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Germination Self-Inhibitors from *Colletotrichum gloeosporioides* f. sp. *jussiaea*

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Three self-inhibitors of conidial germination were isolated from *Colletotrichum gloeosporioides* f. sp. *jussiaea* and identified as (*E*)- and (*Z*)-3-ethylidene-1, 3-dihydroindol-2-one and (2*R*)-(3-indolyl) propionic acid. These compounds were named CG-SI (*C. gloeosporioides*-self-inhibitor) 1, 2 and 3, respectively. The inhibitors reduced the germination of conidia with ED_{50} values of 3 μg/ml for CG-SI 1, 5 μg/ml for CG-SI 2, and 100 to 150 μg/ml for CG-SI 3. Among 16 tested species of anthracnose fungi, CG-SI 1, 2, and 3 were only detected from *C. gloeosporioides* f. sp. *jussiaea* and *C. fragariae*. Activity of the self-inhibitors was greater for germination than for germ tube growth and the effects were greatest against the fungal species that produced them. The evidence indicates that CG-SI 1, 2, and 3 are physiologically important as germination self-inhibitors in these fungi.

Additional keywords; gloeosporone, self-inhibition.

It is well known that fungal spores in dense populations do not germinate at all or only at a reduced rate. This phenomenon is known as self- or auto-inhibition. Despite its common occurrence in more than 60 reported species, the physiological and chemical basis for self-inhibition has been elucidated in only a few cases (Allen 1976). Self-inhibitors have been isolated from such diverse genera as *Dictyostelium* (Abe et al. 1976), *Peronospora* (Leppik et al. 1972), and *Puccinia* (Macko et al. 1970, 1972, 1976; Tsurushima et al. 1984a). These self-inhibitors leach during germination, so that the effects are reversible and generally do not affect hyphal growth (Macko et al. 1976; Tsurushima et al. 1984b).

The existence of self-inhibitors in *Colletotrichum* (or *Glomerella*) was first reported by Lingappa and Lingappa (1965) from conidia and mycelia extracts of *Glomerella cingulata* (= *C. gloeosporioides*), but the inhibitor from these extracts was not isolated or characterized (Lingappa and Lingappa 1967). The first report on isolation and structure determination of a self-inhibitor (gloeosporone) from *C. gloeosporioides* was published by Meyer et al. (1983). The proposed structure of gloeosporone was investigated through total synthesis by Carling and Holmes (1986) and Mortimore et al. (1987), but none of the synthesized compounds gave an identical ^{1}H-NMR spectrum to the isolated natural self-inhibitor. Later, a revised structure including the relative configuration was proposed, based on a single crystal X-ray analysis (Meyer et al. 1987). Total synthesis of natural (–)-gloeosporone was performed by Takano et al. (1988) and Schreiber et al. (1988). Matsushita et al. (1992) and Seebach et al. (1989) have also accomplished the synthesis of (–) and (+)-gloeosporone including epimers, but these compounds were not active as germination inhibitors.

In view of this confusion, the present study was undertaken to reinvestigate whether the germination of conidia of *Colletotrichum gloeosporioides* f. sp. *jussiae* was in fact controlled by self-inhibitors and to characterize them.

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RESULTS

Detection of self-inhibitors.

There was an inverse relationship between conidial concentration and germination percentage. Conidial suspensions were adjusted to 10^4 to 10^7 conidia per milliliter by serial 10-fold dilution in sterile distilled water by counting with a hemacytometer. The resulting germination percentages are shown in Figure 1. The conidial germination of *C. gloeosporioides* f. sp. *jussiaea* was increased by washing to remove putative self-inhibitors (Table 1). The existence of self-inhibitors in these wash fluids was confirmed by a TLC plate assay (Fig. 2).

Isolation of self-inhibitors from *C. gloeosporioides* f. sp. *jussiaea*.

