# Isolation, Purification, and Biological Activity of a Self-Inhibitor from Conidia of *Colletotrichum gloeosporioides*

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## ABSTRACT

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Low concentrations of conidia ($10^4$/ml) of *Colletotrichum gloeosporioides* f. sp. *jussiaea* germinated readily, but high concentrations of conidia ($10^7$/ml) in water or on water agar germinated poorly. Aqueous extracts from high concentrations of conidia inhibited germination of dilute concentrations of conidia. An inhibitor was extracted from these extracts with chloroform. Thin-layer chromatography of the chloroform extracts gave a single spot of inhibition in assays on the chromatograms. Chloroform extracts were taken to dryness, and the inhibitor was crystallized from hexane as fine colorless needles, m. p. 108–110 C. Germination of conidia was reduced by approximately 50% at 2 μg of inhibitor per milliliter. Dilutions of the purified inhibitor or conidial exudates gave germination percentages that paralleled those of dilutions of conidia. Chemical analysis revealed a molecular formula of $C_{18}H_{30}O_5$ and a molecular structure quite different from other known germination self-inhibitors.

*Additional key words*: gloeosporone, dihydro-5-hydroxy-5-(8-pentyl-2-oxocanyl)-acetyl)-2(3H)-furanone

Spores of many fungi germinate readily when dispersed, but poorly or not at all under crowded conditions (3,8). This phenomenon, which is called self-inhibition or autoinhibition, occurs commonly enough among fungi to be considered a general rule and is thought to be an ecological adaptation that ensures spatial and temporal distribution of the species (3,8,10,29).

Spores of several fungi have been found to contain endogenous self-inhibitors, water-soluble organic compounds that leach from the spores and inhibit germination of other spores (1,3,4,6,17,19). Self-inhibitors have been isolated and characterized from such diverse genera as *Dictyostelium* and *Puccinia* (2,3,23). The most studied cases of self-inhibition involve the rust fungi, in which various derivatives of cinnamic acid have been demonstrated to be responsible for self-inhibition. Deuteromycetes have also been shown to contain endogenous self-inhibitors (3). There have been numerous reports of self-inhibition of *Colletotrichum* conidia (9,12–16,27). To date, however, specific metabolites that fit the requirements of self-inhibitors have not been isolated from *Colletotrichum*. Lingappa and Lingappa (12–16) reported detection of putative "alkaloids or alkaloid-like substances" with germination-inhibiting activity from basic fractions of conidial and mycelial extracts of *Glomerella* (= *C. gloeosporioides*), and they also found activity in several crude nonbasic fractions but did not obtain or identify any pure inhibitor from these extracts.

In this paper we report the purification and biological activity of a self-inhibitor from conidia of *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *jussiaea*.

## MATERIALS AND METHODS

An isolate of *C. gloeosporioides* f. sp. *jussiaea* was obtained from leaf lesions of winged waterprimrose (*Jussiaea decurrens* L.) collected at Stuttgart, AR. A monoconidial subculture was selected and maintained on Torula yeast agar (TYA) composed as follows: Torula yeast, 15 g; $K_2HPO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 20 g; and water, 1 L.

Pathogenicity of this isolate was demonstrated, then stock cultures were maintained in soil tubes at 4 C (31). The fungus was transferred weekly on plates of TYA, incubated at 28 C, and periodically renewed from stock cultures.

Liquid cultures used to produce spores for subsequent work were grown in 500-ml Erlenmeyer flasks containing 250 ml of Richard's V-8 solution as described by Boyette (7). Flasks were inoculated with a single plug cut from the leading edge of actively growing colonies on TYA and incubated at 28 C for 7 days on a rotary shaker at 200 rpm.

Conidia for bioassays or inhibitor production were harvested from 7-day-old liquid cultures through eight layers of cheesecloth and concentrated by centrifuguation at 1,050 *g* for 10 min at 20 C. They were washed three times by resuspension and centrifugation in 500, 250, and 250 ml of sterile distilled water. Concentrations of spore suspensions were adjusted densimetrically to $10^7$ spores per milliliter and serially diluted to appropriate concentrations for biological assay.

