Allelopathy of *Aspergillus japonicus* on Crops

Ren Sen Zeng,*a Shi Ming Luo, Mu Biao Shi, Yue Hong Shi, Qiang Zeng, and Hui Fen Tan

**ABSTRACT**

The seeds of some dicotyledons fail to germinate and grow well when contaminated by *Aspergillus japonicus* Saito. One strain of *A. japonicus* isolated from the seeds of contaminated rape (*Brassica campestris* L.) inhibited the seedling growth of rape and radish (*Raphanus sativus* L.) when the fungus was directly inoculated on the seed surface. Metabolites released from the fungus inhibited the seedling germination and seedling growth of rape and radish. The culture filtrate and mycelium acetone (C,H,O) extract inhibited the seedling growth of rape. The major allelochemical of *A. japonicus* was identified by spectroscopic methods as secalonic acid F (SAF). Bioassays showed that SAF at concentration 0.038 mM significantly inhibited the seedling growth of several crops.

The term Allelopathy was coined by Molisch (1937) to refer to the biochemical interactions between all types of plants, including microorganisms (Rice, 1974, 1995). Rice (1974) pointed out that only a small amount of research had been done on the chemical inhibition of higher plants by microorganisms, except for the specialized field of plant pathology. Metabolites of many fungi may have adverse or stimulatory effects on plants (Heisey et al., 1985; Rice, 1995) such as suppression of seed germination, malformation, and retardation of seedling growth (Lynch and Clark, 1984). Many crop seeds are infected by fungi before harvest or during storage (Neergaard, 1979). If conditions are not favorable, then the situation is more serious (Kozakiewicz et al., 1996). Some fungi on the surface of seeds may produce mycotoxins that affect food quality (Betina, 1984), and some may produce phytotoxins that affect seed germination and seedling growth (Neergaard, 1979).

Secalonic acids, which represent a series of ergochromes, are a group of fungal metabolites (Kurobane et al., 1979). They are the stereoisomers and differ only in the placement of substituting groups. The producing strains often produce one or more secalonic acids when they grow on rice (*Oryza sativa* L.), corn (*Zea mays* L.), and rye (*Secale cereale* L.). Secalonic acid D (SAD) is the major mycotoxin of this group of ergochromes (Betina, 1984). Secalonic acid A (SAA) was the first compound that was reported to have a highly potent phytotoxicity. It was isolated from *Pyrenochaeta terrestris*, the pathogen of pink root disease of onion (*Allium cepa* L.) and other species of *Allium* (Steffens and Robeson, 1987). The compound inhibited the onion seedling elongation by 4, 32, 40, 68, and 94% at concentrations of 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M, respectively. *Penicillium oxalicum*, which caused a storage rot of cucumber (*Cucumis sativus* L.) and tomato [*Lycomusセンセーん* oxycoccus L. (L.) Karsten] fruit, also produced SAD and oxalic acid (Jarvis et al., 1990).

The genus *Aspergillus* is a saprophyte that occurs in and on a variety of substrates, including grains, decaying vegetation in the field, and cattle dung (Raper and Fennell, 1965; Tzean et al., 1990, p. 43). It is particularly abundant in soils in the tropics and subtropics. During routine laboratory work, we found that some dicotyledons that failed to germinate or grew poorly were infected by *Aspergillus* sp. In this paper, we report on the allelopathic effects of the fungus on several crops. We have demonstrated that SAF is the major chemical responsible for these effects.

**MATERIALS AND METHODS**

**Isolation and Culture of *A. japonicus***

The strain of the fungus was isolated from the surface of infected radish seeds that failed to germinate and then purified by successive transfers of mycelia tips. Stock cultures were maintained on PDA slants at 28°C for 7 d and then kept at 4 to 5°C thereafter. The fermentative media consisted of: D-glucose, 20; potassium monophosphate (K₂HPO₄), 0.5; magnesium sulfate (MgSO₄), 0.25; corn flour steep liquor, 20; and potato (*Solanum tuberosum* L.) liquor, 200 g L⁻¹. Each 500-mL Erlenmeyer flask contained 100 mL of media. The flasks were incubated at 26°C in the dark for 4 d on a gyratory shaker at 180 rotations min⁻¹.