A conidial suspension of *C. gloeosporioides* f. sp. *jussiaea* which had been adjusted to 10^7/ml in sterilized water (500 ml) was shaken in a 1-liter flask at 28°C at 200 rev/min for 16 h. The extract was centrifuged at 2,000 × *g* for 10 min and the supernatant was retained. The supernatant was extracted with an equal volume of ethyl acetate. The extraction was repeated three times, and the ethyl acetate extracts were combined and concentrated. TLC plate and HPLC analyses indicated that the same active principles were also present in acetone extracts of PSA plate cultures. An acetone extract of plate cul-

tures was accordingly employed for large-scale preparation of the inhibitors. The acetone extract was evaporated under reduced pressure to obtain an aqueous fraction, which was saturated with NaCl and successively extracted three times with ethyl acetate. The pooled ethyl acetate extracts were evaporated to dryness to give a syrup, which was dissolved in chloroform and chromatographed by stepwise gradient elution (chloroform-MeOH, from 100:0, 99:1, and 97:3 to 9:1) on a silica gel column (Kieselgel 60, 230-400 mesh ASTM, E. Merck). Five hundred-milliliter fractions were collected and the active principles were detected in the fractions eluted with chloroform containing MeOH from 1 to 3%. These fractions were combined, evaporated to dryness, and rechromatographed on a reverse-phase ODS column by eluting with methanol-water. The active fraction eluted by methanol-water (1:1, v/v) was purified with HPLC on an ODS column by eluting with methanol-water (55:45, v/v). This resulted in several fractions (peaks 1 to 5), the latter three of which expressed inhibitory activity against conidial germination. Further purification of these active compounds was performed with HPLC on an ODS column by eluting with acetonitrile-water (3:7, v/v) to give three active compounds. Each of these compounds was recrystallized from ethyl acetate-hexane.

Structure of the self-inhibitors from *C. gloeosporioides* f. sp. *jussiaea*.

Physicochemical properties of the isolated compounds (called CG-SI 1, 2, and 3 for *C. gloeosporioides* self-inhibitors) are given as follows. CG-SI 1: MS (EI 70 eV) m/z (%); 159.0643 (M^+, 100%, $C_{10}H_9NO$), 144 (52%), 130 (42%), 103 (5%), ^{1}H-NMR (400 MHz, $CDCl_3$), d; 2.29 (3 H, d), 6.91 (1 H, d), 7.03 (1 H, t), 7.12 (1 H, q), 7.21 (1 H, t), 7.57 (1 H, d), 8.70 (1 H, s). IR (KBr); 1715 cm^{-1} (νOC = 0), CG-SI 2: MS (EI 70 eV) m/z (%); 159 (M^+, 100%), 144 (63%), 130 (53%), 103 (7%). ^{1}H-NMR (400 MHz, $CDCl_3$), d; 2.46 (3 H, d), 6.83 (1 H, d), 6.97 (1 H, q), 6.99 (1 H, t), 7.18 (1 H, t), 7.37 (1 H, d), 7.95 (1 H, s), IR(KBr); 1712cm^{-1} (νC = 0). CG-SI 3: MS (EI 70eV) m/z (%); 190 ($[M+H]^+$, 32%), 189 (M^+, 23%), 175 ($[M+H-\cdot CH_3]^+$, 3%), 144 ($[M-\cdot COOH]^+$, 100%), 117 (9%), 115 (9%). ^{1}H-NMR (400 MHz, CD_3OD), d; 1.57 (3 H, d), 3.97 (1 H, q), 7.00 (1 H, d), 7.08 (1 H, t), 7.15 (1 H, s), 7.33 (1 H, d), 7.66 (1 H, d). $[a]_D = -60°C$ (23°C, CH_3OH).

