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Figs : 5, Photograph 1.

Herbicidal effects of allelochemicals produced by *Aspergillus japonicas* on growth of *Oryza sativa* and *Echinochloa crusgalli*

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ABSTRACT

We isolated, 5- allelochemicals [secalonic acids (H, I, G, D, F)] from the culture broth of *Aspergillus japonicas*. Among them secalonic acid H (I), I (II) and G (III) were isolated for the first time from *Aspergillus*. These secalonic acids showed inhibitory effects on root and shoot growth of rice (*Oryza sativa*) and barnyard grass (*Echinochloa crusgalli*). Secalonic acids H, I and G completely inhibited the root growth of barnyard grass at 0.15-0.3 mM concentrations. Meanwhile, the shoot growth of rice was affected by I and G at 0.038 mM concentrations and inhibited by D and F at 0.15 mM concentrations. Thus natural products produced by microorganisms may serve as an important source for new pesticides.

Key words: Allelochemicals, *Aspergillus japonicas*, barnyard grass, *Echinochloa crusgalli*, herbicidal effects, microbial allelopathy, natural herbicides, *Oryza sativa,* rice, Secalonic acids,

INTRODUCTION

Plants and microorganisms can synthesize and release a variety of secondary metabolites, which may directly or indirectly affect the growth and development of other plants and microorganisms in the vicinity. This phenomenon is termed as allelopathy (21,24), which possesses a wide range of influences in crop production, weed management, and natural herbicide development (1,3,4,6,23). Microorganisms also produce allelochemicals to interfere with plants (14,20). Metabolites of certain fungi show inhibitory or stimulatory effects on plants such as inhibition of seed germination and seedling growth. Isolation and identification of allelochemicals are key steps to understand role of allelopathy in agroecosystems (2,7,8,10,17,19,25). *Aspergillus japonicas* Saito is widely distributed in soils and stored seeds, such as rice, peanut and chili powder (29). In our previous study, secalonic acid, F, oxalic acid and citric acid were isolated from the fermented broth of *A. japonicas* (30), and secalonic acid F showed inhibitory effects on the seed germination and seedling growth of *Oryza sativa*, *Raphanus sativus* and *Brassica campestris* (31).

Recently, we found that acetone extract of *A. japonicas* mycelium after fermentation drastically suppressed the root growth of *R. sativus* by 97.7 % at 500 mg/L concentration. The acetone extract was subjected to silica gel column, eluting with

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chloroform-methanol, to give 8- Fractions. Fraction 3 at 500 mg/L concentration, inhibited the root growth of *R. sativus*, *B. campestris* and *O. sativa* by 96.5 %, 100 % and 92.9 %, respectively (11). However, the chemical constituents with bioactivities in the Fraction 3 were still unknown. Thus, this study aimed to Isolate and identify the chemical components from Fraction 3 and study their herbicidal effects on the growth of *O. sativa* and *E. crusgalli*.

MATERIALS AND METHODS

The fungal strain of *A. japonicas* was isolated from the contaminated radish seeds that failed to germinate as previously described (31). The study was conducted in College of Agriculture, South China Agriculture University, Guangzhou, China (113°15’E, 23°06’N, temperature: 13.5° to 28.7°, annual rainfall: 1736 mm, altitude: 11 m). Seeds of *O. sativa* and *E. crusgalli* were obtained from Department of Agronomy.

Fermentation: *A. japonicas* was cultured in a liquid fermentation medium. One liter medium contained: 200 g potato extract, 20 g glucose, 1 g KH_2PO_4 and 0.5 g $MgSO_4.7H_2O$. The cultures were maintained on a rotary shaker (160 rpm) at 25℃ for 5 days.

