *G. g.* var. *tritici*. A preliminary report has been published (7).

## MATERIALS AND METHODS

**Fungal cultures.** Cultures of *G. g.* var. *tritici* were isolated from infected wheat from Oregon by the method of Davies (6). The other cultures of fungi referred to in the text were isolated from the rhizosphere of wheat plants or obtained from the culture collections of C. M. Leach and E. M. Hansen (Oregon State University, Department of Botany and Plant Pathology).

**Antigen preparation.** A pathogenic isolate of *G. g.* var. *tritici* was cultured in a defined liquid medium under conditions previously described (1,8). Fungal mycelia were harvested, washed several times with sterilized distilled water, and rinsed twice in sterile phosphate-buffered saline (pH 6.8). The washed mycelia were frozen, lyophilized, ground to a powder, and stored at −70 C.

Soluble proteins and cell-wall antigens for *G. g.* var. *tritici* were obtained following conditions described by Abbott and Holland (1). One gram of powdered mycelium was ground in a mortar at 5 C with 10 ml of phosphate-buffered saline (pH 6.8) containing 17% sucrose, 0.1% ascorbic acid, and 0.1% cysteine hydrochloride. The slurry was left at 5 C for 1 hr, then centrifuged at 48,200 *g* for 30 min at 2 C. The supernatant (= soluble protein fraction) was stored in aliquots at −70 C until needed. The pellet was washed in phosphate-buffered saline (pH 6.8), and stored in 1-ml aliquots at −70 C as cell-wall antigens.

**Immunization schedule and antisera preparation.** Antisera were prepared by injecting New Zealand white rabbits intradermally (11) with 0.6 ml of either cell-wall or soluble protein fraction of *G. g.* var. *tritici*. For the first injection, antigens were emulsified with an equal volume of Freund's complete adjuvant. Intradermal injections were made at 12 sites along the back of the rabbit, and 0.1 ml of antigen was injected per site for the first injection. After a rest period of 1 wk, one intramuscular injection was given per week for an additional 2 wk. The latter injections were carried out using 0.5 ml of antigen mixed with an equal volume of Freund's incomplete adjuvant for each injection. A final 1-ml intramuscular injection of antigen:incomplete adjuvant (1:1, v/v) was administered to each rabbit 45 days after the primary injection.

Antibody titer was determined on blood samples from ear bleedings 1 wk after the third injection and once each week over the next 2 wk. A fourth sample was collected 1 wk after the last injection, and a final cardiac bleeding was made 60 days from the first injection. After collection, the blood was allowed to stand overnight to coagulate, then centrifuged, and the serum was collected and stored in 2-ml aliquots at −70 C.

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**Serological detection.** Detection of *G. g.* var. *tritici* associated with roots of wheat was conducted according to the ELISA procedure of Clark and Adams (5). The $\gamma$-globulin fraction obtained from the antiserum was partially purified using the CM Affigel blue procedure (Bio-Rad Labs, Richmond, CA). The $\gamma$-globulin/enzyme conjugates were prepared by mixing 2.5 mg of alkaline phosphatase Type VII (Sigma Chemical Co., St. Louis, MO) with 1-ml (1-mg) of purified $\gamma$-globulin ($A_{280nm}$ = 1.4). The solution was dialyzed three times against 1 L of phosphate-buffered saline (pH 7.4) at 4 C for 24 hr. Dialysis was followed by addition of glutaraldehyde to 0.1% final concentration. After 4 hr of conjugation at 22 C, the unbound glutaraldehyde was removed by dialysis against phosphate-buffered saline.

The ELISA tests were conducted in microtiter plates (Dynatech-Immulon II, Alexandria, VA). The plates were coated with purified globulin at a concentration of 1:100 in standard ELISA coating buffer. The ELISA coating buffer composition was 0.013 M $Na_2CO_3$, $H_2O$, and 0.035 M $NaHCO_3$ (pH 9.6). The coated plates were incubated at 4 C for 24 hr and then washed four times with phosphate-buffered saline (pH 7.4) containing 0.02% of Tween 20. Test samples (200 $\mu$l) described below were added to the wells, and the plates were incubated at 4 C for 24 hr. The test samples were then removed, the plates were washed with phosphate-buffered saline-Tween, and 200 $\mu$l of enzyme-antibody conjugate (1:100) in phosphate-buffered saline containing 0.05% gelatin (pH 7.4) were added.