**Plant Materials**

Seeds of radish, rape, cucumber, corn, and sorghum (*Sorghum vulgare* Pers.) were obtained from a local market in Guangzhou, China. Seeds of hairy beggarticks (*Bidens pilosa* L.) and barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] were collected at the campus of South China Agricultural University.

**Inoculation of *A. japonicus* on Seedling**

Seeds of radish and rape were surface-sterilized with 1 g L⁻¹ mercury chloride (HgCl₂) for 10 min, then with 750 g L⁻¹ ethanol (C₂H₅OH) for 10 s. After sterilization, the seeds were thoroughly rinsed with sterile water. Fifty seeds and one piece of filter paper were each placed in 9-cm petri dishes. Seven mL 20% (V/V) PD was added. The spores of *A. japonicus* were inoculated on the surface of the seeds before incubation. The controls consisted of noninoculated seeds. The dishes were incubated at 25°C in a greenhouse with 10 h of artificial light (250

**Abbreviations:** DMF, dimethyl formamide; EIMS, electron impact ionization mass spectrometry; EtOAc, ethyl acetate; NMR, nuclear magnetic resonance; SAA, secalonic acid A; SAD, secalonic acid D; SAF, secalonic acid F.

R.S. Zeng, S.M. Luo, M.B. Shi, and Y.H. Shi, Inst. of Tropical and Subtropical Ecology, S. China Agric. Univ., Wushan, Guangzhou, 510642, P.R. China; Q. Zeng and H.F. Tan, Elemento Organic Chem. Lab. Nankai Univ., Tianjin 300071, P.R. China. This paper was presented orally at the Second World Congress on Allelopathy (Symposium: Allelopathy in Natural and Managed Ecosystems) held during 9–13 Aug. 1999 at Lakehead Univ., Canada. Received 29 Nov. 1999. *Corresponding author (rszeng@scau.edu.cn).

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Inoculation of *A. japonicus* mass spectrometer. Mass spectra [electron impact ionization (EIMS)] were recorded on a MSHP5988A mass spectrometer.

** Significant at the 0.05 level.

** Significant at the 0.01 level.

μmol photons m$^{-2}$ s$^{-1}$ daily. The root length and seedling height of the tested plant species were measured after 5 d.

### Microporous Membrane Method

A 9-cm disc of preculture *A. japonicus* was placed on 25% (V/V) PDA (containing 2% agar) that was covered with a sterile microporous membrane with a 0.4-μm aperture. Fungal spores were inoculated on the surface of the microporous membrane. A control was made with a disc of the same PDA without fungal spores and a microporous membrane. Five days after incubation at 28°C, the microporous membrane on which the fungi were growing (no fungal growth on the control) was aseptically removed. A piece of sterile filter, 5 mL of sterile water, and 50 surface-sterilized seeds were immediately placed on the top of the medium.

### Treatment

The microporous membrane on which the fungi had grown was removed, and 10 mL of ether was added to a petri dish for 5 h to extract the fungal metabolites. The extract was moved to another dish with filter paper to evaporate the ether. After the ether had completely evaporated, 5 mL of distilled water and 50 sterile seeds were added. The seed germination, root length, and seedling height were measured 5 d after incubation with 10 h d$^{-1}$ artificial light at 25°C for radish and rape and at 28°C for barnyardgrass in triplicated experiments.

### Extract of Fermentative Hyphae

Hyphae that were filtered from 250 mL of liquid culture were extracted with 125 mL of acetone for 48 h. The extract was evaporated to dryness under reduced pressure at 55 to 60°C, and then the residue was dissolved in 250 mL of distilled water. The water solution was used in bioassays on rape.

