

## A Selective Medium for *Gaeumannomyces graminis* var. *tritici*

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### ABSTRACT

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A selective medium (SM-GGT3) for *Gaeumannomyces graminis* var. *tritici* was developed to confirm field diagnosis of take-all. Differentiation of *G. graminis* var. *tritici* from other common soil fungi is based on its melanin pigment formation in the presence of L-DOPA. Other ingredients of the medium are potato-dextrose agar, streptomycin sulfate, dicloran, metalaxyl, and Hoe 00703. A 1% silver nitrate solution was used as a surface-sterilant. There was a 32% increase in take-all isolations from irrigated wheat and barley samples using SM-GGT3 compared with using potato-dextrose agar. There was also a 49% decrease in *Fusarium* spp., *Cochliobolus sativus*, and isolations of sterile mycelia using SM-GGT3.

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Take-all, caused by the soilborne fungus *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici* (Walker), is a yield-limiting root and

crown disease of wheat and barley in most areas of the world. Characteristic field symptoms of take-all include blackened roots, stunted plants with fewer tillers than normal, and prematurely ripened heads. These symptoms are also characteristic of other small-grain diseases such as dryland root rot caused by *Cochliobolus sativus* Ito & Kurib., *Fusarium culmorum* (Smith) Sacc., and *F. graminearum* Schwabe. The one symptom of take-all that normally is not confused with other diseases is the black plate mycelium, which occurs on the basal stem area of severely infected

plants. To confirm a field diagnosis, however, isolation of the fungus is necessary (3).

Previous isolation methods have involved surface-sterilization of infected tissue followed by plating the tissue on potato-dextrose agar, with or without antibiotics (1,5,8,10,13). Because *G. graminis* var. *tritici* normally does not produce asexual or sexual spores in culture, isolation and confirmation of take-all is difficult even if one is familiar with its typical colony characteristics. Also, *G. graminis* var. *tritici* is a slow-growing fungus and is easily overgrown by other common soil and plant tissue organisms (2,4).

One objective of this research was to develop a medium that would allow one to easily isolate, differentiate, and identify *G. graminis* var. *tritici* from infected plant tissue. The second objective was to identify a basic medium that could be developed further for use in soil-assay systems, antagonism studies, inoculum level surveys, and other investigations concerning this fungus in its soil and plant environment. Such a medium was not previously available.

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## MATERIALS AND METHODS

Test compounds were classified as carbon sources or antifungal, antibacterial, and general organic compounds. Carbon sources were incorporated at 100 and/or 1,000  $\mu\text{g/ml}$  into a minimal medium containing 1 g  $\text{KH}_2\text{PO}_4$ , 1 g  $\text{MgSO}_4$ , 3 g  $\text{NaNO}_3$ , 1 mg biotin, 1 mg thiamine, and 20 g Difco agar or purified Oxoid agar in 1 L distilled water. Antibacterial compounds were incorporated into potato-broth agar (PBA). The general organic compounds were screened via incorporation into potato-dextrose agar (PDA). Most of the antifungal compounds were tested at 1, 10, and 100  $\mu\text{g/ml}$  in one or more of the following basic media: PBA, PDA, or the minimal medium described previously. After testing each of the compounds individually for their effect on growth of *G. graminis* var. *tritici*, various combinations in PDA were tested for their combined effect on growth of *G. graminis* var. *tritici* and various test fungi.

