Quantitative Detection of Propagules of Cephalosporium gramineum in Soil

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Michigan Agriculture Experiment Station Journal Series Article No. 6167.
Accepted for publication 29 March 1973.

ABSTRACT

A selective medium was developed which permitted the quantitative detection of propagules of *Cephalosporium gramineum* in natural soil. Essential to the recovery of this relatively slow-growing fungus in the presence of numerous other faster-growing soil microorganisms was the proper balance of wheat leaf extract and copper sulfate in an agar-base medium. Characteristic sporulating colonies of *C. gramineum* were countable within 4-7 days after dilutions of naturally infested soil were applied directly to the medium. Gram quantities of naturally infested field soil yielded up to 30,000 propagules of *C. gramineum*. The propagules were typical free conidia of *C. gramineum*, or of its sporodochial stage, *Hymenula ceralis*.

Phytopathology 63:1198-1201.

Cephalosporium gramineum Nisikado & Ikata is an important soil-borne pathogen of wheat (1). The fungus survives as a vascular parasite in wheat and other gramineous hosts (5, 9), and as a saprophyte on decaying host residues (2, 6). In artificial culture it produces restricted, bacteria-like colonies, and its inherent slow growth has been largely responsible for the inability to isolate this organism directly from soil in which numerous faster-growing organisms abound.

The only real success at isolating *C. gramineum* has been to recover it from the host plants it invades or from host debris where it is already selectively entrapped (3). Because of the inability to directly detect the fungus in soil, we know little about its distribution in soil or its ability to survive apart from host debris. Furthermore, an ability to qualitatively and quantitatively detect the fungus in soil would permit an evaluation of its prevalence as influenced by climatic conditions and/or cultural practices and, perhaps, even allow advance estimates of disease incidence and crop loss. Since selective media have been successfully employed for these kinds of studies involving other soil-borne microorganisms (8), this study sought to develop a medium for the detection of *C. gramineum* in soil. Portions of this study were reported previously (7).

MATERIALS AND METHODS.—Attempts to search out a selective medium for *C. gramineum* involved an evaluation of numerous basal media, media amendments, and incubation techniques. Among the parameters tested were antibiotic amendments such as the cephalosporins, cephalosporin C, cephalexin monohydrate, cephaloridine, and sodium cephalothine (Eli Lilly & Co., Indianapolis). Antibiotic culture fractions from liquid shake cultures of *C. gramineum* were also tested for possible selective inhibition of noncephalosporium organisms. Media amended with plant alkaloids and fungicides (8) were evaluated as was media pH and the temperature of incubation.

Each medium was tested for: (i) the support of linear growth of *C. gramineum* from agar plugs; (ii) the development of colonies of *C. gramineum* from aliquots of sterile or natural soil amended with known quantities of *C. gramineum* conidia; and (iii) the development of *C. gramineum* colonies from naturally infested field soil. The soil used was a clay-loam type from a field at East Lansing that had been in continuous wheat culture for 3 yr and in which each year some 70% of the plants showed Cephalosporium stripe symptoms.

Certain media were further compared for support of conidial germination and subsequent colony development; i.e., for percent recovery of *C. gramineum* and/or of its sporodochial stage, *Hymenula cerealis* Ell. & Ev. (2). This test involved measuring the number of colonies developed on the medium as percent of the number of conidia applied. For this test, quantitative suspensions of conidia of *C. gramineum*, or of *H. cerealis* from potato-dextrose agar (PDA) or naturally infected wheat straw, respectively, prepared in sterile distilled water were used. Spore concentration was determined using a hemocytometer.

RESULTS AND DISCUSSION.—After evaluating numerous media and media amendments without success (nearly all the media tested, with or without antibiotics, failed to sufficiently inhibit the growth of a number of contaminant organisms in the soil) a medium was found which readily detected *C. gramineum* in soils amended with the fungus and in naturally infested field soil. The successful medium had a base of 2% Difco Bacto agar with only two essential amendments: copper sulfate and an extract of green wheat. The proper balance of the growth inhibitory effects of the copper sulfate, and the growth promotive effects of the leaf extract, was critical to the success of the medium.