CG-SI 1 and 2 gave identical mass spectra, which suggested that they were isomers. Slight differences of δ values and almost the same shape of the signals in ^{1}H-NMR spectra of both compounds also supported this inference. Following analysis of ^{1}H-NMR spectra data, the compounds were estimated as 3-ethylidene-1,3-dihydroindol-2-one. Since the synthesis of *E*- and *Z*-forms of 3-ethylidene-1,3-dihydroindol-2-one had been reported by Tacconi et al. (1976), we synthesized them according to their method. ^{1}H-NMR spectra of the natural self-inhibitors were compared with those of the synthetic compounds. CG-SI 1 and 2 were identified as the *E*- and *Z*-forms, respectively (Fig. 3). The third active compound, CG-SI 3, was determined to be 2-(3-indolyl)-propionic acid from mass and ^{1}H-NMR spectra correlation to 3-indolyl acetic acid (Fig. 3). The absolute configuration at the C-2 position was determined to be the *R*-arrangement by comparing its $[\alpha]_D$ value to that reported (Yamano 1961; Yoshida and Sassa 1990). This is the first isolation of CG-SI 1 and 2 from a natural source.

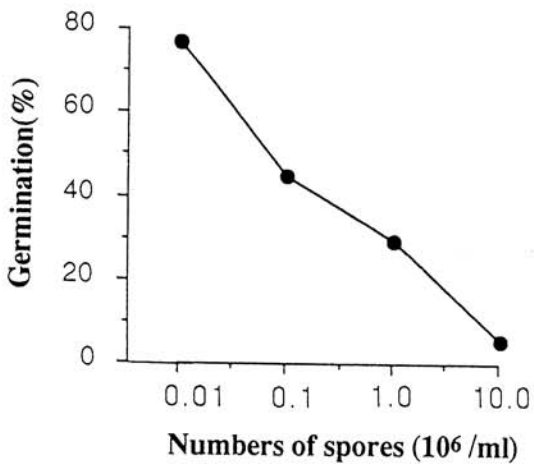


Fig. 1 Relation between germination percentage and spore density of *Colletotrichum gloeosporioides* f. sp. *jussiaea*.

Table 1. Effect of washing on germination of crowded conidia

Treatment	Germination (%) Conc.: 5×10^5 conidia/ml
Nonwashed	56
Washed 1 x	74
Washed 2 x	88

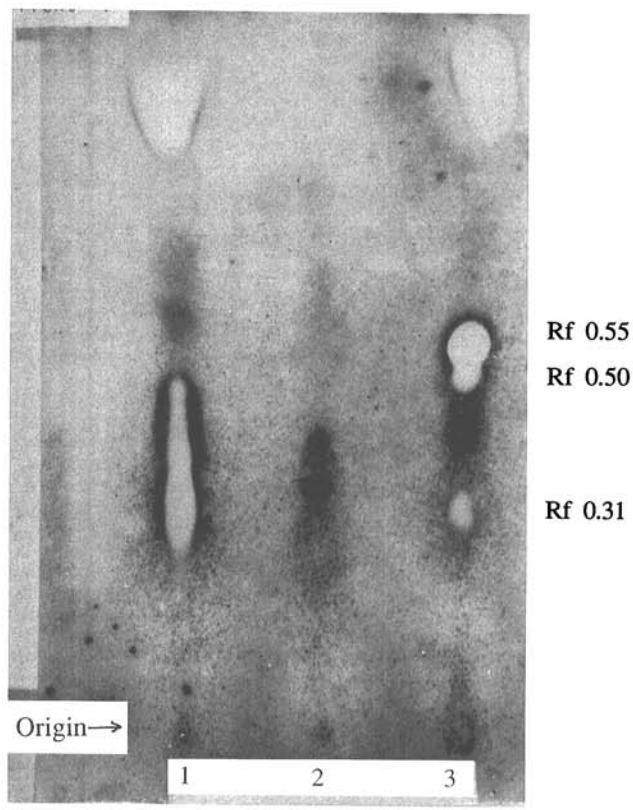
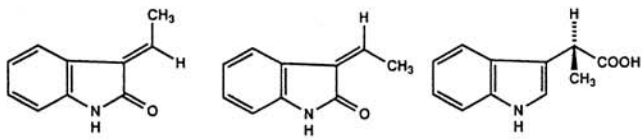


Fig. 2. Self-inhibitors detected on thin-layer chromatography plate sprayed with conidia of *Colletotrichum lagenarium*. Lane 1, *C. lagenarium*; lane 2, *C. malvarum*;. lane3, *C. gloeosporioides* f. sp. *jussiaea*.