Thirty-two isolates of *G. graminis* var. *tritici* from Montana, Washington, Oregon, Indiana, Arkansas, Kansas, Colorado, and Idaho were used in this study. *G. graminis* var. *graminis* and var. *avenae* (E. M. Turner) Dennis, *Phialophora graminicola* (Deacon) Walker, and a *Phialophora* sp. (lobed hyphopodia) were tested to compare their growth responses with those of *G. graminis* var. *tritici*. Other fungi tested (test fungi) were *Penicillium* spp., *Chaetomium* sp., *Aspergillus* sp., *Trichoderma* sp., *Alternaria* sp., *Rhizopus* spp., *Cochliobolus sativus*, *Fusarium culmorum*, *F. graminearum*, *Rhizoctonia solani*, *Rhizoctonia* spp., *Pythium* sp., and *Pseudocercospora herpotrichoides*. These fungi were used because they are common soil saprophytes or soilborne pathogens of small grains. Test fungi were maintained on PDA. A 4- or 7-mm-diameter agar mycelial plug was placed on the test medium plates with a minimum of three replicate test plates per organism. Inoculated plates were maintained at 20–25 C for 5 days before recording the linear amount of fungal mycelial growth from the edge of the inoculum plug. Growth comparisons were made with replicated unamended check plates.

The final medium developed was SM-GGT3, which contained 39 g Difco PDA, 10 mg dicloran, 10 mg metalaxyl, 25 mg 1-(3,5-dichlorophenyl)-3-methoxymethylpyrrolidin-2,4-dione (Hoe 00703), 100 mg streptomycin sulfate, and 500 mg L- $\beta$ -3,4-dihydroxyphenylalanine (L-DOPA) in 1 L distilled water. The amounts given for each chemical are amounts of active ingredient. A sample of Hoe 00703 may be obtained from the laboratory of D. E. Mathre, Montana State University, Bozeman 59717.

To prepare SM-GGT3, dehydrated PDA was added to distilled water,

autoclaved at 121 C for 20 min, and cooled to 50 C. The remaining compounds were added to 10 ml sterile distilled water in a sterile 15-ml tube before incorporating into the molten PDA. The medium was then agitated to suspend any undissolved compounds and poured into sterile petri plates. Plates were stored upside down in plastic bags or metal tins in the dark at 4 C. SM-GGT3 was compared with PDA using naturally infected tissue and in the combat tests and soil-assay tests described.

Mature plants showing characteristic field symptoms of take-all were collected during 1982 from irrigated small-grain fields in Montana (primarily spring wheat). Mature plants were also randomly collected from dryland small grain fields. All plants were stored dry in paper bags at room temperature. The basal stem area or subcrown internode was removed, rinsed thoroughly with water, and cut into 1-cm pieces, which were sterilized for 30 sec in a 1% silver nitrate solution, rinsed 30 sec in sterile distilled water, and blotted dry on filter paper before being placed on SM-GGT3. If there was enough material, at least three plates per location were inoculated. Inoculated plates were maintained at 20–25 C for 5–9 days. Results were tabulated by determining the percentage of tissue pieces that fostered *G. graminis* var. *tritici* growth, with and without pigment production. When possible, the number and identity of any contaminating organisms were determined using microscopy and selective media.

To determine the effectiveness of SM-GGT3 in detecting *G. graminis* var. *tritici* and eliminating common soilborne fungi such as *F. culmorum*, combat tests comparing the ability of two organisms to grow on specific media were conducted. The subcrown internode and basal culm tissue naturally infected with *G. graminis* var. *tritici* was washed thoroughly and cut into 1- or 2-cm pieces. Half of the pieces were soaked in sterile distilled water for 1 hr and the remaining pieces were soaked in a dense *F. culmorum* spore suspension for 1 hr. The pieces were then blotted dry and incubated for 2 days in sterile petri plates. When incubation was completed, all pieces were surface-sterilized with a 1% silver nitrate solution for 30 sec, rinsed in sterile distilled water for 30 sec, blotted dry on filter paper, and plated on SM-GGT3 or PDA.

Although SM-GGT3 was developed primarily as a medium for isolating *G. graminis* var. *tritici* from tissue, preliminary experiments were conducted to determine its value as a soil-assay medium for *G. graminis* var. *tritici*. SM-GGT3 was compared with PDA containing 100  $\mu\text{g/ml}$  streptomycin sulfate using two different soils. A silty clay-loam soil was collected from a field at the A. H. Post Agricultural Research Station, Bozeman, MT. A sandy clay soil

was collected at Ulm, MT, from a field infested with take-all. Ground *G. graminis* var. *tritici*-infected oat-kernel inoculum was added to both soils at a rate of 1 g of inoculum per 100 g of soil (dry weight basis) and tumbled for 5 min in a rotating seed treater.