### Isolation and Identification of Growth Inhibition Compounds

All reagents and solvents were of analytical grade, and the silica gel for chromatography was 300 to 400 mesh. The mycelium was separated from 3000 mL of liquid culture by filtration and dried for 48 h at 58 to 60°C. Thirty-eight grams of dried mycelium was continuously extracted with ethyl acetate [C$_2$H$_4$O$_2$] (EtOAc) for 3 d. The EtOAc was evaporated to dryness under reduced pressure at 55 to 60°C.

An elemental analysis for C, H, and N was taken with a CHNCORDERD MT-3 elemental analyzer. The melting points were determined with a Yanaco MP-500 apparatus and were uncorrected. Infared spectra were obtained with a Shimadza-IR 435 infrared spectrophotometer. $^1$H nuclear magnetic resonance (NMR) and $^{13}$C NMR were recorded on a Bruker AC-P200 spectrometer (200 MHz), using cadmium chloride (CDCl$_3$) as the solvent and tetramethyl silicane as the internal standard. Mass spectra [electron impact ionization mass spectrometry] (EIMS) were recorded on a MSHP5988A mass spectrometer.

### Bioassays

Seeds were germinated before treatment with different concentrations of SAF solutions. Ten seeds were placed in each beaker (50 mL) and kept in 5-mL SAF solutions at 28°C for 12 h of day and 12 h of night. After 4 d, the root and shoot lengths were measured. Secalonic acid F was dissolved in 1.5 g L$^{-1}$ dimethyl formamide (DMF) and 1 g L$^{-1}$ Tween 80 solution. The ultraviolet absorption of SAF dissolved in 1.5 g L$^{-1}$ DMF did not change compared with that of SAF in 950 g L$^{-1}$ ethanol solution. Previous work showed that DMF and Tween 80 did not affect the phytotoxicity of SAF (unpublished data, 1999). All treatments consisted of at least three replicates.

### Statistical Analysis

Bioassay data were analyzed by the student’s t-test at the 0.05 and 0.01 level. To compare the effects of different concentrations of SAF, the data were subjected to an analysis of variance, and significant treatment differences were tested at the 0.05 level using the LSD test.

### RESULTS AND DISCUSSION

#### Effects of *A. japonicus* Inoculation on Seedling Growth

When seeds of rape, radish, and cucumber were inoculated with *A. japonicus*, the seedling growth was significantly inhibited (Table 1). The root lengths of rape, radish, and cucumber were 18.6, 27.5, and 25% of the control, respectively.

Because the experiment was conducted in sterile conditions, the only difference was that the treated seeds were inoculated with *A. japonicus*. This shows that *A. japonicus* may affect seedling growth when it is on the seed surface. Seed germination was not affected by direct inoculation with *A. japonicus*. In the liquid culture experiment, we found that the color of the culture liquid was white during the first 48 h but then became yellow. The bioassays showed that the white culture liquid possessed negligible phytotoxicity. There was no significant phytotoxic difference between 96- and 144-h culture liquid, so the fungus only produces phytotoxins after 2 d and does not need to sporulate before producing phytotoxins. *A. japonicus* cannot affect germination be-

### Table 1. Effects of *A. japonicus* inoculation on seedling growth.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rape</th>
<th>Radish</th>
<th>Cucumber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root length (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.3</td>
<td>2.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Inoculation of <em>A. japonicus</em></td>
<td>0.8</td>
<td>1.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Significant at the 0.05 level.
** Significant at the 0.01 level.

### Table 2. Effects of metabolites released from *A. japonicus* on seed germination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rape</th>
<th>Radish</th>
<th>Cucumber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>97.4 ± 1.2</td>
<td>89.4 ± 1.2</td>
<td>94.6 ± 2.0</td>
</tr>
<tr>
<td>Treatment</td>
<td>44.6 ± 3.0**</td>
<td>7.4 ± 4.2**</td>
<td>86.6 ± 4.6*</td>
</tr>
</tbody>
</table>

* Significant at the 0.05 level.
** Significant at the 0.01 level.
Table 3. Effects of metabolites released from *A. japonicus* on seedling growth.