Biological activities of CG-SI 1, 2, and 3.

ED_{50} values of germination inhibitory activity of the isolated self-inhibitors, CG-SI 1, 2, and 3, were estimated to be 3, 5, and 100 to 150 µg/ml, respectively, with the microscope slide assay using conidia of *C. gloeosporioides* f. sp. *jussiaea*. The antibiotic spectra of these self-inhibitors were broad, compared to self-inhibitors from rust fungi (Allen 1976). Among eight species of anthracnose fungi, two species of *Cochliobolus* and *Fusarium oxysporum* f. sp. *cucumerinum*, conidial germination of three species of *C. gloeosporioides* and *C. fragariae* were totally inhibited at 10 ppm CG-SI 1 or 2 (Table 2). At concentrations above 30 ppm of these compounds, conidial germination of other *Colletotrichum* spp. and *F. oxysporum* f. sp. *cucumerinum* were inhibited, too. The action of CG-SI 1 and 2 on conidia of *Colletotrichum* species was reversible; i.e., conidia treated with the self-inhibitors recovered their germination capacity after washing with water. This suggests that the natural biological effect of the self-inhibitors is closely related to spore dormancy.

The activities of the self-inhibitors, CG-SI 1 and 2, isolated by our group from *C. gloeosporioides* f. sp. *jussiaea*, were compared with those of natural (–)-gloeosporone, the antipode (+)-gloeosporone, 13-epigloeosporone, and 7-epigloeosporone, previously synthesized by Takano et al. (1988) and by our group (Matsushita et al. 1992). Results of the microscope slide bioassay (Table 3) indicated that activities of the gloeosporones were 30-fold weaker than those of CG-SI 1 and 2. This suggests that the biological role of gloeosporone is ambiguous in *C. gloeosporioides* f. sp. *jussiaea*. Based on these observations, we performed qualitative and quantitative analyses of gloeosporone by high-performance liquid chromatography in the culture broth of the fungus. Unfortunately, however, we failed to detect the compound in organic extracts prepared according to the procedure reported by Meyer et al. (1983) or in the ethyl acetate extracts prepared from conidia or agar plate cultures by our methods.



CG-SI 1 CG-SI 2 CG-SI 3

Fig. 3. Chemical structures of germination self-inhibitors from *Colletotrichum gloeosporioides* f. sp. *jussiaea*.

Table 2. Inhibitory activity of CG–SI 1 and 2 against conidial germination of various fungi

		Germination (%)[a]			
		Conc. (µg/ml)			
Fungus	**Sample**	**10**	**3**	**1**	**0**
Colletotrichum gloeosporioides f. sp. *jussiaea*	CG–SI 1	0	40	81	82
	CG–SI 2	0	68	80	82
C. gloeosporioides f. sp. *aeschnomene*	CG–SI 1	0	46	80	80
	CG–SI 2	0	62	81	80
C. gloeosporioides isolate 7696	CG–SI 1	0	47	83	81
	CG–SI 2	0	65	79	81
C. malvarum	CG–SI 1	5	60	84	81
	CG–SI 2	14	79	82	81
C. graminicola	CG–SI 1	96	94	96	95
	CG–SI 2	96	96	96	95
C. dematium	CG–SI 1	88	90	90	88
	CG–SI 2	87	84	85	88
C. fragariae	CG–SI 1	0	48	80	82
	CG–SI 2	0	60	82	82
C. lagenarium	CG–SI 1	13	64	95	95
	CG–SI 2	92	97	94	95
Cochliobolus miyabeanus	CG–SI 1	32	80	92	93
	CG–SI 2	85	91	90	93
Cochliobolus sativus	CG–SI 1	32	76	89	88
	CG–SI 2	80	89	89	88
Fusarium oxysporum f. sp. *cucumerinum*	CG–SI 1	3	65	91	94
	CG–SI 2	12	91	93	94

[a] Germination percentages were measured by the microscope slide assay.