**Biological assay.** To assess germination of conidia at various concentrations, spore suspensions were diluted and plated on 2% water agar in 60-mm-diameter plastic petri plates. Triplicate plates were inoculated with 0.2 ml of spore suspension containing either $10^7$, $10^6$, $10^5$, or $10^4$ conidia per milliliter. Plates were incubated at 28 C for 4.5 hr, and germination was determined at ×100 magnification. Spores were considered germinated when germ tubes were longer than the width of the spore (12,20). Two hundred spores from random fields were counted per plate.

The time required for maximum germination of conidia was determined by incubation of conidia on plates of 2% water agar at 28 C for 1, 2, 3, 4, 10, and 20 hr. Germination of three replicates was determined for each time period.

The germination of freshly harvested conidia incubated in cell-free exudates from germinating conidia was assayed. Washed conidia ($10^7$/ml) were dispersed in 0.6-ml aliquots on 2% water agar in 9.0-cm-diameter petri plates. Control plates received sterile distilled water. Plates were incubated for 20 hr at 28 C in

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polyethylene bags, and the agar surface was rinsed three times with 3.0-ml aliquots of sterile distilled water. Rinses were combined in a capped 150-ml centrifuge bottle, and conidia and mycelial fragments were removed by centrifugation at 8,000 *g* for 20 min at 4 C. Supernatants were assayed with freshly harvested conidia from 7-day-old cultures. Conidia were added (to a final concentration of $10^5$ conidia per milliliter) to 10-fold serial dilutions of each of the supernatants. Sterile distilled water and rinses from uninoculated plates served as controls.

In some experiments, exudates were filtered through a 1.2 $\mu$m sterilizing membrane prior to bioassay, the pH of the conidial exudates and control rinses was determined, and conidial exudates were aerated by bubbling compressed air through them for 1 hr prior to bioassay.

Inhibitory exudates from conidia were extracted with chloroform and bioassayed. Conidial exudates or agar rinses (10 ml each) were extracted three times with 3-ml portions of spectral-grade chloroform. The chloroform was removed and evaporated to dryness under a stream of air. Before the extracted exudates or agar rinses were bioassayed, nitrogen was bubbled through them until no odor of chloroform could be detected and then for another 30 min.

**Purification of inhibitory fraction(s).** Thin-layer chromatography was used to aid identification of inhibitory material(s) in the conidial exudates. Chloroform extracts from 100 ml of exudate were redissolved in 0.5 ml of chloroform and spotted onto a 250 $\mu$m layer of Silica Gel $PF_{254}$ (EM Reagents; Brinkmann Instruments, Westbury, NY 11590). Plates were developed for 10 cm in chloroform/diethylamine (9:1, v/v, tank saturation) and air dried, and spots were detected by UV light, iodine vapor, or phosphomolybdic acid reagent (25). Biologically active compounds in the chloroform extracts were located by biological assay on the chromatograms as follows: air-dried plates were further dried overnight at 80 C in a mechanical convection oven, cooled to room temperature, sprayed with a suspension of conidia ($10^5$/ml) until uniformly moist, and incubated at 28 C for 18 hr in polyethylene bags lined with wet paper towels. Incubated plates were sprayed with cotton blue in lactophenol and germination was assessed by microscopic observation at ×100. Plates spotted with either chloroform extracts or chloroform, but not developed in solvent, were similarly assayed.

Isolation of an inhibitory compound located on thin-layer chromatograms was accomplished by crystallization of chloroform-extracted materials from *n*-hexane. Spore exudates were prepared by incubation of $10^7$ conidia per milliliter in 250 ml of 0.22 M dextrose in 500-ml Erlenmeyer flasks for 16 hr at 28 C on a rotary shaker at 200 rpm. Germlings were removed by filtration through eight layers of cheesecloth and ungerminated conidia were removed by centrifugation at 2,000 *g* for 20 min at 20 C. Supernatants were taken to dryness in vacuo at 40 C. The dried residue was extracted three times with 15-ml portions of chloroform. Extracts were combined and evaporated to dryness under a stream of compressed air at room temperature, and the residue was taken up in a minimal volume of hot (64 C) hexane. Insoluble material was removed by centrifugation at 400 *g*. Crystals obtained after cooling were concentrated by centrifugation, recrystallized from *n*-hexane, and subjected to thin-layer chromatography as above. To determine if the compound was pure, additional solvent systems were employed: chloroform/methanol (99:1, v/v) and chloroform alone (plates prepared with 1N NaOH rather than $H_2O$) (25). Spots were visualized as before. The melting point of the crystals was determined on glass coverslips with a Fisher-Johns melting point apparatus and corrected for stem exposure.