The soil-suspension-assay method consisted of 1 g of the soil/inoculum mixture suspended in 100 ml of sterile distilled water equivalent to a  $10^{-2}$  dilution. Other dilutions used were  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$ . After shaking for 1 min, 0.1 ml of each dilution was spread over the plate with a sterile bent-glass rod. Four plates of each medium were used for each dilution.

With the sieved-soil technique, about 150 g of soil/inoculum mixture was dry-sieved through a set of four sieves with openings of 2.36, 1.70, 0.85, and 0.25 mm. Twelve pieces of organic matter, primarily straw residue, were selected from each set of sieved material, including the material that passed through the 0.25-mm sieve, and placed directly on the test plates. Again, four plates of each medium were used for each size of sieved material. There was some difficulty in selecting pieces of organic matter smaller than 0.25 mm. If this occurred, soil particles picked up with the organic matter were also used for inoculating these plates.

## RESULTS AND DISCUSSION

Two of Tsao's suggestions (16) were used to develop SM-GGT3—selective inhibition and selective differentiation by pigmentation. *Gaeumannomyces* uses L-DOPA to produce a melanin pigment that easily distinguishes it from most other soil organisms. Selective inhibition was accomplished with the compounds dicloran, metalaxyl, Hoe 00703, and streptomycin sulfate. Streptomycin sulfate is an inexpensive and readily available inhibitor of gram-positive and gram-negative bacteria (7).

Metalaxyl in SM-GGT3 selectively inhibited Oomycetes (6). This was necessary because Oomycetes such as *Pythium* spp. are root parasites and common soil inhabitants.

Dicloran was used because of its reported ability to inhibit *Rhizopus* (9), a fungal genus commonly encountered in soil. Although *Rhizopus* was never isolated from any of the plant tissue cultured, growth of *Rhizopus* from pure-culture inoculations on the selective medium and colonies of *Rhizopus* that originated from soil dilutions or soil particles were not extensively inhibited. One reason for the failure to isolate *Rhizopus* from root or stem tissue could be the use of a 1% silver nitrate solution as a surface-sterilant when isolating from plant tissue. With addition of 10  $\mu\text{g/ml}$  silver nitrate to PDA, *Rhizopus* was inhibited 25–30% versus no inhibition with 75  $\mu\text{g/ml}$  dicloran. This indicates that it was the silver nitrate surface-

sterilant that inhibited growth of *Rhizopus* on SM-GGT3 and not the antifungal compounds; however, dicloran is still useful for inhibiting susceptible species of *Rhizopus*.

At 25 µg/ml, Hoe 00703 completely inhibited pure cultures of *F. culmorum*, *F. graminearum*, *C. sativus*, and *R. solani*, with only minor inhibition of *G. graminis* var. *tritici*. *F. culmorum*, *F. graminearum*, *C. sativus*, and *R. solani* are common root and basal culm pathogens of small grains, and in general, these and other species of these fungi are common soil inhabitants. Plant tissue isolation studies with a previous medium (11) indicated that *F. roseum* and *C. sativus* accounted for the largest percentage of non-*G. graminis* var. *tritici* isolations. Asher (2) stated that fast-growing fungi, such as *Fusarium* species, were a leading cause in failing to isolate *G. graminis* var. *tritici* from infected plant tissue. This necessitates complete inhibition of *Fusarium* because it grows at a significantly faster rate than *G. graminis* var. *tritici*. Without Hoe 00703, the value of SM-GGT3 as a selective medium would be greatly diminished.