Distribution of CG-SI 1, 2, and 3 in different species of anthracnose fungi.

The distribution of 3-ethylidene-1,3-dihydroindol-2-one and 2-(3-indolyl)propionic acid as self-inhibitor(s) within the genus *Colletotrichum* was surveyed using the TLC plate assay and HPLC techniques. The results shown in Table 4 indicate that the compounds are restricted to only two species, *C.*

Table 3. Inhibition of germination of conidia by CG–SI 1 and 2 and synthesized gloeosporones

	Germination(%)[a]			
	Concentration (µg/ml)			
Compound	**100**	**30**	**10**	**3**
CG–SI 1	0	0	0	40
CG–SI 2	0	0	0	68
(–)–gloeosporone	49	82		
(+)–gloeosporone	44	81		
7–epigloeosporone	45	82		
13–epigloeosporone	48	81		
Water (control)	82			

[a] Germination percentages were measured by the microscope slide assay using conidia of *C. gloeosporioides* f. sp. *jussiaea*.

Table 4. Content of CG–SI 1 and 2 and detection of CG–SI 3 in cultures of various anthracnose fungi

Anthracnose fungus	**CG-SI 1[a]**	**CG-SI 2[a]**	**CG-SI 3[b]**
Colletotrichum gloeosporioides f. sp. *jussiaea*	149 µg	63 µg	++
C. gloeosporioides f. sp. *aeschnomene*	–[c]	–	–
C. gloeosporioides isolate 7696	–	–	–
C. malvarum	–	–	–
C. graminicola	–	–	–
C. dematium	–	–	–
C. fragariae	38 µg	5 µg	+
C. lagenarium	–	–	–
C. lindemuthianum IFO 5260	–	–	–
Glomerella cingulata IFO 6445	–	–	–
G. cingulata IFO 7478	–	–	–
G. fructigena IFO 5951	–	–	–
G. gossypii IFO 6181	–	–	–
G. glycines IFO 7384	–	–	–
G. tucumanensis IFO 7395	–	–	–
G. fusarioides IFO 8831	–	–	–

[a] Quantitative analyse were performed by HPLC–UV detection.
[b] Spots on TLC–plates were detected with the van Urk–Salkowsky reagent.
[c] "–" indicates below limit of detection (ca. 0.1 µg for CG SI–1 and CG SI–2).

gloeosporioides f. sp. *jussiaea* and *C. fragariae*, among the collected 16 species. The results of the TLC plate assay indicate that several substances having different R_f values participate in self-inhibition among other species of anthracnose fungi. Isolation and structural studies of these compounds are in progress.

Time of action of CG-SI 1 and 2.

The appearance of germ tubes from conidia began to be observed after about 4 h from the time that conidia were suspended in water (Fig. 4). After about 2 h, treatment with CG-SI 1 or 2 totally inhibited the subsequent appearance of germ tubes. After about 3 h, however, the percent inhibition of germination by treatment of CG-SI 1 or 2 was reduced to about 63 and 77%, respectively (Fig. 4). Treatment with CG-SI 1 or 2 after about 4 h did not inhibit germ tube appearance at all, although only 12.5% of the conidia germinated during the experiment. This result shows that CG-SI 1 and 2 inhibit initial germ tube emergence.

DISCUSSION

We investigated the germination self-inhibition phenomenon in conidia of *C. gloeosporioides* f. sp. *jussiaea* since dense suspensions of this fungus exhibit strong germination inhibition in water. Washing the conidia repeatedly with water reduced germination inhibition and the washings showed inhibitor activity. These results indicated that water-soluble inhibitors present in the conidia may be responsible for self-inhibition.