After the ELISA plates were incubated for 9 hr at 4 C, the excess conjugate was removed by washing in phosphate-buffered saline-Tween; the enzyme substrate, P-nitrophenyl phosphate, was added at a concentration of 1 mg/ml in diethanolamine-sodium carbonate buffer (pH 9.8) and incubated at 22 C for 30–60 min. The enzymatic reaction was terminated by the addition of 50 $\mu$l of 3M NaOH to each well, and quantitative measurements of the enzymatic activity (ELISA values) were read (at $A_{405nm}$) by using an ELISA reader (Chromo-scan Model 307).

**Culture of wheat seedlings and inoculation with microorganisms.** Wheat seeds (Hill-81) were surface-sterilized in 0.2% sodium hypochlorite for 10 min, washed three times in sterilized distilled water, and dried with sterile air. Seeds were coated with 1% methylcellulose (25 centipoises) (Sigma Chemical Co.) alone or coated and mixed with one of two bacterial strains (A and B). These strains were isolated from soil (Oregon State University, experimental field plot at Hyslop Field Lab) suppressive to *G. g.* var. *tritici* and were antagonistic to the pathogen in-vitro (eighth-strength potato-dextrose agar, PDA). Both strains were gram-negative rods, and catalase and citrate positive. Strain A was oxidase positive and motile, whereas strain B was oxidase negative and nonmotile. Neither one of the strains was fluorescent when plated on King's medium B (KMB) (9). Strain A was tentatively identified as a nonfluorescent *Pseudomonas* species and strain B as an *Acinetobacter* species (2). No attempts were made to further identify these strains.

An aqueous suspension of the bacteria used to inoculate the seed was spread onto KMB, and the plates were incubated for 48 hr at 25 C. Bacterial growth was scraped from the plates and suspended uniformly in 10 ml of an aqueous suspension of 1% methylcellulose. Two hundred wheat seeds were added to each bacterial-methylcellulose suspension and gently agitated for 3–5 min to ensure a uniform coating of the seeds with the suspension. Samples of the coated seeds were assayed to determine the mean number of colony-forming units (cfu) of bacteria (13). Coated seeds yielded an average of $10^7$ or more colony-forming units per seed.

Cone-shaped plastic tubes (Ray Leach Container Co., Canby, OR), $4 \times 21$ cm, were used to grow the wheat seedlings. The lower 12 cm of the tubes were filled with sterile perlite (horticulture grade, Supreme Co., Portland, OR), on which was added a 5-cm layer of sterile sand mixed with 1-mm particles of oat grains colonized by *G. g.* var. *tritici* (2.5, 5, or 10 mg of inoculum per gram of sand). Inoculum particles of oat grains were colonized by *G. g.* var. *tritici* prepared as previously described by Wilkinson et al (14). Three wheat seeds coated with methylcellulose alone or mixed with antagonistic bacterium A or B were placed on the infested sand in each tube. A layer of sterilized perlite 2 cm thick was then added to cover the seeds, and tubes were placed in a growth chamber at $15 \pm 2$ C with a 16-hr photoperiod. The tubes were watered daily with tap water. Starting 3 days after seeding and then every 48 hr thereafter, 10 ml of Hoagland II solution was added per tube.

**Sample preparation for ELISA.** Insoluble antigens from the cell wall and the soluble antigen fraction of *G. g.* var. *tritici* (that were used initially as immunogens) were diluted in sterilized distilled water ($10^{-1}$–$10^{-8}$) and used as the known standards. Unknown test samples obtained from the roots of wheat seedlings were prepared as follows. Whole roots of wheat seedlings were harvested every 2 days, starting the sixth day after planting. The roots were washed in running distilled water, blotted with paper toweling, weighed, and homogenized in an 18- $\times$ 150-mm curved bottom tube containing enough sterilized distilled water to obtain a 1:10 dilution of the root tissues. Homogenization was accomplished with a polytron homogenizer type PT 10/35 (Brinkman Instrument, Westbury, NY). The homogenate preparation was further diluted to $10^{-2}$ with sterilized distilled water and used in ELISA.

