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Novel Fungitoxicity Assays for Inhibition of Germination-Associated Adhesion of *Botrytis cinerea* and *Puccinia recondita* Spores

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***Botrytis cinerea* and *Puccinia recondita* spores adhere strongly to polystyrene microtiter plates coincident with germination. We developed assays for inhibition of spore adhesion in 96-well microtiter plates by using sulforhodamine B staining to quantify the adherent spores. In both organisms, fungicides that inhibited germination strongly inhibited spore adhesion, with 50% effective concentrations (EC_{50}s) comparable to those for inhibition of germination. In contrast, fungicides that acted after germination in *B. cinerea* inhibited spore adhesion to microtiter plates only at concentrations much higher than their EC_{50}s for inhibition of mycelial growth. Similarly, in *P. recondita* the ergosterol biosynthesis inhibitors myclobutanil and fenbuconazole acted after germination and did not inhibit spore adhesion. The assays provide a rapid, high-throughput alternative to traditional spore germination assays and may be applicable to other fungi.**

Various methods have been used to measure the sensitivity of filamentous fungi to antifungal compounds. Measurement of the inhibition of spore germination is one such technique. Since spore germination occurs very rapidly for many fungi, this approach frequently allows a fungitoxic effect to be measured within a few hours rather than one or more days, as is typical for methods based on inhibition of mycelial growth. A major disadvantage of spore germination tests for measuring fungitoxicity is the need for labor-intensive microscopic evaluation to assess germination. This problem has essentially precluded the use of germination assays in large-scale fungicide screening operations designed to discover new antifungal compounds.

Germination assays are also very useful for evaluating the mechanism of action of antifungal compounds. For many compounds, spore germination is the growth stage that is most sensitive to inhibition. For example, the strobilurin class of fungicides, which block electron transport at the cytochrome bc_1 complex of the mitochondrial electron transport chain, are extremely potent inhibitors of spore germination but much less active as inhibitors of mycelial growth (19). On the other hand, many fungicides have little or no effect on spore germination but strongly inhibit mycelial growth. Examples of fungicides that typically act after germination in filamentous fungi include antimicrotubule agents, e.g., carbendazim and *N*-phenylcarbamates, which inhibit nuclear division (22), and inhibitors of ergosterol biosynthesis (4, 20). Consequently, comparison of the potency of a compound as an inhibitor of germination with its activity in a mycelial growth assay can provide preliminary information on its mode of action.

Botrytis cinerea is the causal agent of grey mold disease on a variety of fruits, vegetables, and field crops (1). *Puccinia recondita* f. sp. *tritici*, the causal agent of wheat leaf rust, is one of the most economically important pathogens of wheat (24). Because it is an obligate pathogen, methods for measuring in vitro fungitoxicity based on inhibition of mycelial growth cannot be used. However, *P. recondita* uredospores will germinate in vitro, and spore germination tests have been used to measure fungitoxicity (2). In this report we describe novel assays for inhibition of spore germination in *B. cinerea* and *P. recondita*, which are based on the ability of the germinating spores to adhere to polystyrene microtiter plate wells and are amenable to high-throughput screening.

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MATERIALS AND METHODS

Fungicides. Technical-grade (unformulated) fungicides were used in all experiments. Chlorothalonil, folpet, carbendazim, and carboxin were obtained from Riedel de Haen, Hannover, Germany. Kresoxim-methyl, azoxystrobin, fluazinam, pyrimethanil, thifluzamide, fenbuconazole, and myclobutanil were synthesized at Rohm and Haas Company, Spring House Technical Center.

Preparation of *B. cinerea* spores. A grape isolate of *B. cinerea* (strain B123, obtained from P. Leroux, Institut National de la Recherche Agronomique, Paris, France) was grown at room temperature on potato dextrose agar (Difco Laboratories, Detroit, Mich.) under fluorescent lights in 9-cm diameter petri dishes for 10 to 14 days. Sterile water (20 ml) was added to each plate, and the surface was scraped gently with a sterile loop to release the spores. The resulting spore suspension was filtered through glass wool to remove any mycelial fragments and diluted with sterile water to the desired concentration.