The second of Tsao's suggestions used in developing SM-GGT3 was selective differentiation by pigmentation. Noting that *G. graminis* var. *tritici* infection of wheat is associated with dark runner hyphae called macrohyphae (13,21) and that melanin has been extracted from *G. graminis* var. *tritici* hyphae (17), an attempt was made to enhance this natural hyphal pigmentation. L-Tyrosine, an amino acid precursor of melanin, induced *G. graminis* var. *tritici* to produce a brown pigment in the medium. This pigmentation permits selective differentiation of *G. graminis* var. *tritici* similar to the selective medium for *Streptomyces scabies* (12). Of the four tyrosine analogues tested, L-DOPA induced the darkest pigment formation without inhibiting growth of *G. graminis* var. *tritici*.

All isolates of *G. graminis* var. *tritici* tested produced a melanin pigment on SM-GGT3. *G. graminis* var. *avenae*, *G. graminis* var. *graminis*, *P. graminicola*, and a lobed hyphopodial *Phialophora* sp. also produced a melanin pigment of the same intensity as *G. graminis* var. *tritici*. This was not surprising, however, considering the close taxonomic relationship of these fungi (22). Some *R. solani* isolates produced a melanin pigment in the presence of L-DOPA in PDA but SM-GGT3 completely inhibited *Rhizoctonia* species. If *Rhizoctonia* growth should occur, macroscopic and microscopic examinations could easily distinguish *Rhizoctonia* from *G. graminis* var. *tritici*.

To distinguish *G. graminis* var. *tritici* from *G. graminis* var. *graminis*, *G. graminis* var. *avenae*, and *P. graminicola* will be more difficult and will probably

require production of perithecia in culture. However, *G. graminis* var. *tritici* is considered the main cause of take-all of wheat. Another possible way to distinguish between varieties of *G. graminis* would be with chemical additives to SM-GGT3. For instance, the sulfur-containing amino acids cysteine and cystine reportedly inhibit *G. graminis* var. *avenae* growth while stimulating *G. graminis* var. *tritici* (18–20).

To determine the practical use of SM-GGT3, 34 small-grain plant samples were collected in 1982. Mature plants showing characteristic field symptoms of take-all were collected from irrigated fields. Mature plants were also randomly collected from dryland fields. Silver nitrate was used as the surface-sterilant. Using SM-GGT3 as the isolation medium, 31% of all subcrown internode or basal stem tissue pieces tested produced *G. graminis* var. *tritici* growth and pigmentation, 16% produced other fungal growth, and 53% did not produce growth of any organism (Table 1).

*G. graminis* var. *tritici* was isolated from four of the 10 dryland wheat samples and from none of the dryland barley samples. Separating the irrigated samples from the total sample group, 51% of the tissue pieces yielded *G. graminis* var. *tritici* and pigment, 7% produced other fungal growth, and 43% did not produce growth of any organism

(Table 1). Six of the irrigated small-grain samples apparently were not infected with *G. graminis* var. *tritici*—three barley and three wheat samples. Table 2 is a comparative summary of the results obtained on SM-GGT3 and PDA using the same isolation techniques for irrigation samples. It is apparent that SM-GGT3 would be the superior medium for isolating *G. graminis* var. *tritici* from plant tissue. Seven irrigated wheat samples collected in 1981 were also tested on SM-GGT3 with the same isolation techniques. *G. graminis* var. *tritici* was isolated from all seven samples.

Comparisons were made of the amount of fungal growth originating from plant tissue after 5 days on PDA and SM-GGT3. *G. graminis* var. *tritici* growth was inhibited only slightly, if at all, on SM-GGT3. *C. sativus* did not grow on SM-GGT3 even though the average colony on PDA was 24 mm in diameter. The *F. roseum* group produced 60-mm-diameter colonies on PDA but were reduced 85–90% on SM-GGT3, and *F. roseum* colonies seldom grew at all. *Rhizoctonia* species were isolated only once on PDA and were never isolated on SM-GGT3. Although unidentified sterile mycelia grew on SM-GGT3, diametric growth was restricted to less than 15 mm. *Rhizopus* contaminated one plate of PDA and one plate of SM-GGT3. Bacterial growth was either completely