Extraction and isolation: After the fermentation, *A. japonicas* mycelia were collected and extracted with acetone (3×3.5 L, 7 days each) at room temperature. The extracts were pooled and dried under vacuum. A 20 g dried extract was obtained. The acetone extract was mixed with silica gel (1:3) and then applied to a silica gel column, eluting with chloroform-methanol (v/v, 97:2), to yield eight fractions (Fractions 1-8). Fr. 3 (6.3 g) was chromatographed over a MPLC column, eluting with cyclohexane-ethyl acetate (9:1), to give 8-subfractions (Fr. 3.1-3.8). Fr.3.6 (1.94 g) was subjected to MPLC column (cyclohexane-ethyl acetate-formic acid, 100:10:1), then purified by silica gel column (petroleum ether-acetone, 7:1) to afford compounds **I** (12 mg), **II** (17 mg), and **III** (33 mg). Fr.3.8 (865 mg) was purified by MPLC column repeatedly (petroleum ether-acetone, 5:1), to yield **IV** (14 mg) and **V** (43 mg).

Experimental procedures

1D and 2D spectra were run on Bruker DRX-500 and spectrometers operating at 400 MHz for ^1H, and 100 MHz for ^{13}C, respectively. IR spectrum was measured on a Bruker Tensor 27 spectrometer with KBr pellets. EI-MS was measured at VG Auto Spec-3000. Column chromatography (CC) was performed by using silica gel (200-300 mesh) (Qingdao Marine Chemical and Industrial Factory, China). Medium pressure liquid chromatography (MPLC) was done on a silica gel column (Buchi, Switzerland). FPQ artificial climate box (Ningbo Fulai, China). Fractions were monitored by TLC and spots were visualized by heating the silica gel plates sprayed with 10% sulphuric ethanol solution. Fractions were evaporated to dryness by rotary evaporation (Shanghai Chuangmeng Biological, China).

Biological activity assay

9.57 mg of each compound of secalonic acids [H (**I**), I (**II**), G (**III**), D (**IV**) and F (**V**)] was dissolved in 50 mL volumetric bottles to get 0.3 mM concentration, and then diluted to 0.01, 0.019, 0.038, 0.075, 0.15 and 0.3 mM concentrations. The herbicidal effects of the isolated allelochemicals on *O. sativa* and *E. crusgalli* were evaluated by small glass method as previously described (31). The filter paper was laid on the glass beads of 50 mm diameter, which were laid on the bottom of 50 mL beaker (Photograph 1).

Photograph 1. The petridish photograph of bioassay test

Compounds were dissolved in methanol, added to the sheet of filter paper in the beaker, and the methanol was evaporated to dryness. The seeds were placed on the filter paper after it was moistened with 5 mL distilled water. The beaker was placed into culture room at 25-28℃ and 9 h light daily in an artificial climate box. There were three replicates for each concentration. The length of roots and shoots was measured after *O. sativa* was cultured for 9 days, and *E. crusgalli* were cultured for 5 days. SPSS14.0 software was used to analysis the experimental data.

RESULTS AND DISCUSSION

Identification of allelopathic substance

After fermentation, the mycelia of *A. japonicas* were extracted with acetone. The dried extract was applied to a silica gel column, eluting with chloroform-methanol, to give eight fractions (Fr.1-8). Fr. 3 (6.3 g) was applied to silica gel column and medium pressure liquid chromatography (MPLC) column, to yield five compounds, identified as secalonic acids H (**I**) (5), I (**II**) (5), G (**III**) (15), D (**IV**) (16), and F (**V**) (30) (Fig. 1) by comparing their spectroscopic data with literature values. The secalonic acid H, I and G were isolated for the first time from *Aspergillus*.