Preparation of *P. recondita* uredospores. Wheat plants, cultivar 'Fielder', were inoculated with an aqueous mist of a suspension of uredospores at 10^5 spores per ml in distilled water. The inoculated plants were kept in a dew chamber for 24 h and then placed in a greenhouse for 20 to 30 days. Leaves with sporulating lesions were excised and transferred to a glass jar containing a 0.05% aqueous solution of Tween 20. The jar was shaken to dislodge the uredospores, which were then collected by filtering the liquid through cheesecloth into 50-ml polypropylene centrifuge tubes. The tubes were left undisturbed for 30 min at 4°C to let the spores settle, and then the supernatant was carefully removed with a pipette. The spores were resuspended in 0.05% Tween 20, and the suspension was adjusted to a density of 4×10^5 spores per ml.

Adhesion of *B. cinerea* spores to chamber slides. Chamber slides (two-well, Lab-Tek Permanox chamber slides, obtained from Nalge Nunc International, Naperville, Ill.) received 5 μl of the fungicide dissolved in dimethyl sulfoxide (DMSO) or DMSO alone (controls), immediately followed by the addition of 500 μl of Sabouraud dextrose broth (SDB) (Difco). Spore suspension (500 μl) at 2×10^5 spores per ml was added, the well contents were mixed gently, and the slides were incubated at 25°C for 0 to 8 h. To determine the number of unbound spores, the contents of each well were transferred to a 20-ml cell-counting vial. Each well was washed by adding 1 ml of Isoton II counting fluid (Coulter Electronics Limited, Luton, United Kingdom), mixing briefly, and adding the wash mixture to the vial. Additional counting fluid (14 ml) was added to each vial, which was capped and inverted gently five times before the spore concen-

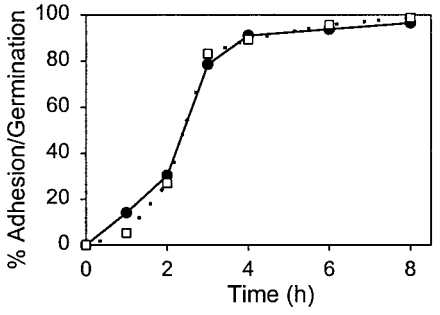

FIG. 1. Time course of adhesion (●) and germination (□) of *B. cinerea* spores in chamber slides. Values are means of two replicates.

tration was measured by counting in a Multisizer IIE cell counter (Coulter Electronics), using a sampling volume of 2 ml and a 4- to 15-μm aperture. To evaluate germination visually, spores were fixed in 5% glutaraldehyde by adding 77 μl of 70% glutaraldehyde to chamber slide wells. The percent germinated spores was determined by microscopic examination of 100 spores for evidence of germ tube emergence.

Microtiter plate *B. cinerea* spore adhesion assay. Stock solutions of fungicides were prepared in DMSO and diluted with SDB such that the DMSO concentration after dilution was ≤2%; then 1:1 dilutions were prepared in 96-well polystyrene microtiter plates (catalog number 9055; Dynex Technologies, Inc., Chantilly, Va.) by serial dilution in 100-μl aliquots of SDB. Spore suspension (100 μl at 2×10^5 spores per ml) was then added to the wells. Control wells that received 100 μl of SDB without fungicide and 100 μl of spore suspension, as well as controls that received 100 μl of SDB without fungicide and 100 μl of water instead of spore suspension, were included in each plate. DMSO did not affect spore adhesion or germination at the final concentrations used in the assay (≤1%). Plates were incubated in the dark at 25°C, and then the medium containing most of the unbound spores was removed by inverting the plate onto an adsorbent pad and forcefully tapping the plate sharply on the pad several times to remove as much liquid as possible.