**Biological assay with purified inhibitor.** A 1.0-mg sample of crystals dissolved in 1.0 ml of 95% ethanol at 50 C was added to 250 ml of sterile distilled water at 50 C. A 0.4% solution of ethanol in water served as control. Freshly harvested conidia from 7-day-old cultures were added (to a final concentration of 105 conidia per milliliter) to 10-fold serial dilutions of inhibitor and 4.0 ml was dispersed over the surface of sterile plastic petri plates (60 × 20 mm). Inoculated plates were incubated at 28 C for 4.5 hr, then lactophenol cotton blue was added to terminate germination and aid assessment at ×100 magnification.

## RESULTS

There was an inverse relationship between conidial concentration and germination percentage. Germination of $10^4$ conidia per milliliter plated on water agar was over 75% but that of $10^7$ conidia per milliliter was less than 20% (Fig. 1).

Reduction of spore germination in crowded conditions was duplicated by adding conidia to cell-free exudates from conidia germinated on water agar. Germination was significantly reduced by spore exudates to an average of 17% compared with 80 and 82% for those germinated in agar rinses or water, respectively ($P > 0.05$). Spore exudates had pH values ranging from 6.3 to 6.8, the same as those of agar rinses or water. Aeration of exudates did not reduce inhibition.

Extraction of exudates with chloroform removed inhibition. Germination of conidia in exudates after chloroform extraction was equal to that in unextracted controls. Extraction of the agar rinses with chloroform was without effect (Table 1).

**Purification of inhibitory fraction(s).** A chromatogram of chloroform-soluble material from spore exudates is shown in Fig. 2. Five major spots were detectable when visualized with shortwave UV light and/or spray reagents after development in chloroform/diethylamine (9:1, v/v).

TABLE 1. Effect of chloroform extraction on the inhibition of germination by aqueous spore exudates[a]

| | Spore germination (%) in: | | | | |
|---|---|---|---|---|---|
| | | Agar rinse | | Spore exudate | |
| Experiment | $H_2O$ | Before extraction | After extraction | Before extraction | After extraction |
| I | 78[b] | 78 | 79 | 10 | 75 |
| II | 93 | 94 | 94 | 32 | 94 |
| III | 87 | 86 | 86 | 40 | 76 |
| Mean | 86 A | 86 A | 86 A | 28 B | 81 A |

[a]Means followed by the same letter are not significantly different as determined by a Duncan's new multiple range test ($P = 0.05$).
[b]Average of three replications.

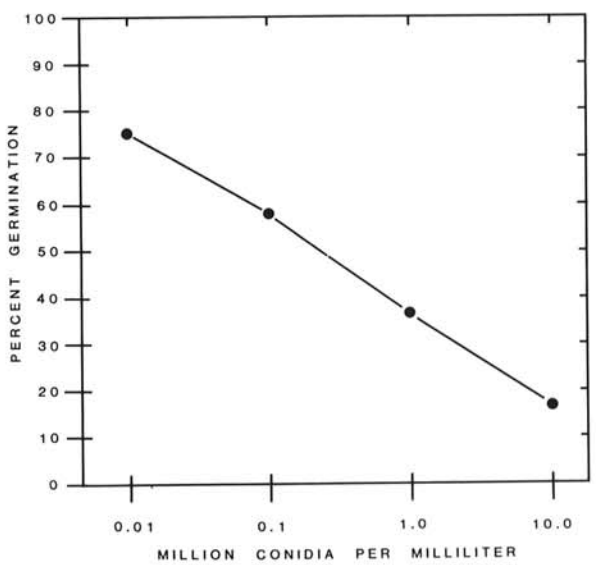


**Fig. 1.** Relationship between spore concentration and percent germination. Conidia were incubated on 2% water agar at 28 C for 4.5 hr. Each point represents the average of 600 conidia, 200 per replicate.