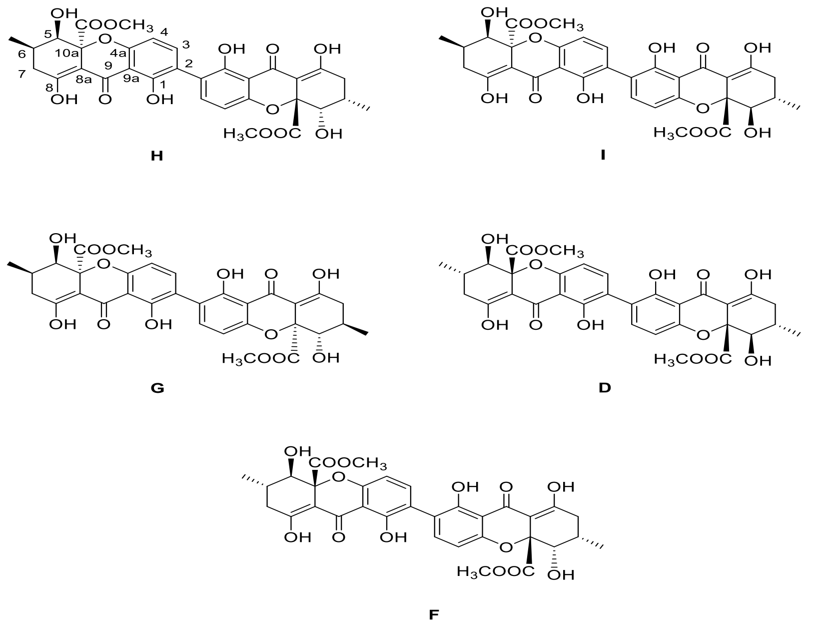

Figure 1. Structures of compounds isolated from *Aspergillus japonicas*

Biological activity

The effects of compounds H, I, G, D and F on the root and shoot growth of *O. sativa* are shown in Fig. 2 and 3. Secalonic acid G promoted the root growth of rice at low concentration (0.01 mM). There were no effects of H, D and F at 0.01 mM concentration on rice root growth. These five secalonic acids inhibited the root growth of *Oryza sativa* at higher concentrations (0.075 mM). Secalonic acid G showed the most inhibitory effect. However, these compounds showed less effects on shoot growth (Fig. 3). There were no influences of H on shoot growth of *Oryza sativa* at 0.01 to 0.3 mM concentrations. Secalonic acids I and G inhibited the shoot growth at 0.038 mM concentration. However, the two compounds showed significantly less inhibitory effects at $\geq$ 0.075 mM concentrations than their effect at 0.038 mM concentration. The possible reason is that the two compounds were not fully dissolved at $\geq$ 0.075 mM concentrations, leading to fewer active molecules in the solution. Secalonic acids D and F inhibited the shoot growth in a concentration-dependent manner.

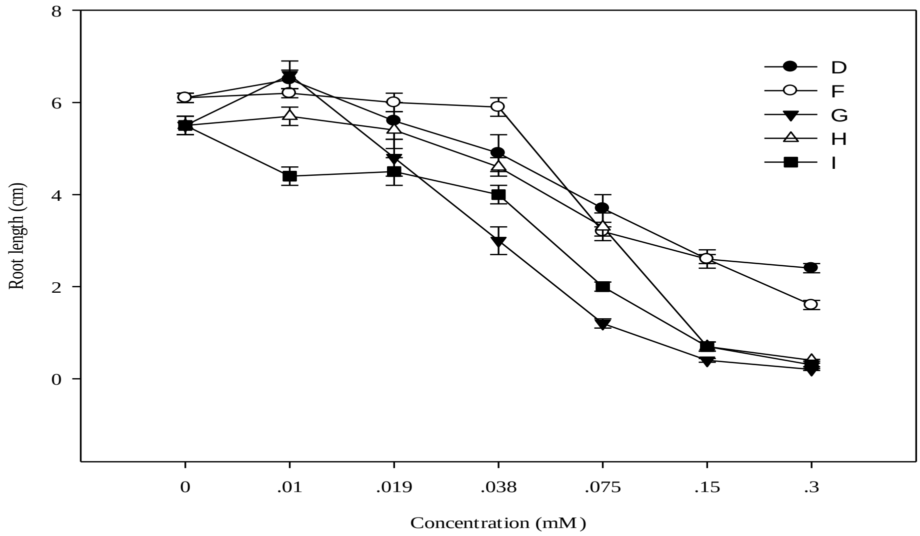

Figure 2. Effects of secalonic acids (D, F, G, H, I) on the root growth of *Oryza sativa*