The extract was prepared from 100 g of leaf blades harvested from 10- to 30-day-old wheat seedlings (*Triticum aestivum* L.) grown in composted soil. Extracts of the cultivars 'Genesee' or 'Ionia' were most frequently used, but a satisfactory medium was prepared from an extract of seedlings developed from a bulked seed line mixture. Likewise, extracts prepared from Genesee seedlings developed in greenhouses (23 ± 3 C) or in growth chambers [20 ± 1 C with 15,070 lx (1,400 ft-c) of mixed fluorescent

and incandescent light during alternate 12-hr periods] proved equally useful.

Leaves to be extracted were placed in 1 liter of distilled water, heated to boiling, and maintained at a medium boil with occasional stirring for 10 min. The hot, boiled mixture was poured (not squeezed) through two layers of cheesecloth and the liquid volume adjusted to 1 liter with distilled water. Such extracts were used fresh or stored at −10 C. At this writing, extracts proved useful after 6 months in cold storage.

Twenty grams of Difco Bacto agar were added to each liter of fresh or thawed extract and the resultant mixture autoclaved for 10 min at 121 C or merely heated sufficiently to melt the agar. Prior to solidification of the agar, $CuSO_4 \cdot 5H_2O$ was added in the amount of 0.8-1.2 g/liter depending on the age of the seedlings used for the extract (Fig. 1). The range of 0.8-1.2 g/liter was the range of optimum copper sulfate concentrations required to counter-balance the growth promotive effects of leaf extracts prepared from seedlings of different age. Copper sulfate concentrations in the green wheat agar (GWA) were effective within the range of 0.5-2.0 g/liter of medium. However, above 1.2 g/liter all microbial growth was markedly inhibited and above 2.0 g/liter all growth was eliminated. Likewise, copper sulfate concentrations below 0.8 g/liter permitted excessive growth of contaminant organisms, and below 0.5 g/liter contaminant growth was massive and *C. gramineum* was not distinguishable. Optional additions of 1-10 mg of pentachloronitrobenzene (PCNB)/liter were also made prior to agar solidification.

Fifteen to 20 ml of warm GWA were poured into 100 × 15-mm plastic petri plates. The plates were covered and allowed to stand for 5-7 days to permit evaporation of excess moisture and better facilitate the distribution of soil suspension on the medium surface. Plates not used at this time remained usable, if stored at room temperature in sealed plastic bags.

Soil tested for *C. gramineum* had a moisture level of 20%. It was coarse-screened, mixed thoroughly, and diluted 100- or 1,000-fold (w/w) with distilled water. Each GWA plate received a 1-ml aliquot of soil suspension and was rotated to uniformly distribute the suspension on the medium surface.

Plates thus inoculated were incubated on a laboratory bench at room temperature for periods up to 2 weeks. Isolated, characteristic colonies of *C. gramineum* developed within 3 days and after 4-7 days could be counted at low magnification with a stereo microscope. The *Cephalosporium* colonies were submerged and compact with prominent radiating arms (sometimes curved as in a pinwheel) covered with mounds of conidia (Fig. 2). These characters readily distinguished the *Cephalosporium* colonies from those of any contaminant organisms on the plates. Production of conidia was not as abundant on GWA as on noncopper media like PDA or PDAS (PDA + 50 mg streptomycin/liter). However, the conidia produced on GWA were characteristic of the *Cephalosporium* genus in terms of their size (2-4 × 4-10 μ) and their production in mucilagenous heads at the ends of inconspicuous conidiophores.

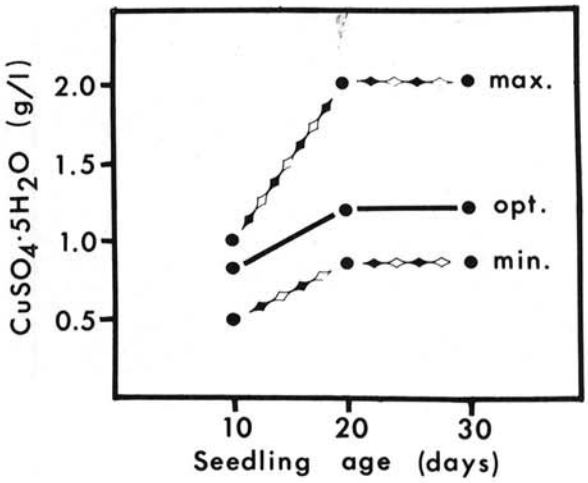

Fig. 1. The maximum, minimum, and optimum effective concentrations of copper sulfate to be added to leaf extracts prepared from 'Genesee' wheat seedlings of different ages.