The self-inhibitor of *C. gloeosporioides* f. sp. *jussiaea* was previously characterized as gloeosporone (Meyer et al. 1983; Lax et al. 1985). We re-examined the same isolate according to the procedure reported by Meyer et al. (1983) but could not detect gloeosporone. However, we detected three active principles in washings and cultures of the conidia and identified these self-inhibitors, CG-SI 1, 2, and 3, as (*E*) and (*Z*)-3-ethylidene-1,3-dihydroindol-2-one and (2*R*)-(3-indolyl) propionic acid. This result is consistent with a report that self-inhibitors from *C. gloeosporioides* were alkaloids or alkaloid-like substances by Lingappa and Lingappa (1967). We also compared CG-SI 1 and 2 with natural (–)-gloeosporone for conidial germination. The inhibitory activity of CG-SI 1 and 2 was about 30 times that of gloeosporone. Based on the results, we conclude that CG-SI 1 and 2 are the physiological germination inhibitors in *C. gloeosporioides* f. sp. *jussiaea*.

Recently a self-inhibitor of germination was isolated from the conidial mucilage of *C. graminicola*, which was identified as mycosporine-alanine (Leite and Nicholson 1992). Germination self-inhibition is a common phenomenon within species of *Colletotrichum* (*Glomerella*), and it is assumed that endogenous self-inhibitors regulate their conidial germination (Edgerton 1910; Lingappa and Lingappa 1967; Vasudeva et al. 1961). However, only in two species of *C. gloeosporioides* f. sp. *jussiaea* and *C. graminicola* have the self-inhibitors been separated and identified. Therefore, self-inhibitors might also be present in other *Colletotrichum* species. We could better understand conidial germination regulation systems by determining the chemical structure of self-inhibitors from many species of *Colletotrichum*.

The action of the self-inhibitor of the stem rust fungus has been reported in detail (Hess et al. 1975). It was suggested that the self-inhibitor prevented only digestion of the pore plug of spores and that therefore the inhibitor was not effective on germ tube elongation. CG-SI 1 and 2 inhibited especially initial germ tube emergence from conidia of *C. gloeosporioides* f. sp. *jussiaea*. CG-SI 1 and 2 might have the same mode of action as the self-inhibitor of stem rust uredospores. We are currently investigating the mode of action of the self-inhibitors.

In the case of rust fungi and *Peronospora tabacina*, self-inhibitors are generally highly specific for the producing species (Macko et al. 1976; Tsurushima et al. 1984b; Leppik et al. 1972). Similarly, only two species of *C. gloeosporioides* f. sp. *jussiaea* and *C. fragariae* produced CG-SI 1 and 2 among the collected 16 species and these same isolates were totally inhibited at a concentration 10 ppm. Thus, CG-SI 1 and 2 also show specificity in activity. This specificity suggests that use of inhibitor analogs may facilitate the biological control of these pathogens.

Since CG-SI 1 and 2 showed inhibitory activity against several other *Colletotrichum* spp. and another pathogen, *F. oxysporum* f. sp. *cucumerinum*, at higher concentrations, these compounds might also have nonspecific activity as well as specific activity. The study of the structure-activity relations and the modes of action of self-inhibitors should give us many ideas for disease control of crops.

MATERIALS AND METHODS

Fungi and culture condition.

The 16 species of anthracnose fungi used are as follows: *C. gloeosporioides* f. sp. *jussiaea* Penz., *C. gloeosporioides* f. sp. *aeschynomene* Penz., *C. gloeosporioides* isolate no. 7696, *C. malvarum* Southw, *C. graminicola* Wilson, *C. dematium* Persoon ex Fries, and *C. fragariae* Brooks were provided by G. Templeton at the University of Arkansas. *C. lindemuthia-*