**Table 1.** Results of isolations from 1982 irrigated and dryland wheat and barley tissue suspected of being infected with *Gaeumannomyces graminis* var. *tritici* (*Ggt*) using the selective medium SM-GGT3

| No. field sources <sup>a</sup> | Tissue pieces sampled | No. tissue piece isolations yielding |                         |                        |                    |                   |
|--------------------------------|-----------------------|--------------------------------------|-------------------------|------------------------|--------------------|-------------------|
|                                |                       | <i>Ggt</i>                           | <i>Fus</i> <sup>b</sup> | <i>Cs</i> <sup>c</sup> | Other <sup>d</sup> | None <sup>e</sup> |
| 15 ISW                         | 141                   | 87                                   | 1                       | 0                      | 3                  | 50                |
| 7 DSW                          | 68                    | 3                                    | 8                       | 0                      | 10                 | 47                |
| 1 IWW                          | 10                    | 0                                    | 0                       | 0                      | 0                  | 10                |
| 3 DWW                          | 24                    | 2                                    | 3                       | 0                      | 1                  | 18                |
| 4 IB                           | 34                    | 7                                    | 3                       | 0                      | 5                  | 19                |
| 4 DB                           | 40                    | 0                                    | 5                       | 0                      | 11                 | 24                |
| IB, ISW, IWW                   | 185                   | 94                                   | 4                       | 0                      | 8                  | 79                |
| DB, DSW, DWW                   | 132                   | 5                                    | 16                      | 0                      | 22                 | 89                |
| Total                          | 317                   | 99                                   | 20                      | 0                      | 30                 | 168               |
| Percent                        | ...                   | 31                                   | 6                       | 0                      | 10                 | 53                |

<sup>a</sup>I = irrigated, D = dryland, B = barley, SW = spring wheat, and WW = winter wheat.

<sup>b</sup>*Fusarium* spp., primarily of the *F. roseum* group.

<sup>c</sup>*Cochliobolus sativus*.

<sup>d</sup>This group consisted of sterile mycelia (septate and nonseptate hyphae) and a few bacteria.

<sup>e</sup>No growth of any organism.

**Table 2.** Comparison of the selective medium (SM-GGT3) with potato-dextrose agar (PDA) in isolation of *Gaeumannomyces graminis* var. *tritici* (*Ggt*) from 1982 irrigated wheat and barley tissue suspected of being infected with *Ggt*

| Medium  | Percentage of total tissue pieces yielding |                         |                        |                    |                   |
|---------|--|-------------------------|------------------------|--------------------|-------------------|
|         | <i>Ggt</i>                                 | <i>Fus</i> <sup>a</sup> | <i>Cs</i> <sup>b</sup> | Other <sup>c</sup> | None <sup>d</sup> |
| PDA     | 19   | 21                      | 6                      | 29                 | 25                |
| SM-GGT3 | 51   | 2                       | 0                      | 5                  | 43                |

<sup>a</sup>*Fusarium* spp., primarily of the *F. roseum* group.

<sup>b</sup>*Cochliobolus sativus*.

<sup>c</sup>This group consisted of sterile mycelia (septate and nonseptate hyphae) and bacteria.

<sup>d</sup>No growth of any organism.

inhibited on SM-GGT3 or restricted to a small area immediately surrounding the tissue piece.

Because the *F. roseum* group was the most common non-*G. graminis* var. *tritici* group isolated with SM-GGT3, a combat test experiment was conducted to determine the efficiency of SM-GGT3 in detecting *G. graminis* var. *tritici* in the presence of *F. culmorum*. The naturally infected take-all plant tissue soaked only in sterile distilled water yielded 67% *G. graminis* var. *tritici* with pigment and 0% *F. culmorum* on SM-GGT3. The naturally infected tissue soaked in *F. culmorum* spores yielded 17% *G. graminis* var. *tritici* and 17% *F. culmorum* on SM-GGT3 versus 100% *F. culmorum* and 0% *G. graminis* var. *tritici* on PDA. The *F. culmorum* colonies on SM-GGT3 were restricted to less than 10 mm in diameter versus 60 mm on PDA.