## RESULTS

Antiserum from the rabbit injected with cell-wall antigen of *G. g.* var. *tritici* had a much higher titer and was more specific than the antiserum prepared against the soluble antigen fraction; partially purified $\gamma$-globulin from this higher-titered antiserum was used throughout this study. Antiserum from the first bleeding gave ELISA absorbance values of 0.35 and 0.18 at a dilution of $10^{-3}$ and $10^{-4}$, respectively. The second and third bleedings, gave 0.45, 0.16 and 0.70, 0.23, respectively, at the same dilutions. A known sample of antigens of *G. g.* var. *tritici* (2.5 mg of inoculum per 5 ml of sterilized distilled water) was detectable at $10^{-6}$ and $10^{-7}$ dilutions. Antiserum from the third bleeding and an antibody-alkaline phosphatase conjugate prepared from the first bleeding were used for all subsequent ELISA work.

Several fungal genera isolated from soil and/or rhizosphere of field-grown wheat cross-reacted with antisera of *G. g.* var. *tritici* used in ELISA. The soluble antiserum fraction reacted with *Rhizopus* sp., *Trichoderma* sp., *Fusarium graminearum*, *F. culmorum*, *F. oxysporum*, *Phoma* sp., *Cephalosporium* sp., *Chaetomium* sp., *Colletotrichum* sp., *Mucor* sp., *Rhizoctonia* sp., *Penicillum* sp., and *Gliocladium* sp. In contrast, cell-wall antisera reacted positively with five fewer fungal species, namely *Rhizopus* sp., *Trichoderma* sp., *F. graminearum*, *F. roseum*, *Phoma* sp., *Colletotrichum* sp., *Rhizoctonia* sp., and *Gliocladium* sp.

ELISA values indicated that a maximum level of antigens was associated with wheat roots 6 days from planting at each of the three levels of inoculum used (Fig. 1A–C). At 8 days from planting, the ELISA values dropped rather sharply, and then increased to 0.39, 0.35, and 0.28 for 10, 5, and 2.5 mg of inoculum per gram of sand, respectively, at 10 days from planting (Fig. 1A–C). ELISA values for all inoculated plants continued to decline steadily from 10 days after seeding to 14 days. ELISA values of the healthy roots and buffer were much lower than those from samples of the infected roots (Fig. 1A–C).

When ELISA values of the individual treatments were averaged over time, the means were 0.34, 0.30, and 0.25 for 10, 5, and 2.5 mg of inoculum per gram of sand, respectively. A correlation coefficient of $r$ = 0.998 was calculated between the latter mean values and log values of the inoculum concentrations used. Standardization of ELISA values of the individual treatments and calculation of the means over time, resulted in a mean value of 0.52, 0.49, and 0.39 for 10, 5, and 2.5 mg of inoculum per gram of sand, respectively (Fig. 2).

ELISA values of roots infected with *G. g.* var. *tritici* were less reduced when wheat seeds were coated with the antagonistic strains (before planting into infested sand), especially with the antagonist B (Fig. 2) than when coated with methylcellulose. For example, at 2.5 mg of inoculum per gram of sand, ELISA values of infected roots were 0.29 or 0.24 when antagonist A or B, was

present (Fig. 2). Similarly, the ELISA values were 0.44 or 0.32 when A or B was present at 10 mg of inoculum per gram of sand (Fig. 2). The same pattern was observed at 5 mg of inoculum per gram of sand (Fig. 2).

## DISCUSSION

Results from our system show that antigens of *G. g.* var. *tritici* associated with roots of winter wheat seedlings can be detected readily by ELISA. The technique is so sensitive that quantitative measurements of *G. g.* var. *tritici* on roots can be obtained accurately and precisely, even in homogenates of plant extracts. In contrast to infected tissues, ELISA values of the healthy roots and buffer were very low. These results are similar to those reported for other microbial-plant systems (3,10). The extreme sensitivity of ELISA enabled the detection of antigens of *G. g.* var. *tritici*.

Equally important, the antigens could be detected within the roots as early as 6 days from planting. These data suggest that ELISA can be used for rapid field diagnoses of take-all on wheat roots, and use of the method for detection of *G. g.* var. *tritici* in crop residue from soil samples is being investigated.