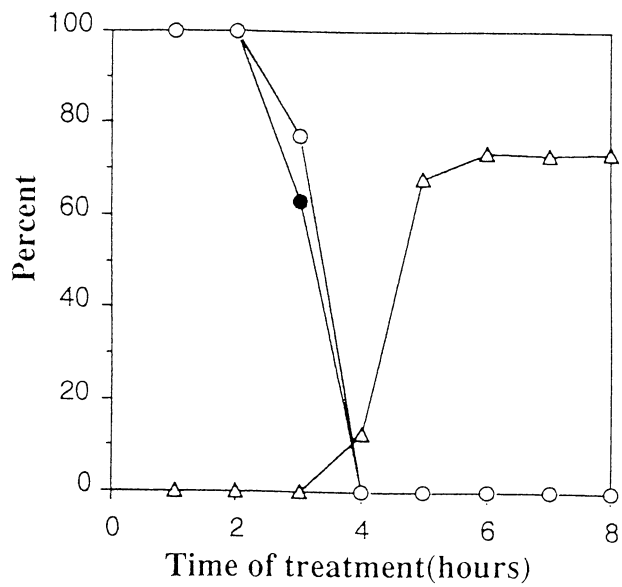


Fig. 4. Effect of treatment time with CG-SI 1 and 2 on germination of *Colletotrichum gloeosporioides* f. sp. *jussiaea*; filled bullet, percentage of germination inhibition by CG-SI 1 (10 ppm); open bullet, percentage of germination inhibition by CG-SI 2 (10 ppm); open triangle, percent germination (control).

num Briosi et Cavara IFO 5260, *Glomerella cingulata* Spaulding et Schrenk IFO 6445, *G. cingulata* IFO 7478, *G. fructigena* Saccardo IFO 5951, *G. gossypii* Edgerton IFO 6181, *G. glycines* Lehman et Wolf IFO 7384, *G. tucumanensis* von Arx. et Müler IFO 7395, and *G. fusarioides* IFO 8831 were obtained from the Fermentation Institute, Osaka. *G. cingulata* is the teleomorph of *C. gloeosporioides* and this name is used by the IFO. *C. lagenarium* Ellis et Halsted was provided by T. Tani, Kagawa University. All strains were cultured and maintained on potato-sucrose agar (PSA) medium.

An isolate of *C. gloeosporioides* f. sp. *jussiaea* provided by G. Templeton was selected for isolation and chemical study of the spore germination self-inhibitor. The fungus was obtained from leaf lesions of winged water primrose (*Jussiaea decurrens* L.) collected at Stuttgart, Ark., in 1977.

Collection of conidia.

Each strain of anthracnose fungus was cultured on PSA medium (prepared from 200 g of potato, 20 g of sucrose, 30 g of agar and 1 liter of water) for 7 days at 24°C in darkness. Conidia for bioassay or inhibitor production were harvested from 7-day PSA plate cultures by mild brushing after addition of distilled water, filtered through three layers of gauze and collected by centrifugation at 1,500 rpm for 5 min at 0°C. The conidia were washed two times by resuspension in water and centrifugation.

TLC plate bioassay.

Detection of inhibitory activity against conidial germination was carried out by a thin-layer chromatography (TLC) plate bioassay. A portion of concentrated ethyl acetate extract of the supernatant was spotted onto a silica gel TLC plate and developed by benzene-ethyl acetate-methanol (50:50:1, v/v/v). After removal of solvents from the TLC plate by air-drying, dilute PSA culture medium (which had been mixed with conidia of *C. lagenarium* at 50°C in an appropriate dilution just before use) was uniformly sprayed on the TLC plate. The plate was incubated at 24°C in darkness for 36 h in a moist chamber. Inhibitory activity was recognized as colorless spots against a dark background of hyphae from germinated spores (see Fig. 2).

Germination tests.

The activity of the self-inhibitor was quantitatively investigated at each concentration under a microscope by estimation of the percentage of germination of conidia incubated for 20 h at 24°C in darkness.

To estimate time of action of the germination self-inhibitors, CG-SI 1 and 2, were added at 1-h intervals to water suspensions of conidia. The germination percentage was determined immediately before addition of the self-inhibitor. The inhibition percentage was determined after 20 hr total germination time.

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