Contaminant soil organisms that were inhibited but not eliminated on the GWA plates were identified as *Fusarium, Candida, Aureobasidium, Trichoderma, Verticillium, Penicillium* spp., and yeasts. One or more of these organisms were of consequence only in occasional soil samples and in such cases their growth was inhibited further by the addition of PCNB to the GWA (Fig. 3). The GWA also supported *Cephalosporium gregatum* Allington & Chamberlain, but this organism was not encountered in the wheat field soil.

In repeated tests, GWA inoculated with 1 ml of 10^{-3} dilutions of natural soil yielded up to 30 *Cephalosporium* colonies per plate indicating the presence of up to 30,000 propagules of this fungus per gram of soil. Conidia of such colonies recovered from soil were fully pathogenic when inoculated to wheat seedlings (9), and the colonies were

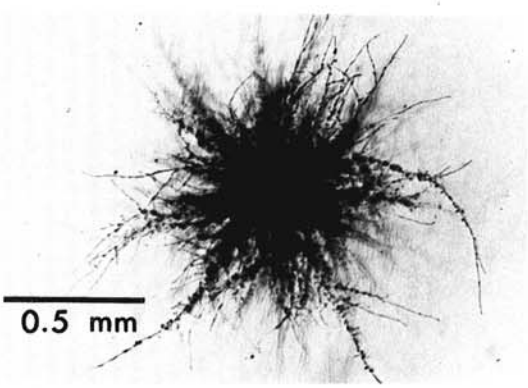

Fig. 2. Seven-day-old colony of *Cephalosporium gramineum* on GWA.

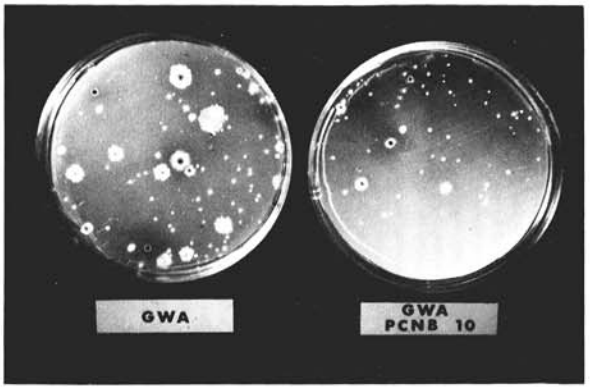

Fig. 3. Inhibitory effect of 10 mg of pentachloronitrobenzene (PCNB)/liter of green wheat agar (GWA) on the growth of contaminant fungi. Plates are shown 7 days after inoculation with 1 ml of a 10^{-3} suspension of wheat field soil. Small, white colonies on both plates are *Cephalosporium gramineum*.

indistinguishable from cultures obtained by conventional methods from infected plant parts.

To determine whether the detection of such high propagule numbers in soil might be contiguous with the profuse sporulation of the fungus (2, 9), a study of the nature of the propagules recovered from soil and of the efficiency of propagule recovery on GWA was undertaken. By searching out fungal germlings on GWA plates (newly inoculated with soil suspensions) and thereafter monitoring their development, it was possible to discern that the propagules in natural soil that gave rise to *C. gramineum* colonies were individual conidia. These conidia were similar to those produced on artificial media (1), in host plants (9), or in sporodochia of *H. cerealis* (2) in terms of morphology and color. They were at no time observed in association with soil particles or with organic debris.

To determine the number of propagules in soil in more absolute terms, the efficiency of the soil dilution and plating techniques was examined and GWA was compared to noncopper media for support of conidial germination and subsequent colony development. For these studies quantitative preparations of conidia of *C. gramineum* or *H. cerealis* from 7- to 10-day-old stock cultures on PDA or from sporodochia on moist, freshly collected wheat straw, respectively, were used. Germination was measured at intervals over a 5-day period on duplicate plates of media inoculated with 1 ml of a concentrated suspension of conidia in water (10^5 conidia/ml). Within 4-12 hr, germination of conidia of both fungi was initiated on all the media tested. Initially, the GWA appeared to reduce the germination rate compared to that on noncopper media, but after 36-48 hr all media supported equivalent germination percentages. By 48 hr, germination on GWA, PDAS, and GWA minus copper routinely ranged from 95-100% for conidia of both fungi.