<table>
<thead>
<tr>
<th></th>
<th>Rape</th>
<th>Radish</th>
<th>Cucumber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root length</td>
<td>Shoot height</td>
<td>Root length</td>
</tr>
<tr>
<td>Control</td>
<td>2.5 ± 0.6</td>
<td>1.6 ± 0.4</td>
<td>2.9 ± 1.0</td>
</tr>
<tr>
<td>Treatment</td>
<td>0**</td>
<td>0**</td>
<td>0.3 ± 0.3**</td>
</tr>
</tbody>
</table>

* Significant at the 0.05 level.
** Significant at the 0.01 level.

cause seed germination of these crops only requires 1 to 2 d. In nature, if the fungus remained on the seed surface for a sufficient amount of time, then it could inhibit seed germination.

**Effects of Metabolites Released from *A. japonicus* on Seed Germination**

In order to separate the effects of allelopathy and disease of *A. japonicus*, microporous membranes were used to separate the fungus and its growth substrate. The seed germination of the three crops was significantly inhibited by the metabolites released from the fungus (Table 2). The germination rates of rape, radish, and cucumber were inhibited by 54.2, 91.7, and 8.5%, respectively. Thus, the strongest effects were found on radish germination.

Table 3 show that although some seeds germinated, they stopped growing immediately after germination. The inhibition of rape root growth was more than 96%. Because there was no fungal body left on the substrate, the inhibition was only caused by the fungal exudation. The inhibition was even higher than that resulting from direct inoculation of the fungus on the seed surface. This suggests that a certain period is necessary for the fungus to produce enough phytotoxins.

**Effects of Fermented Broth of *A. japonicus* on Seedling Growth**

Fermented broth that was incubated for 4 d was filtered and diluted (×4). To exclude the effects of the broth pH, the pH of the dilution was adjusted to 7 with 1 M of sodium hydroxide (NaOH). The diluted extract (pH 2.13) and dilution (pH 7) were used for bioassay. The seedling growth of rape and radish were significantly inhibited by the culture filtrate of the fungus. The rape seedling inhibition was especially high (Table 4). Although the seedling growth was significantly improved if the broth was adjusted to a pH of 7, it was still significantly inhibited compared with control. This indicated that the inhibitory effects of the fermented broth were not caused by a low pH alone. The acetone extract of the fermentative hypha also inhibited the seedling growth of rape (Table 5).

**Isolation and Identification of Allelochemicals**

The EtOAc extract of 38 g of dried mycelia was evaporated to dryness to yield 1.2 g of yellow precipitate. The inhibition of rape root growth was more than 96%. One thousand milliliters of fermented broth was evaporated at 55 to 60°C to yield 1.2 g of yellow precipitate. The precipitate was fractionated by chromatography on a column of silica gel H using a petroleum ether (boiling point of 60–90°C)–EtOAc gradient (10:1–1:5) for elution. Nine hundred and thirty milligrams of yellow needles were obtained, which had the following properties: Melting point of 238 to 242°C (from acetone); [α]D +201.5; ultraviolet maximum (ethanol) of 337 and 268 nm; infrared (KBr) of 3417 (−OH), 1746 (−COOCH3), 1725 (−CO), 1689, and 1568 cm⁻¹; EIMS of (relative intensity) 638.45 (calculation of 638.1635 for C32H30O14: 638.1635) (20), 579 (100), 561 (10), 501 (15), 395 (3), 377 (9), 260 (21), 151 (25), and 123 (17) m/z; and an elemental composition of 59.78, 4.71, and 35.51% for C, H, and O. Data of the 13C NMR and 1H NMR are shown in Table 6. The compound was identified as SAF compared with data reported by Andersen et al. (1977). Fig. 1 shows the structure of SAF, which was also obtained from both fermented broth and rice meal culture.