Germination (or inhibition) of conidia on thin-layer plates was detectable by microscopic examination after spraying the plates with lactophenol cotton blue. Chromatographed exudate extracts inhibited germination only in a spot centered at $R_f = 0.6$. No inhibition was noted at any position along the track spotted with chloroform (control) or in the areas between tracks. Chloroform spotted on a silica gel plate (but not chromatographically developed) did not affect germination compared to an unspotted area, whereas germination was inhibited in areas that had received aliquots of chloroform extracts of conidial exudates.

Crystallization of a material with an $R_f = 0.6$ was accomplished by dissolving chloroform extracts in boiling hexane and allowing the solution to cool to room temperature. Fine colorless needles that crystallized were collected by centrifugation, washed successively with hexane, and recrystallized. They melted at 108-110 C (corrected). The crystals were slightly soluble in water, but soluble in numerous organic solvents. Successive recrystallizations yielded a product that chromatographed as a single spot on thin-layer chromatography in the three solvent systems described. The purified fraction is compared chromatographically to the initial extract in Fig. 2.

Approximately 5 mg of chloroform-soluble material was obtained per liter of supernatant extracted. Production of purified inhibitory crystals from solution followed the scheme shown in Fig. 3, with a yield of approximately 2 mg of fine colorless needles per liter of supernatant.

**Biological assay with purified inhibitor.** Biological assay of the purified crystals at a concentration of 4 mg/L in 0.4% ethanol reduced germination of conidia to 2% compared to 50% in 0.4% ethanol after 4.5 hr of incubation. The few conidia that did germinate in inhibitor solutions appeared to germinate normally.

In Fig. 4, the percent germination of conidia in dilutions of purified inhibitor is compared with germination in dilutions of exudates or conidia. Inhibition was reduced with increasing dilution in all cases. The amount of pure inhibitor required to reduce germination to 50% of controls was approximately 2 $\mu$g/ml. The slopes of the regression lines were not significantly different.

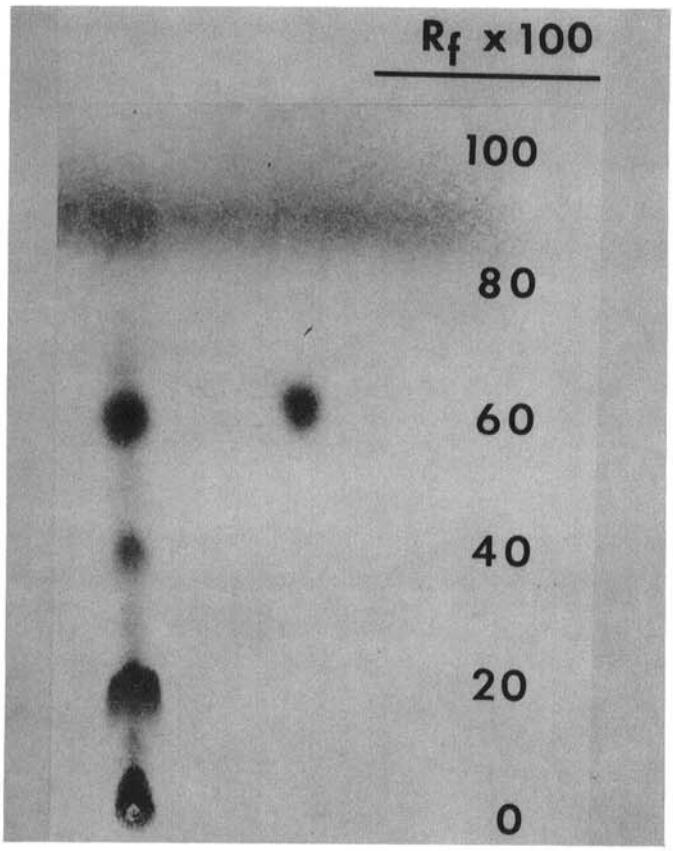


**Fig. 2.** Thin-layer chromatogram comparing chloroform extracts of spore exudates of *Colletotrichum gloeosporioides* f. sp. *jussiaea* with purified spore inhibitor. Compounds were detected with phosphomolybdic acid reagent after development in chloroform/diethylamine (9:1, v/v). Track A, chloroform extract; Track B, inhibitor crystallized from hexane.