Spore adhesion was quantified with the protein stain sulforhodamine B (SRB) by a procedure that is used to quantify adherent mammalian cells in cytotoxicity assays (21). Spores were fixed by the addition of 100 μl of 10% trichloroacetic acid and incubation at 4°C for 1 h. The trichloroacetic acid was removed, the wells were washed five times with water, and the plates were allowed to dry in a chemical hood. Residual unbound spores were removed during this fixation and washing step. If desired, at this stage the plates could be sealed with Parafilm and stored for up to 1 week. Spores were stained by the addition of 100 μl of a 0.4% solution of SRB in 175 mM acetic acid to each well and incubation for 30 min at room temperature. The stain was removed by aspiration, and the plates were washed four times with 175 mM acetic acid with a Maxline microtiter plate washer (Molecular Devices Corporation, Sunnyvale, Calif.), with a 2-s soak in 175 mM acetic acid between washes. After a final manual wash with a wash bottle containing 175 mM acetic acid to remove residual stain that could splash on the upper sides of the wells, the plates were air-dried at room temperature for 1 h and could be stored at 4°C for several days at this stage if desired. The protein-

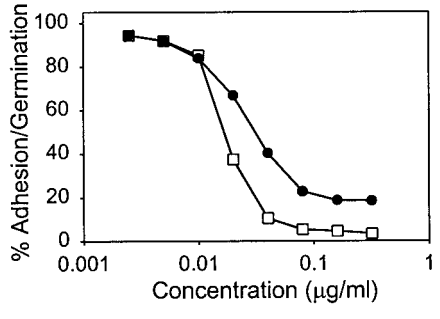

FIG. 2. Inhibition of *B. cinerea* spore adhesion (●) and germination (□) in chamber slides by kresoxim-methyl. Spores were treated with the fungicide for 5.5 h before evaluation of adhesion and germination. Values are means of two replicates.

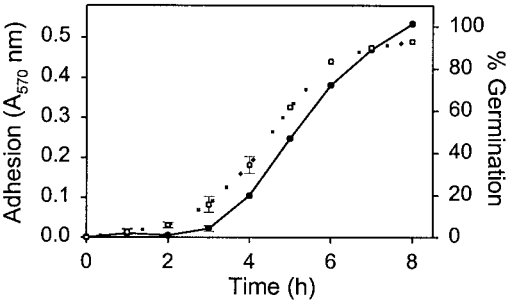

FIG. 3. Time course of adhesion (●) and germination (□) of *B. cinerea* spores in microtiter plates. Adhesion was quantified by SRB staining of adherent germlings. Values are the means of four replicates ± standard deviations. Error bars are not shown for standard deviations that are less than 10% of the value of the point.

bound dye was extracted into the medium by adding 200 μl of 10 mM unbuffered Tris base (pH 10.1) to each well and shaking the plates for 10 min with a gyratory shaker at 250 rpm. The resulting pink color in the wells was quantified by measuring the absorbance at 570 nm with an HTS 7000 Plus plate reader (Perkin Elmer Corporation, Norwalk, Conn.). Inhibition of adhesion was determined by comparing the absorbance values in wells containing the fungicide with the absorbance in control wells containing spores without fungicide, and 50% effective concentrations (EC_{50}s) were determined from dose-response curves.

Microtiter plate *P. recondita* spore adhesion assay. Stock solutions of fungicides were prepared in DMSO and diluted with 0.05% Tween 20 such that the DMSO concentration after dilution was 1%, and then 1:1 dilutions were prepared in 96-well polystyrene microtiter plates by serial dilution in 50-μl aliquots of 0.05% Tween 20. Spore suspension (50 μl at 4×10^5 spores per ml) was then added to the wells. Control wells without fungicide and controls lacking both fungicide and spores were included in each plate. DMSO did not affect spore adhesion at the final concentrations used in the assay (≤0.5%). The plates were left undisturbed for 15 min to allow the spores to settle to the bottom of the wells and then shaken at 100 rpm on a gyratory shaker at 19°C. Unbound spores were removed, and the bound spores were quantified by SRB staining, as described above for *B. cinerea*.

***P. recondita* germination assay.** To evaluate effects of fungicides on germination, spores were incubated for 4 h with fungicides in microtiter plates as described above. The well contents were fixed by adding 20 μl of 30% glutaraldehyde to each well, and the percentage of germinated spores was determined by microscopic examination of spores (100 spores per well in four replicate wells) for evidence of germ tube emergence. EC_{50}s for inhibition of germination were determined from dose-response curves.

