

Bioassay of Fungitoxic Compounds on Thin-Layer Chromatograms with *Pythium* and *Phytophthora* species

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ABSTRACT

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The bioautographic technique for detecting fungitoxic compounds on thin-layer chromatograms was modified for use with species of Phycomycetes by using *Phytophthora capsici*, *Phytophthora megasperma* var. *sojiae*, and *Pythium aphanidermatum* as examples. The modification makes possible the detection and assay of materials specifically fungitoxic to phycomycetes and may be of value in the demonstration of naturally occurring antifungal compounds in diseases caused by Phycomycetes. Methods are described for preparing suspensions of encysted zoospores and spraying these onto chromatograms in dilute V-8 juice agar. Fungitoxic

spots were visualized by immersing the plates in a suspension of charcoal in water. The charcoal adhered to the mycelium, but not to the smooth agar surface of the inhibition zones. The method was illustrated by bioassaying the fungicides metalaxyl, furalaxyl, and propamocarb, which are specific for Phycomycetes, and the phytoalexins, glyceollin and capsidiol. With *Phytophthora megasperma* var. *sojiae*, 25 ng of metalaxyl could be detected. *Pythium aphanidermatum*, but not the *Phytophthora* spp., was sensitive to propamocarb. Thus, even within the Phycomycetes, selection of appropriate species for bioassay may be important.

A bioautographic technique for detecting fungitoxic compounds on paper chromatograms was first described by Weltzien (10). This was modified by Dekhuijzen (2,3) and adapted subsequently for thin-layer chromatography by Peterson and Edgington (6) and Homans and Fuchs (5). In the latter form, thin-layer chromatograms are sprayed with a suspension of spores of the assay fungus in a nutrient medium and, after suitable incubation, fungitoxic spots are distinguished as zones of growth inhibition. The technique is simple, rapid, and sensitive and has been used in numerous studies for the detection of phytoalexins, fungicides, and their breakdown products (2-6).

A number of assay fungi have been used (2-6), but we have not seen any previous reports of the use of phycomycetes. There may be two reasons for this: zoospores are more fragile and less readily obtained than the conidia of the more commonly used fungi such as *Cladosporium* spp.; also, the mycelia of the phycomycetes are not pigmented and hence inhibition zones on chromatograms are difficult to discern. The choice of assay fungus may not be critical for broad spectrum fungitoxic compounds, but with the recent introduction of experimental and commercial fungicides such as the acylalanines, which are highly specific for phycomycetes (1,7), it is important that the technique be extended to include fungi from this group. In this paper we describe a procedure we now use routinely. The method is illustrated by assays of three fungicides and two phytoalexins against three species of phycomycetes.

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MATERIALS AND METHODS

Phytophthora capsici Leonian, *Phytophthora megasperma* Drechs. var. *sojiae* Hildeb. and *Pythium aphanidermatum* (Edson) Fitzp. were grown on V-8 juice agar plates at 25 C. Methods for zoospore production by *P. capsici* and *P. megasperma* var. *sojiae* have been described previously (8,9).

Yields from *P. capsici* were $>10^6$ zoospores per milliliter and two or three cultures were sufficient for each chromatogram. Yields from *P. megasperma* var. *sojiae* were lower, 1 to 3×10^5 zoospores per milliliter and four or five cultures were required. Zoospores were obtained from *P. aphanidermatum* by flooding 5- to 7-day-old cultures with sterile distilled water for 15-18 hr and then replacing the water at 30-min intervals until the release of zoospores began. Yields were usually $2.5-5 \times 10^5$ /ml and three or four cultures were used for each chromatogram.

Prior to being sprayed onto chromatograms, zoospores were encysted and then suspended in dilute V-8 juice agar as follows. V-8 juice was neutralized with CaCO₃ (1 g in 50 ml) clarified by centrifugation at 20,000 g for 15 min and filtration through Whatman No. 1 filter paper, mixed with 0.1 M CaCl₂ (1:10, v/v), and sterilized by autoclaving. Zoospores were encysted by adding 10 ml of this medium to 90 ml of zoospore suspension containing neomycin sulfate (25 µg/ml final concentration) and shaking the mixture on a rotary shaker at 100 rpm for 2-4 hr at room temperature. Encysted and germinated zoospores were collected by centrifuging the mixture at ~100 g for 3 min. They were resuspended in sterile distilled water containing 40 µg/ml neomycin sulfate; a volume of 12 ml was used for each chromatogram to be sprayed. To this, 8 ml of clarified V-8 juice containing 2% agar (v/v) and β-sitosterol (1 mg/100 ml, previously

autoclaved and equilibrated at 50 C) was added with continuous mixing on a Vortex mixer. Using a Grumbacher (airbrush-type) atomizer the entire mixture was sprayed immediately and as uniformly as possible onto one chromatographic plate, forming a layer approximately 0.5 mm thick. The chromatograms were incubated for 24–30 hr at 25 C in closed Pyrex trays lined with moist paper. Zones of inhibition were visualized by immersing the chromatograms in a 3–4% suspension of finely ground charcoal (Nuchar Chemical Div., West Virginia Pulp and Paper Co., New York, NY) in water, with gentle swirling to completely cover the surface. The chromatogram was drained and dried at room temperature.