To test the efficiency of the soil dilution and plating techniques, 2×10^5 conidia in concentrated aqueous suspension were added per gram fresh weight of soil to yield a fluid soil paste. The amended soil paste was thoroughly mixed, subsequently diluted 1,000-fold, and plated (1 ml/plate). The number of developing *C. gramineum* colonies was expressed as percent of the theoretical 200 propagules applied per plate (Table 1).

As with percent conidial germination, GWA supported propagule recovery, and subsequent colony development, as well as the nonselective media. Although recoveries varied somewhat from experiment to experiment, the recovery of *C. gramineum* generally exceeded that of *H. cerealis*, and, similarly, recovery from water or sterile soil suspensions normally exceeded that from natural soil suspensions. In a typical experiment (Table 1), the efficiency of recovery of conidia of *C. gramineum*

TABLE 1. Percent recovery[a] of *Cephalosporium gramineum* on different media inoculated with conidia of *C. gramineum* or *Hymenula cerealis* (the sporodochial stage of *C. gramineum*) suspended in water, sterile soil, or natural soil

		Media			
Conidia	Suspension	PDAS[e]	GWA[e]	GWA minus copper	GWA minus wheat extract
C. gramineum[b]	Water	103	81	94	0
	Sterile soil	110	103	98	0
	Natural soil	ND[d]	58	ND	0
H. cerealis[c]	Water	35	22	27	0
	Sterile soil	77	34	55	0
	Natural soil	ND	21	ND	0

[a] Percentages calculated from counts of developing colonies after 5 days growth on triplicate plates of each medium to which 1-ml aliquots of suspension equivalent to 200 conidia were applied.

[b] From 10-day-old stock cultures on PDA.

[c] From sporodochia on moist, freshly collected wheat straw.

[d] ND = no determination, because of excessive growth of contaminant microorganisms.

[e] PDAS = potato-dextrose agar + 50 mg streptomycin/liter; GWA = green wheat agar (the extract of 100 g of wheat seedling leaves, 0.8 - 1.2 g $CuSO_4 \cdot 5H_2O$, and 20 g of agar/liter of medium.

and *H. cerealis* from natural soil suspensions was 58 and 21%, respectively.

Although these quantitative tests helped substantiate that GWA compared to nonselective media offers no appreciable disadvantage to either conidial germination or subsequent colony development, they also revealed an inherent difference in the recoverability of conidia of *C. gramineum* and *H. cerealis* from dilute aqueous suspensions. While the explanation of this phenomenon is under investigation and beyond the scope of the present study, the difference may be inherent to the substrates on which the propagules are produced and hence to the nutritional status of the conidia of each morphological form of the fungus.

Since GWA readily supports the germination and subsequent colony development of both morphological forms of the wheat pathogen, and since both forms produce conidia abundantly (1, 2, 9), the recovery of several thousand propagules (conidia) per gram of infested soil is not unreasonable. In fact, numbers greater than 30,000/g might be expected when one considers (as our preliminary measurements have shown) that some 4 million conidia can be washed from a single sporodochium of *H. cerealis* and that hundreds of such sporodochia can be produced on the residue of one infected wheat plant. Furthermore, the system of GWA inoculated with 1 ml of 10^{-2} or 10^{-3} soil dilutions is best employed when propagule numbers are high; i.e., greater than 1,000/g of soil. However, this does not preclude the use of more concentrated soil suspensions to detect smaller numbers of *Cephalosporium* propagules. In such cases, excess soil could be washed from the medium after incubation to facilitate counting any *C. gramineum* colonies which would be held intact by virtue of being submerged in the medium. In this same light, quantitative measurements of propagule numbers may be further refined by putting soil suspensions through settling columns (4), a procedure that would preferentially eliminate soil debris and microbial propagules larger than those of *C. gramineum*.

The selectivity of GWA for *Cephalosporium* is based on the proper balance of its growth inhibitory factor, copper sulfate, and its growth promotive factor, wheat extract. The growth inhibitory effects of the copper sulfate become markedly evident when it is used in excess of the optimum concentration, 0.8-1.2 g/liter, or when used at optimum concentrations but in the absence of wheat extract (Table 1).

The system for the selective quantitative recovery of *C. gramineum* from soil described herein shows promise for use in detailed studies of *Cephalosporium* populations in soil. It may be possible also, to extend the usefulness of GWA to studies of *C. gregatum* in soybean field soil.

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