Poison agar assay for mycelial growth of *B. cinerea*. Fungicides were dissolved in DMSO at 3.2 mg/ml and diluted in DMSO to give a series of 1:3 dilutions. Then 125 μl of each dilution was added to 25-ml aliquots of molten malt extract agar (20 g of malt extract, 20 g of glucose, 1 g of peptone, and 20 g of agar per liter) at 50°C, which were poured immediately into 9-cm-diameter petri dishes. Two replicate plates were used for each treatment. A mycelial plug (7 mm in diameter) cut from the growing edge of a *B. cinerea* culture on PDA was transferred to each plate. Colony diameters were measured after growth for 3 days in the dark at 25°C, and EC_{50}s were determined from dose-response curves.

RESULTS

***B. cinerea* spore adhesion.** *B. cinerea* spores adhered firmly to plastic chamber slides in a time-dependent manner, with >90% of spores adhering after incubation for 4 h. The timing of spore germination corresponded with that of adhesion (Fig. 1). The strobilurin fungicide kresoxim-methyl, which is a potent inhibitor of spore germination, inhibited spore adhesion in a dose-dependent manner, which was quite similar to its dose-response curve for inhibition of germination (Fig. 2). Spore germination took slightly longer in microtiter plates than in chamber slides, but the timing of spore adhesion, as quantified by SRB staining, corresponded to germination (Fig. 3). Also,

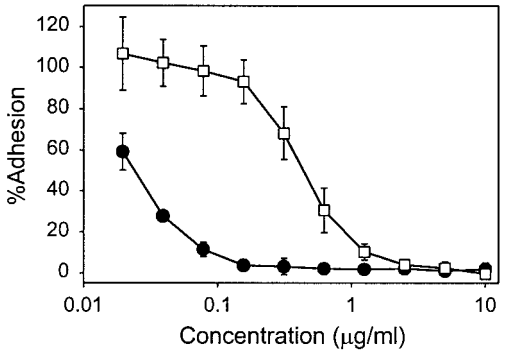

FIG. 4. Inhibition of *B. cinerea* spore adhesion in 96-well microtiter plates by kresoxim-methyl (●) and azoxystrobin (□) as measured by SRB staining of adherent germlings. Spores were treated with the fungicide for 5.5 h before evaluation of adhesion. Values are the means of four replicates ± standard deviations. Error bars are not shown for standard deviations that are less than 10% of the value of the point.

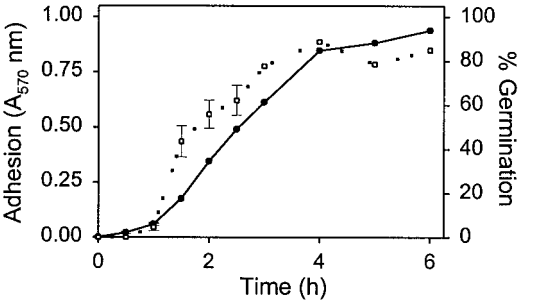

FIG. 5. Time course of adhesion (●) and germination (□) of *P. recondita* spores in microtiter plates. Adhesion was quantified by SRB staining of adherent germlings. Values are the means of four replicates ± standard deviations. Error bars are not shown for standard deviations that are less than 10% of the value of the point.

similar to the results from chamber slide experiments, spore adhesion in microtiter plates was strongly inhibited by strobilurin fungicides (Fig. 4).

We evaluated the spore adhesion assay as an alternative to a traditional germination test for measuring antifungal activity with various commercial fungicides. We compared these compounds with respect to their ability to inhibit spore adhesion in both chamber slides and microtiter plates, their potency in a standard germination assay in chamber slides, and their activity in a poison agar assay for mycelial growth (Table 1). For each compound tested, EC_{50}s from the adhesion assays were similar. All of the compounds that inhibited spore adhesion also were potent inhibitors of germination, and EC_{50}s for inhibition of germination were comparable to those for inhibition of spore adhesion. Carbendazim and pyrimethanil had little or no effect on germination but were potent inhibitors of mycelial growth. Neither of these compounds inhibited spore adhesion to chamber slides, and they inhibited adhesion to microtiter plates at concentrations much higher than their EC_{50}s for inhibition of mycelial growth.