Thin-layer chromatography was performed on silica gel (0.2 mm) precoated plastic sheets (20 × 20 cm) (Polygram, SIL NH-R, Machery-Nagel, Postfach 307, 5160 Düren, West Germany). Three solvent systems were used: A, ethyl acetate, *t*-butanol (95:5, v/v); B, benzene, methanol (95:8, v/v); C, chloroform, acetone, ammonium hydroxide (50:50:1, v/v). Plates were thoroughly air-dried to remove solvents before spraying with the inoculum mixture.

Metalaxyl (DL-methyl-*N*-[2,6-dimethylphenyl]-*N*-[2-methoxyacetyl]alaninate) and furalaxyl (DL-methyl-*N*-[2,6-dimethylphenyl]-*N*-[2-furoyl]alaninate) were analytical grade supplied by Ciba-Geigy Canada Ltd., Mississauga, Ont. Propamocarb (propyl-[3-(dimethylamino)propyl]carbamate monohydrochloride) was a commercial formulation (66.5% a.i.) obtained from Nor-Am Agricultural Products Inc., Naperville, IL 60098. Glyceollin and capsidiol were available from previous studies (8,9).

RESULTS AND DISCUSSION

Inhibition zones on chromatograms appeared as clear white spots against a black background, due to the retention of charcoal particles by the surrounding surface growth of mycelium (Fig. 1).

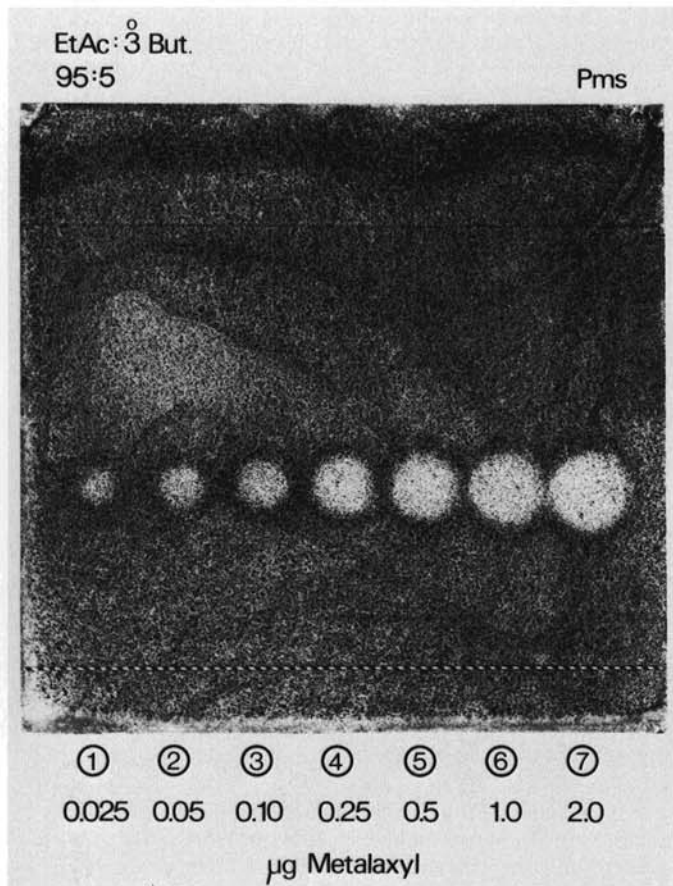


Fig. 1. Bioautograph of the systemic fungicide, metalaxyl with *Phytophthora megasperma* var. *sojae* (Pms) as the assay organism. Chromatography was on silica gel precoated plastic sheets (ethyl acetate:tertiary butanol, 95:5) and powdered charcoal was used to distinguish inhibition zones.