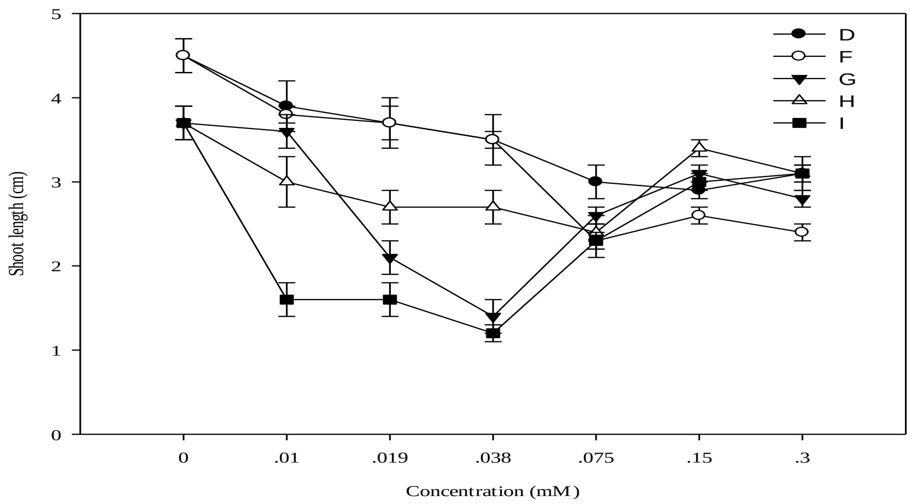

Figure 3. Effects of secalonic acids (D, F, G, H, I) on the shoot growth of *Oryza sativa*

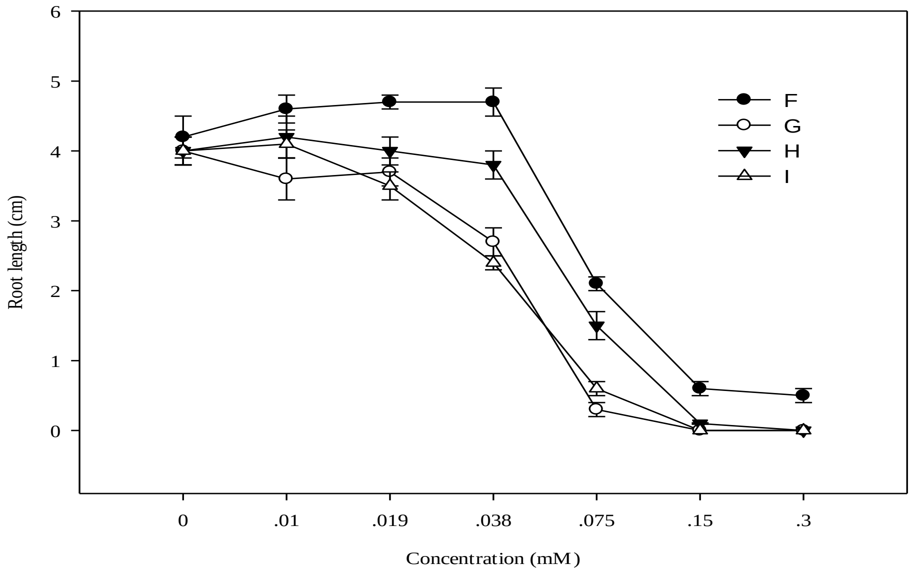

Figure 4. Effects of secalonic acids (F, G, H, I) on the root growth of *Echinochloa crusgalli*