Using the soil-suspension-assay method, *G. graminis* var. *tritici* was not isolated from either soil with either PDA containing 100 µg/ml streptomycin sulfate (PDAS) or SM-GGT3. *Rhizopus* and bacterial colonies were the only organisms growing on PDAS with the soil/inoculum mixture from Ulm. Only *Rhizopus* grew on SM-GGT3. With the Bozeman soil/inoculum mixture, a greater variety of organisms grew on PDAS. Only a few colonies of a sterile fungus with nonseptate hyphae and a bacterial species grew on SM-GGT3.

With the sieved-soil technique, *G. graminis* var. *tritici* was isolated on both PDAS and SM-GGT3 from the Bozeman mixture when organic residue from all five sieve sizes was used. *Rhizopus*, *Fusarium* spp., *Penicillium* spp., and a sterile fungus with nonseptate hyphae were also isolated with PDAS. Only *Rhizopus*, a sterile fungus with nonseptate hyphae and a bacterium were isolated on SM-GGT3. *G. graminis* var. *tritici* was not isolated from the Ulm soil/inoculum mixture, and all plates (PDAS and SM-GGT3) were covered completely by *Rhizopus*.

The advantages of SM-GGT3 over PDA and PDA containing antibiotics are substantial. The compounds for SM-GGT3 are inexpensive and relatively easy to obtain. Also, the medium is not difficult to produce. The shelf life of the medium is at least 3 mo. SM-GGT3 is selective against bacteria, Oomycetes, and many common soil and plant-parasitic and saprophytic Ascomycetes and Basidiomycetes—particularly those causing small-grain root and stem diseases. This medium allows *G. graminis* var. *tritici* to grow without competing with other fungi and bacteria. It is then easy to differentiate it via melanin

pigmentation, which diffuses beyond the mycelium. *G. graminis* var. *tritici* growth and colony characteristics do not appear to be altered by the selective medium. *G. graminis* var. *tritici* also infected wheat seedlings from colonized SM-GGT3 agar disks (M. E. Juhnke, unpublished). Thus, one could quickly fulfill Koch's postulates using SM-GGT3 as the isolation medium.

The only obvious disadvantage of SM-GGT3 is its inability to completely inhibit *Rhizopus*. *Rhizopus* was never a problem in isolating from plant tissue when this medium was used with silver nitrate as a surface-sterilant. However, in soil assays for *G. graminis* var. *tritici*, *Rhizopus* was and will be a problem until a suitable inhibitory compound is identified.

Papavizas and Lumsden (14) were able to significantly improve a selective medium for isolation of *Trichoderma* from soils infested with *Rhizopus* by adding the surfactant alkaryl polyether alcohol (APA). In their trials, dicloran and metalaxyl did not inhibit *Rhizopus*, even at 200 µg a.i./ml. Quintozene reduced *Rhizopus* but also inhibited *Trichoderma*. These results are similar to those obtained in this study except that APA was also inhibitory to *G. graminis* var. *tritici*.

*G. graminis* var. *tritici* is commonly detected in soils indirectly via bioassays that do not permit detection of avirulent *G. graminis* var. *tritici* isolates (15). Use of SM-GGT3 should allow detection of both virulent and avirulent isolates. Past experiments by Asher (2) have shown a lack of correlation between virulence and natural pigmentation. If this holds true for chemically induced pigmentation in a selective medium (SM-GGT3), virulent and avirulent isolates could be detected easily and isolated for use in pathogenicity studies.

It is believed that future work concerning *G. graminis* var. *tritici* isolation, either from plant tissue or from soils, should be based on SM-GGT3. Such a selective medium would help to quantify the amount of *G. graminis* var. *tritici* inoculum and so provide a basis for long-term forecasting of take-all. SM-GGT3 also could be used in a plant disease clinic as a diagnostic medium for isolation of *G. graminis* var. *tritici* to confirm take-all.

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