Charcoal did not adhere to the smooth agar surface either in spots where growth was inhibited or in control plates that did not receive inoculum. Without the charcoal, spots could not be distinguished clearly because of the lack of contrast between the thin layer of mycelium and the silica gel. Initial attempts to increase the contrast by using dyes were unsuccessful due either to similarities in staining of areas of growth and growth inhibition, or to damage to the chromatogram. Charcoal presumably could be used in bioassays to visualize unpigmented fungi other than phycomycetes. Clear inhibition zones were produced by each of the compounds against at least one of the test fungi, suggesting that the method may be extended to other species of phycomycetes. Provided the thin-layer plates were thoroughly dried after removal from the solvents, the assays were not affected adversely by any of the solvent systems used. It does not seem to be essential therefore to use only the more volatile solvent systems recommended by Homans and Fuchs (5). A further difference from their method was the inclusion of agar in the spray mixture as described by Peterson and Edgington (6). This prevents the problems alluded to by Homans and Fuchs (5) that occur when chromatograms become too wet during spraying. Thus, there is no tendency for the inoculum to run and streak over the chromatogram surface and hence uneven growth, dilution of water-soluble material and distortion of spots is avoided.

Metalaxyl (*R*_f: A, 0.41; B, 0.23; C, 0.60) and furalaxyl (*R*_f: A, 0.48) produced large inhibitory zones when bioassayed against *P. megasperma* var. *sojae* or *P. capsici* (Fig. 1). As little as 25 ng of metalaxyl and 50–100 ng of furalaxyl could be detected using *P. megasperma* var. *sojae*. Sensitivity of *P. capsici* was of the same order, but at the lowest concentrations, spot size was more variable. *Pythium aphanidermatum* was much less sensitive than the *Phytophthora* spp. to metalaxyl, 250–500 ng being required for distinct spots. Propamocarb, which remained at the origin in solvent systems A and B, and moved only slightly in solvent system C, was much less active than the acylalanines. Relatively, however, *P. aphanidermatum*, inhibited by 10–25 μg, was very much more sensitive than *P. megasperma* var. *sojae*, which was not inhibited by 400 μg. Specificity of this type together with the general selectivity of these compounds for phycomycetes, stresses the importance not only of extending the bioassay to the phycomycetes, but of choosing appropriate species for assay.

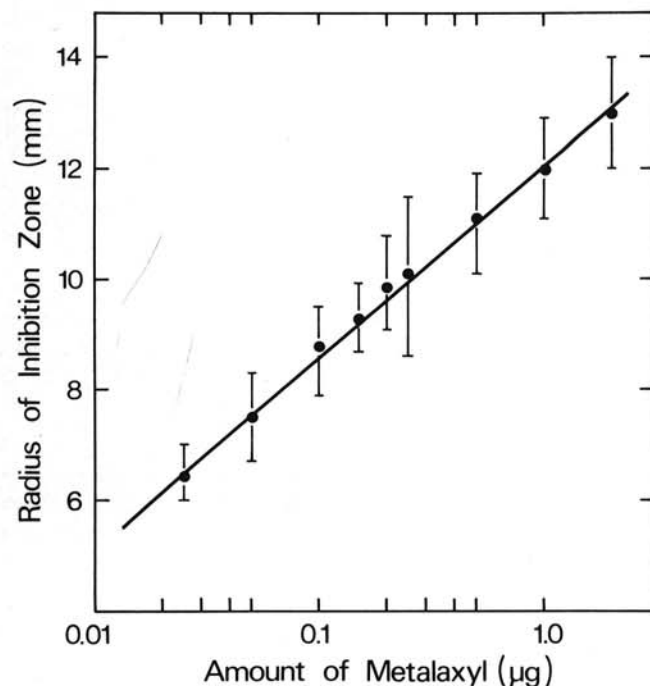


Fig. 2. Semilogarithmic relationship between radii of inhibitory zones obtained with *Phytophthora megasperma* var. *sojae* (Fig. 1) and amounts of metalaxyl applied. The standard deviations were estimated from at least four different measurements.

Both phytoalexins produced inhibitory zones in bioassays against *P. megasperma* var. *sojae*. Capsidiol produced large spots (25 mm diameter) with the lowest amount tested (10 μ g), whereas glyceollin produced relatively small spots even with 100 μ g. We have found the method very useful for bioassay of extracts of plant material for the detection of systemic fungicides and phytoalexins produced during infection.

Plots of the radii of inhibition zones against the logarithm of amount of compound applied gave a straight-line relationship (Fig. 2) in agreement with other reports (4,6). Considerable replication would be required, however, if the method were to be used for precise quantitative determination, for standard errors were appreciable. Incubation time and the size of the spot applied at the origin were eliminated as causes of the variation, which appeared to be due mainly to difficulties in measuring the radii of the inhibitory zones, which were diffuse at the edges.

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