One thousand milliliters of fermented broth was evaporated under reduced pressure at 55 to 60°C to yield 200 mL of solution. The solution was cooled at 4°C. After 48 h, 1.45 g of colorless crystal formed. The crystals were extracted with EtOAc three times, and
the extract was evaporated to dryness to yield 1.13 g of oxalic acid.

**Biological Activities of Secalonic Acid F**

Secalonic acid F significantly inhibited the seedling growth of the test crops at 0.038 mM (Table 7). The roots of rape seedling stopped growing after germination at 0.075 mM of SAF, and the roots of all tested crop seedlings could not grow at 0.3 mM of SAF.

**CONCLUSION**

The allelopathy of *A. japonicus* plays an important role in inhibiting the germination and seedling growth of some crops. If crop seeds are infected by the fungus and the storage conditions are humid with high temperatures, those crops may fail to germinate or may grow badly because of the allelopathy of the fungus.

We report the isolation of SAF from *A. japonicus* for the first time. The compound is abundant in the fungal mycelia. The fungus may influence the seed germination and seedling growth of some cereal crops by means of releasing SAF.

Secalonic acid F and SAA have been demonstrated to be phytotoxic while other secalonic acids (e.g., SAD) have been demonstrated to be toxic in mammalian systems (Vev and Bolon, 1990) and are recognized as myco-toxins, elaborated by food spoilage from *P. oxalicum*, *Aspergillus ochraceus*, and *Aspergillus aculeatus* (Kozakiewicz et al., 1996). The phytotoxicity of the other ergochromes has not been demonstrated; however, in view of their close structural resemblance to SAF and SAA, they would be expected to possess significant phytotoxicity.

More research is needed to determine which of crops will be affected by the allelopathy of *A. japonicus*. Details about the mode of action of secalonic acids against higher plants and attention to the human and mammalian toxicity of SAF will also be required.

**ACKNOWLEDGMENTS**

We thank the National Natural Science Foundation of China (39770136), Guangdong Provincial Natural Science Foundation of China (990682, 960426), and the National Laboratory of Elemento-Organic Chemistry, Nankai University, for financial support.

**REFERENCES**


**Table 6.** 

<table>
<thead>
<tr>
<th>Conc. of SAF</th>
<th>Chemical shift (ppm)</th>
<th>Chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>¹³C NMR</td>
<td>¹H NMR</td>
</tr>
<tr>
<td>0 (control)</td>
<td>4.2a†</td>
<td>2.1a</td>
</tr>
<tr>
<td>0.038</td>
<td>1.6b</td>
<td>1.5b</td>
</tr>
<tr>
<td>0.075</td>
<td>0.3b</td>
<td>1.1bc</td>
</tr>
<tr>
<td>0.15</td>
<td>0b</td>
<td>1.0c</td>
</tr>
<tr>
<td>0.30</td>
<td>0b</td>
<td>0.5d</td>
</tr>
</tbody>
</table>

† Numbers with different letters within a column are significantly different at the 0.05 level according to Duncan’s multiple-range test.