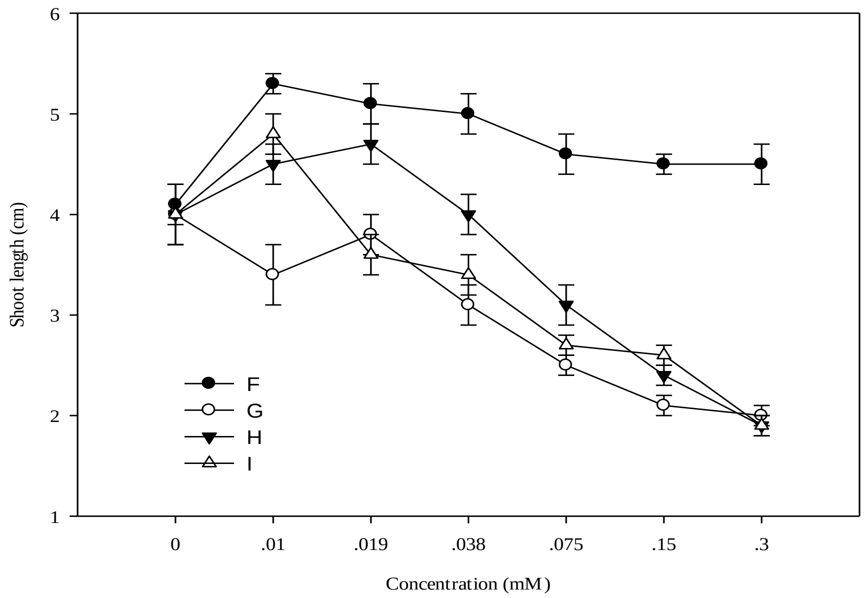

Figure 5. Effects of secalonic acids (F, G, H, I) on the shoot growth of *Echinochloa crusgalli*

Herbicidal effects of the secalonic acids H, I, G, and F on barnyard grass were evaluated (Fig. 4 and 5). H and F at 0.01-0.075 mM concentrations and I and G at 0.01 and 0.019 mM concentrations did not significantly affected the barnyard grass roots (Fig. 4). However, the higher concentrations of these compounds were inhibitory to the root growth. Secalonic acids H, I and G completely inhibited the root growth of barnyard grass at 0.15-0.3 mM concentrations. The Secalonic acids H, I, G and F at 0.15 mM concentration inhibited the root growth by 97.5 %, 100 %, 100 % and 88.1 %, respectively. For shoot growth, the H, I and F were stimulatory at low concentration (0.01 mM) (Fig. 5). However, H, I, and G significantly inhibited the shoot growth at concentrations ≥ 0.075 mM, while F had no influence on shoot growth.

Weeds are the main cause of crop yield reduction. Although traditional chemical herbicides control is effective, but it causes an array of environmental problems. Weed control through allelopathy is a safe, economical and effective method, and had become a research hotspot. Some allelochemicals may be developed as natural herbicides and serve as a template for synthesis of new herbicides (13,22,26). Unfortunately, mass production of plant allelochemicals is costly, and in many cases it is impossible. However, allelochemicals produced by microorganisms can be easily obtained by fermentation and the cost could be acceptable.

In this study, five allelochemicals, secalonic acids (H, I, G, D, F), were isolated from the culture broth of *A. japonicas*. The herbicidal effects of these compounds were examined on the growth of *E. crusgalli* and *O. sativa*. Secalonic acids, a series of ergochrome pigments, commonly exist in a group of food-borne fungal metabolites (9). Secalonic acids show extensive bioactivities, such as antibacterial (12,28), anticancer (27) and neuroprotective effect (18). Secalonic acid D significantly inhibited the growth of sarcoma-180 cell and D3M-16 breast cancer cell (27). Secalonic acid F markedly inhibits the growth of *O. sativa*, *R. sativus* L, and *B. campestris* L (31). The secalonic acids H, I and G showed higher herbicidal activity against barnyard grass than secalonic acid F. Our previous study showed that secalonic acid F inhibits the plant growth by weakening the protective ability of plant tissues against membrane lipid peroxidation and damaging the membrane system of plant cells, resulting in the ultrastructure destruction of chloroplasts, mitochondria, and nuclei (32). Our results showed that secalonic acid G at 0.01 mM concentration, promoted the root growth of *O. sativa*, while it inhibited the root and shoot growth of *E. crusgalli*, suggesting that the compound shows selective bioactivity and could be used as microbe-derived natural herbicide and plant growth regulator.

CONCLUSIONS

We determined the effects of 5- secalonic acids (H, I, G, D, F) isolated and identified from the culture broth of *A. japonicas,* on the growth of *E. crusgalli* and *O. sativa*. These compounds inhibited the root and shoot growth of *E. crusgalli* and *O. sativa*. At the 0.075 mM concentration, these compounds significantly inhibited the root growth of *O. sativa*. The I and G compounds at 0.038 mM concentrations, inhibited the shoot growth of *O. sativa*. The roots of *E. crusgalli* almost could not grow at the secalonic acids H, I and G compounds concentrations of 0.15-0.3 mM. The results provided the basis for the development and utilization of secalonic acids (H, I, G, D, F) as herbicides.

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