ELISA values obtained for individual treatments were high at the beginning and then declined. This pattern may have occurred because the ratio of pathogen to host (roots) was higher at 6 days after seeding, when relatively little root tissue had developed, then declined as roots were produced relatively more rapidly than they were infected. Thus, the amount of antigens found per gram of root tissue was diluted with time under the idealized growth conditions used in this experiment. This may change, however, once the organism is well established or the host is under stress. Daily watering and addition of Hoagland II solution were essential to provide wheat seedlings with the moisture and nutrients they needed. Even so, the stunted root growth of inoculated plants 18 days after seeding suggests that seedlings would not survive for longer periods of time with such severe infection by *G. g.* var. *tritici*.

The use of three different inoculum concentrations (2.5, 5, and 10 mg per gram of sand) yielded different ELISA values (Fig. 1A–C). The correlation between the ELISA values and proportion of the added inoculum was significant ($P \geqslant 0.05$). This indicates that in this model system, the amount of *G. g.* var. *tritici* associated with roots can be measured precisely and accurately.

Samples of root tissues from plants grown in the presence of *G. g.* var. *tritici* always yielded much higher ELISA values than healthy plants. However, values from infected tissues were significantly less when a biological control agent was coated onto the wheat seed before planting in the infested sand. For example, reductions of 17, 25, and 53% were found when bacterium A was used at 5, 10, and 2.5 mg of inoculum per gram of sand, respectively (Fig. 2). Even less disease occurred when antagonistic bacterium B

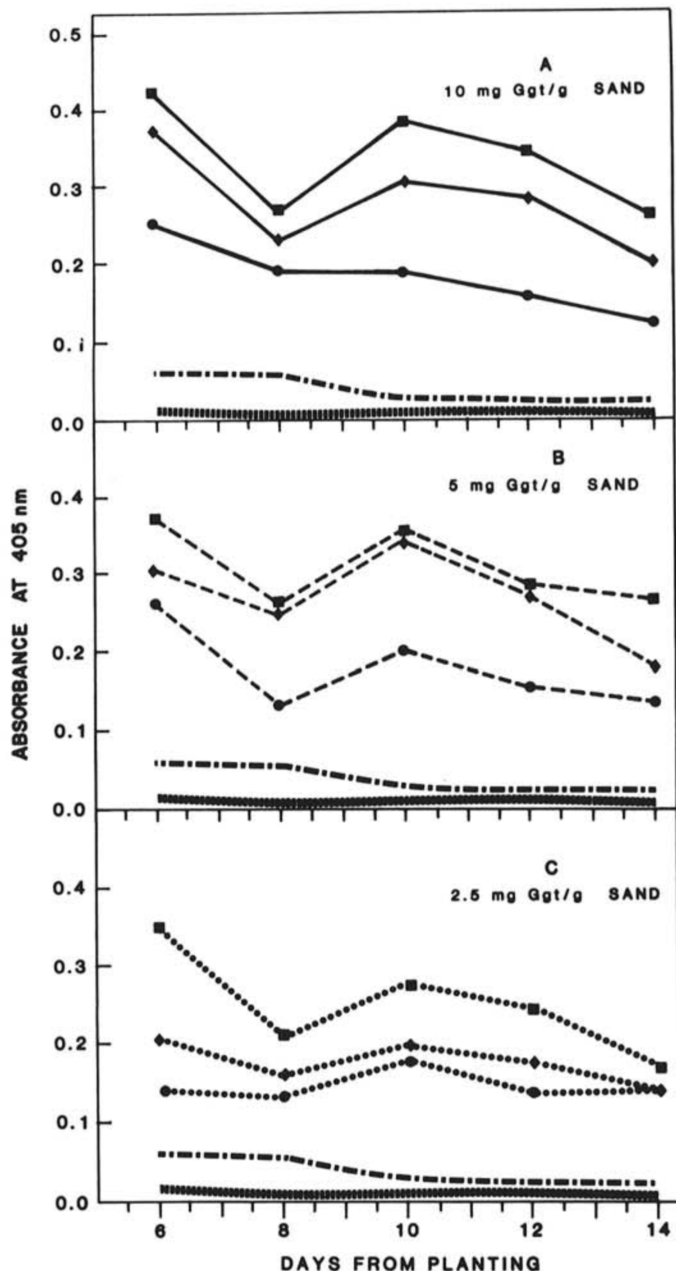


**Fig. 1.** Enzyme-linked immunosorbent assay (ELISA) values ($A_{405nm}$) of antigens of *Gaeumannomyces graminis* var. *tritici* present in homogenate preparation of winter wheat roots grown in sand infested with particles of oat grains colonized by *G. g.* var. *tritici* between 6 and 14 days after planting. **A, B, and C** represent wheat seedlings grown in medium infested with 10, 5, and 2.5 mg of inoculum per gram of sand, respectively. Symbols: ■ = roots of wheat seedlings planted in a medium infested with *G. g.* var. *tritici*; Δ and • roots of wheat seedlings where biological control agents A and B, respectively, were used to coat the seeds before planting in a medium infested with *G. g.* var. *tritici*. The two bottom lines in each figure represent absorbance for roots grown in noninfested medium (upper) and buffer (lower). Each point represents the mean of 24 wells.