**Table 7.** Effects of secalonic acid F (SAF) on seedling growth.

<table>
<thead>
<tr>
<th>Conc. of SAF</th>
<th>Rait</th>
<th>Shoot length</th>
<th>Radish</th>
<th>Root length</th>
<th>Shoot length</th>
<th>Rice</th>
<th>Root length</th>
<th>Shoot length</th>
</tr>
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<tr>
<td>mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>4.2a†</td>
<td>2.1a</td>
<td>5.4a</td>
<td>2.7a</td>
<td>4.3a</td>
<td>2.4a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.038</td>
<td>1.6b</td>
<td>1.5b</td>
<td>5.3a</td>
<td>2.3b</td>
<td>3.2b</td>
<td>1.7ab</td>
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<td></td>
</tr>
<tr>
<td>0.075</td>
<td>0.3b</td>
<td>1.1bc</td>
<td>5.9a</td>
<td>2.1c</td>
<td>3.3b</td>
<td>1.4b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>0b</td>
<td>1.0c</td>
<td>0.5b</td>
<td>1.1c</td>
<td>1.1c</td>
<td>1.4b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0b</td>
<td>0.5d</td>
<td>0.2b</td>
<td>1.0c</td>
<td>0.2e</td>
<td>0.5b</td>
<td></td>
<td></td>
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</table>
ANY noxious annual and perennial weeds have been regarded as species with allelopathic potential and can severely affect crop survival and productivity (Putnam and Duke, 1978; Rice, 1979; Qasem, 1994). Allelochemicals produced by plants may be released into the surrounding environment in sufficient amounts with enough persistence to affect neighboring and successional species (Akram et al., 1990).

Different studies showed that some allelopathic agents are volatile, emanated from different plant parts (Oleszek, 1987; Bradow and Connick, 1988); others indicated that they exuded from roots to the root zone and interfered in root growth and functions (Rovira, 1969; Qasem and Hill, 1989) or inhibit seed germination (Rovira, 1969). Plant residues and their decomposition products are also implicated in virtually all biochemical processes (Patrick et al., 1963; Bhowmik and Doll, 1984). Some allelochemicals are water soluble leached from foliage parts by rain, mist, dew, or fog drip (Lovett and Lynch, 1979; Qasem, 1994), leading to the monospecies stands that several perennial weeds form in nature (Rice, 1984).

However, the inhibitory materials may be autoinhibitory or heteroinhibitory (Kumari and Kohli, 1987), some can be highly selective (Stachon and Zimdahl, 1980; Sahid and Sugau, 1993), and their effect is concentration dependent (Qasem, 1993).

White top [Cardaria draba (L.) Desv.] and Syrian sage (Salvia syriaca L.) are perennial rhizomatous and root creeping weeds belonging to cruciferae and labiatae families, respectively. They are widespread in cultivated fields in Jordan and invade field crops as well as orchards. Their deep, penetrating, hard, and extensive creeping roots make them difficult to eradicate. If the weeds were left uncontrolled, they soon colonize a large area, choking the other plants present. Both are strong competitors for soil moisture in arid regions, and their growth increased with increasing water consumption (Al-Ahmed, 1982; Qasem and Abu-Imaileh, 1983).

Furthermore, both weed species have been reported to possess high allelopathic activity against crops, including wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.) (Qasem and Abu-Imaileh, 1985; Qasem, 1993, 1994). When shoot and root extracts, water leachates, and dried residues of both weeds were added to the soil, all inhibited germination, growth, and development of these crops.

The objective of the present work was to investigate any possible role of allelopathy mechanism in the interference between these two common and noxious weed species (through their possible volatile materials, root exudates, foliage leachates, and shoot residues) on germination and growth of their associated vegetable crops.

MATERIALS AND METHODS

Laboratory Experiments

Experiment 1. Effect of Root Exudates

Ten-cm diameter plastic pots were filled with 500 g of soil mixture (clay/sand/peat, 3:1:1 of a pH 7.7) and planted with rhizomes of both weed species, separately. After emergence, seedlings were thinned to 10 per pot irrigated with tap water when needed and left to grow for 2 mo before being harvested. The soil was loosened, cleaned up from weed roots, and then mixed with an equal volume of distilled water and thoroughly shaken for 2 h on a shaker. The mixture was passed through filter paper and immediately assayed for phytotoxicity. For the control treatment, 500 g of weed-free soil was mixed with the same volume of distilled water and then similarly treated before used.

The effect of soil filtrate was studied by placing 20 seeds of cabbage (Brassica oleracea L. var. Capitata cv. Pronzwick), carrot (Daucus carota L. cv. Natus), cucumber (Cucumis sati-