## DISCUSSION

The germination of conidia of *C. gloeosporioides* f. sp. *jussiaea* is reduced by crowding. This behavior is consistent with that of many fungi, including species of *Colletotrichum* (9,12–16,20,27,32). Aqueous exudates from dense conidial suspensions of *C. gloeosporioides* mimic the effect of crowding when applied to dilute suspensions of conidia. This property parallels that found for uredospores of the rust fungi in which endogenous self-inhibitors leach from the spores and block germination en masse (1–3,6, 18,22). These results indicate that an endogenous self-inhibitor from conidia of *C. gloeosporioides* is responsible for reduced germination under crowded conditions.

The finding that extensively washed conidia germinate readily when dispersed in dilute concentration in sterile deionized water indicates that conidia are nutritionally self-sufficient. Inhibition by exudates from dense concentrations of conidia was not due to differences in pH, or to volatile materials that could be removed by aeration.

We isolated the inhibitor from aqueous solution, and not by direct organic solvent extraction of tissue as was reported with *G. cingulata* (14). Our mild extraction procedure circumvents Allen's criticism that with direct organic solvent extraction of fungi, extraction artifacts may be responsible for germination inhibition (3).

We have isolated from chloroform extracts a highly purified, crystalline compound (single spot in three chromatographic systems and a narrow melting-point range) that inhibits germination of uncrowded conidia. The dilution/activity profile of this purified crystalline material parallels that of conidial exudates and of dilutions of conidia themselves. This is evidence that the purified compound is responsible, either solely or primarily, for inhibition caused in dense concentrations of conidia by conidial exudates (24,26). It also fulfills the requirement, set forth by Allen for designation of a compound as a self-inhibitor, that concentration/activity relationships be similar to those expected in vivo (3).

The purified inhibitor from *C. gloeosporioides* inhibited germination by 50% at 2 mg/L. Two milligrams of purified

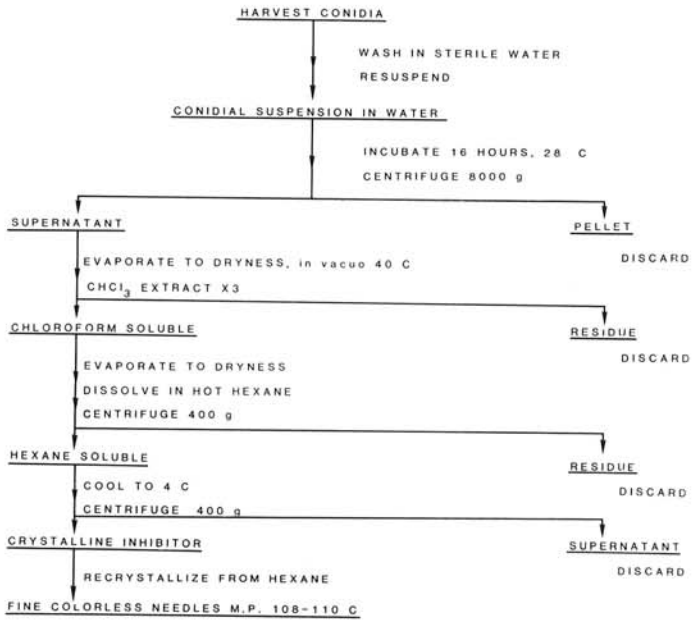


**Fig. 3.** Scheme for purification of crystalline spore germination inhibitor from *Colletotrichum gloeosporioides* f. sp. *jussiaea*.

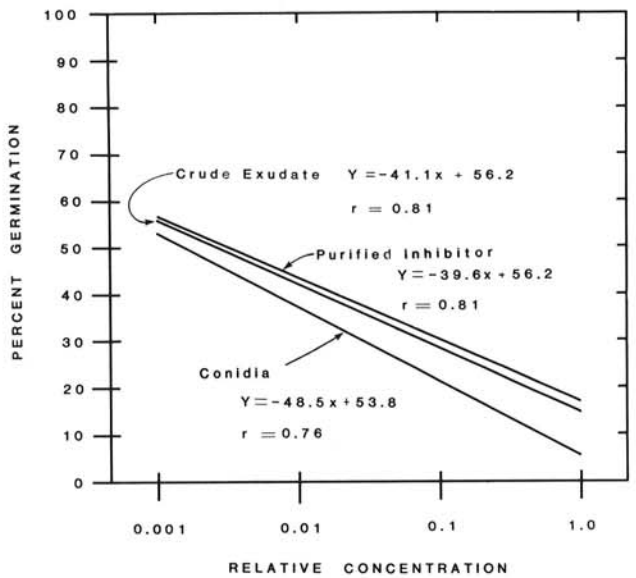


**Fig. 4.** Relationship between percent germination of *Colletotrichum gloeosporioides* f. sp. *jussiaea* and the concentration of conidia, conidial exudates, or purified inhibitor. A concentration of 1.0 represents $10^7$ conidia per milliliter, undiluted exudates from conidia incubated on water agar, or 4 $\mu$g of purified inhibitor per milliliter.