***P. recondita* spore adhesion.** The microtiter plate spore adhesion assay developed for *B. cinerea* could also be used for *P. recondita* with slight modifications. Spores germinated equally well in Czapek Dox broth and in water containing 0.05% Tween 20, and the latter was chosen for our studies since it reduced clumping of the spores. The plates were shaken on a gyratory shaker during germination, since this increased the percentage of germinated spores. After the spores had been added to the microtiter plates it was beneficial to leave the plates undisturbed for 15 min to allow the spores to settle to the bottom of the wells before they were placed on the shaker. When this step was omitted, some clumps of germinated spores formed that were lost from the wells during subsequent washing steps.

In time course experiments, >80% of spores had germinated by 4 h and longer incubation did not increase the number of germinated spores. The maximum length of germ tubes after 4 h was approximately seven times the spore diameter, and lengths did not increase with longer incubation. The time course for spore adhesion as measured by SRB staining paralleled that of germination (Fig. 5), and microscopic examination of the adherent spores showed that all had germinated.

As in the case of *B. cinerea*, adhesion of *P. recondita* spores was potently inhibited by the strobilurin kresoxim-methyl (Fig. 6). Commercial fungicides effective against wheat leaf rust were compared with respect to their ability to inhibit *P. recondita* spore adhesion in microtiter plates and their ability to inhibit germination (Table 2). The compounds which inhibited spore adhesion (kresoxim-methyl, thifluzamide, carboxin, fluazinam, and chlorothalonil) were also potent inhibitors of germination, and EC_{50}s for inhibition of germination were comparable to those for inhibition of spore adhesion. In contrast,

TABLE 1. Inhibition of *B. cinerea* spore adhesion, germination, and mycelial growth by various fungicides

Compound	EC_{50} (μg/ml) for inhibition of[a]:			
	Adhesion to 96-well plates	Adhesion to chamber slides	Germination	Mycelial growth
Kresoxim-methyl	0.023 ± 0.003	0.035	0.02	0.28
Azoxystrobin	0.44 ± 0.10	0.28	0.32	3.1
Chlorothalonil	0.043 ± 0.003	0.089	0.067	0.35
Folpet	0.19 ± 0.02	0.22	0.15	5.5
Fluazinam	0.026 ± 0.004	0.071	0.028	0.033
Carbendazim	6.8 ± 1.4	>25	>25	0.045
Pyrimethanil	6.3 ± 0.36	>25	8.6	0.48

[a] Spores were treated with the fungicide for 5.5 h before evaluation of adhesion or germination. Values for inhibition of adhesion to 96-well plates are means of four replicate assays ± standard deviations. Values for inhibition of adhesion to chamber slides and inhibition of germination are means of two replicate assays. Inhibition of mycelial growth was evaluated by measuring the increase in colony diameter after growth for 3 days. Values are means of two replicate assays.

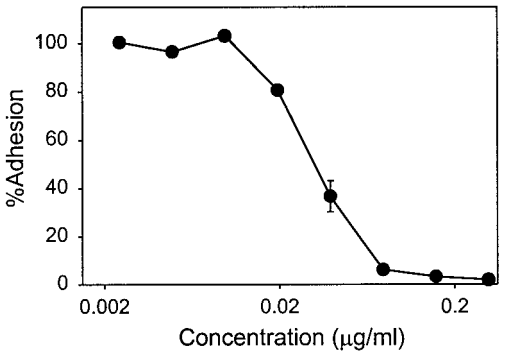

FIG. 6. Inhibition of *P. recondita* spore adhesion in 96-well microtiter plates by kresoxim-methyl as measured by SRB staining of adherent germlings. Spores were treated with the fungicide for 4 h before evaluation of adhesion. Values are the means of four replicates ± standard deviations. Error bars are not shown for standard deviations that are less than 10% of the value of the point.

fenbuconazole and myclobutanil did not inhibit germination or adhesion.