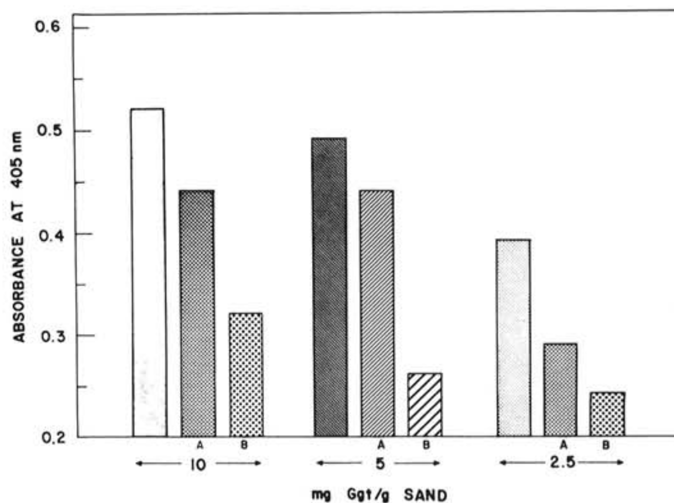


**Fig. 2.** Enzyme-linked immunosorbent assays (ELISA) values of antigens of *Gaeumannomyces graminis* var. *tritici* averaged by inoculum concentration over the five assay times shown in Figure 1. Each bar represents the mean of 24 replications/treatment/assay. The range of the mean absorbance for the buffer controls (not shown) over the five assays was 0.001–0.007, with an overall average of 0.005. To calculate the value of each bar, ELISA values for each assay were standardized as follows: The mean absorbance of a treatment was multiplied by the mean absorbance of its buffer control and divided by 0.005, the calculated average of all the buffer values. The unlabelled bar at each concentration represents absorbance of roots of wheat seedlings planted in a medium infested with *G. g.* var. *tritici*. A and B represent the biological control agents used to coat wheat seeds before planting in a medium infested with *G. g.* var. *tritici*. The mean absorbance of the uninfected roots was 0.032 (not shown).

was used. In ELISA values, absorbance for roots of seeds coated with bacterium B were 78, 79, and 62% less at 2.5, 5, and 10 mg of inoculum per gram of sand, respectively, than values for infested, untreated roots (Fig. 2). It is noteworthy that this ELISA test could detect the difference in the amount of antigens present in root homogenate between roots protected with different biological control agents and also discriminate between the relative activity of the two agents. These data suggest another way to evaluate rapidly and quantitatively large numbers of potentially antagonistic microorganisms to *G. g.* var. *tritici* in an *in-plantae* test. Under the conditions of this experiment, such evaluation could be achieved in less than 10 days from planting.

Our polyclonal antisera to soluble hyphal antigens cross-reacted with a number of other fungal genera. However, the number of fungi that cross-reacted was reduced considerably when the cell-wall fraction was used as an immunogen, as also observed by Holland and Choo (8). Specificity of the cell-wall antiserum was further increased by cross-adsorption with the cross-reacting fungal antigens. The high titer of our antiserum probably achieved by the intradermal immunization technique (11), was essential to successfully cross-adsorb the antisera. However, intradermal immunization with the soluble fraction failed to give as high titer or specificity as with the cell-wall fraction.

Although our polyclonal antiserum worked well in our system and produced excellent results when used by W. C. Mueller at the University of Rhode Island (*personal communication*), greater specificity and an unlimited quantity of the antiserum is desirable to optimize diagnostic capabilities under field conditions. We think the potential exists for increasing specificity further with the use of monoclonal antibodies. Observations that support this conclusion include the fact that specificity was enhanced by using hyphal cell-wall as immunogens and by cross-adsorption of the antisera.

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