inhibitor was recovered from 1 L of exudate, indicating that the purified inhibitor was active in concentrations expected to exude from the spores. Thus, another attribute of self-inhibitors (3) —that the suspected molecule is active at expected in vivo concentrations— was met and supported the hypothesis that this material is the naturally occuring self-inhibitor. It is active at concentrations of $6.1 \times 10^{-6}$ M, a concentration comparable to the $ED_{50}$ of inhibitors from *Syncephalastrum, Dictyostelium*, and *Hemileia* spp. (5,11,22,23,28,30).

Isolation of the crystalline inhibitor agrees with the results of Lingappa and Lingappa (12) that chloroform-soluble material is responsible for self-inhibition in conidia of *C. gloeosporioides* (12). However, since they did not isolate any pure substances it is impossible to say whether this same inhibitor was present in their isolates. Since our inhibitor is not nitrogenous (see below), it cannot be one of the presumed "alkaloid-like" substances from *G. cingulata* (14), but it might correspond to the activity they found in crude nonbasic fractions (13). The morphogenetic effects reported for *G. cingulata* (15) were not noticed in our study, but different concentrations, purity, or assay procedures could account for this difference. In their studies, Lingappa and Lingappa (15) used 1 mg of crude inhibitor per milliliter, which is 250 times higher than the concentrations used in our work.

Chemical investigation of this self-inhibitor, dihydro-5-hydroxy-5((8 pentyl-2-oxocanyl)-acetyl)-2(3H)-furanone, for which we have proposed the trivial name gloeosporone, is described elsewhere (21). It has the molecular formula $C_{18}H_{30}O_5$ and has been assigned the stucture shown in Fig. 5 on the basis of NMR, IR, and mass spectrometric evidence (21). The relative and absolute configurations at its three chiral centers have not yet been determined. We have found no evidence for the presence of other stereoisomers with inhibitory activity in the conidial extracts.

This structure is quite different from those of other known self inhibitors. It is the only example of a self-inhibitor which is the internal $\gamma$-lactol of a 4,5-dioxo carboxylic acid. The unbranched $C_{18}$ carbon skeleton and the eight-membered ether ring are also unique.

It has requently been stated that the self-inhibitors isolated from rust fungi have potential for biological control of disease (1–3). Since the inhibitor isolated in this study also has high biological activity and inhibits several *Colletotrichum* spp. and at least one pathogenic *Fusarium* species (*unpublished*), it also has the potential for use as a fungistat or a model compound for the development of fungistatic compounds. Further research will be

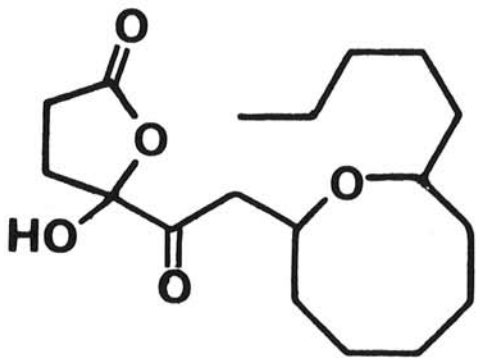


**Fig. 5.** Proposed molecular structure of gloeosporone, the self-inhibitor from conidia of *Colletotrichum gloeosporioides*.

needed to ascertain its mode of action and whether or not it has a role in pathogenesis other than through germination inhibition and to determine whether this or similar compounds are responsible for germination inhibition of other *Colletotrichum* species.

The final criterion for demonstration of a molecule suspected of being an endogenous self-inhibitor, ie, the demonstration that synthetic compound is active, awaits the successful synthesis of this compound. Until then, the first three criteria have been met and support the hypothesis that gloeosporone is the self-inhibitor from this isolate of *Colletotrichum*.

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