DISCUSSION

The ability of fungi to adhere to leaves and other substrata is well documented and is thought to represent an important early event in plant-microbe interactions (3, 13). The adhesion of spores and germlings is commonly associated with the production of an extracellular matrix (5, 6, 8, 14, 17, 18, 23). There are two distinct stages of adhesion for *B. cinerea* (9, 10). The first stage, immediate adhesion, occurs upon spore hydration (9) and involves hydrophobic forces that are easily disrupted. The second stage, delayed adhesion, involves secretion of an extracellular matrix containing carbohydrate and protein and is coincident with germination (10). In the present study, we found that the timing of *Botrytis* spore adhesion paralleled that of germination (Fig. 1 and 3), suggesting that the adhesion process measured by our assay corresponds to delayed adhesion (10). We did not detect the earlier stage of spore adhesion (9), presumably because the forces involved in immediate adhesion were too weak to withstand the washing procedures we employed.

As in the case of *Botrytis*, the timing of *P. recondita* spore adhesion to polystyrene corresponded to that of germination.

TABLE 2. Inhibition of *P. recondita* spore adhesion and germination by various fungicides

Compound	EC_{50} (μg/ml) for inhibition of[a]:	
	Adhesion	Germination
Kresoxim-methyl	0.032 ± 0.002	0.057 ± 0.001
Thifluzamide	0.087 ± 0.010	0.11 ± 0.01
Carboxin	0.13 ± 0.02	0.12 ± 0.01
Fluazinam	0.50 ± 0.01	0.52 ± 0.04
Chlorothalonil	0.89 ± 0.13	0.76 ± 0.05
Myclobutanil	>10	>10
Fenbuconazole	>10	>10

[a] Spores were treated with the fungicide for 4 h in 96-well plates before evaluation of adhesion or germination. Values are means of four replicate assays ± standard deviations.

To our knowledge, there are no previous reports of adhesion involving *P. recondita*. However, the involvement of an extracellular matrix or adhesion pad has been observed in adhesion of the related common maize rust fungus *Puccinia sorghi* (5) and the bean rust fungus *Uromyces viciae-fabae* (6, 8, 14, 23), suggesting that a similar mechanism may be responsible for adhesion in the present study.

Whether a fungicide inhibits germination or acts after germination is determined by its mechanism of action and whether the affected cellular process is required for germination. The compounds that were potent inhibitors of germination-associated spore adhesion in our study either are multisite inhibitors, e.g., chlorothalonil and folpet (7), or interfere with respiration, e.g., kresoxim-methyl, azoxystrobin, fluazinam, thifluzamide, and carboxin (7, 12, 19). The lack of inhibition of *B. cinerea* spore germination and adhesion by carbendazim and pyrimethanil shows that these fungicides affect processes that are not essential for germination. Similarly, the lack of inhibition of *P. recondita* spore adhesion by myclobutanil and fenbuconazole, which are highly active against wheat leaf rust (11, 16), is consistent with the fact that such inhibitors of ergosterol biosynthesis typically act after germination (4, 20).

The ability of fungicides to inhibit germination can provide information about potency and the potential mechanism(s) of action. Traditional germination assays, in which germination is evaluated by microscopic observations, are too labor-intensive to be used in screening large compound libraries for discovery of new antifungal agents. While computer-aided image analysis has been used as an alternative to visual evaluation of spores (15), it requires sophisticated equipment and has not been widely applied. In contrast, our spore adhesion assays provide a simple, rapid, and efficient means to evaluate inhibition of spore germination within a few hours and are amenable to high-throughput screening. We have also performed the *B. cinerea* assay in 384-well plates (results not shown). Because the assays are rapid, they can detect fungitoxicity of inhibitors that are susceptible to inactivation as a result of chemical instability or fungal metabolism more readily than assays based on the inhibition of mycelial growth, which typically involve treatment of the fungus for one or more days. We think that our assays for *B. cinerea* and *P. recondita* will be applicable to other filamentous fungi provided that appropriate conditions for spore germination are used. In preliminary experiments, we have also found a temporal correlation between germination and spore adhesion to polystyrene for *Colletotrichum lagenarium*, the causal agent of cucumber anthracnose (R. A. Slawecki and D. H. Young, unpublished data).

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