Variation in Chemotactic Preferences of *Aphanomyces cochlioides* Zoospores to Flavonoids

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The zoospores of the phytopathogenic *Aphanomyces cochlioides* are chemotactically attracted by a host-specific flavone, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone), and repelled from the mammalian estrogens or estrogenic compounds. This study further examined the responses of *A. cochlioides* zoospores to some flavonoids structurally related to cochliophilin A or compounds known as phytoestrogens. The bioassay revealed that some synthetic flavones (such as 6-methyl-4'-methoxyflavone, 3-hydroxy-4'-methoxyflavone, 7-hydroxy-5-methylflavone, 3-hydroxy-4'-methoxy-6-methylflavone) elicited attractant activity at concentrations as low as picomolar (10 pM), which was an 100-fold lower concentration than that of the threshold concentration of the host-specific attractant cochliophilin A. Apparently, a hydrophobic B-ring with an alkylated (methylated) A-ring or a methoxylated B-ring with an unsubstituted A-ring in the flavone skeleton played a significant role in higher attractant activity. On the other hand, all known estrogenic flavonoids (such as equol, 3'- or 8-prenylated naringenins) displayed potent repellent activity toward zoospores. Surprisingly, zoospores were attracted by non-estrogenic 6-prenylated naringenin indicating that repellent activity is linked to the estrogenic activity of the phytoestrogens.

Key words: Chemotaxis, Flavonoids, Peronosporomycete Zoospore

Introduction

Zoospores of a damping-off pathogen are attracted by the host-specific flavone cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone) released from sugar beet and spinach roots (Horio *et al.*, 1992). The attracted zoospores form a mass and then rapidly encyst on the potential infection sites of the host triggered by the host signal, and subsequently germinate to give hyphal germ tubes within 30 min (Islam *et al.*, 2002, 2003). Then the germlings penetrate the host tissue directly or *via* appressoria to initiate infection. Although this pre-infectional process guided by the kairomone cochliophilin A has been shown in several reports (Islam *et al.*, 2003; Islam, 2008a), very little is known about the structural requirements of flavonoids for attractant activity of zoospores. In an earlier study, Kikuchi *et al.* (1995) tested some flavonoids structurally similar to cochliophilin A and observed that A-ring oxygenation at C-5 and C-7 positions of the flavone skeleton is significant for higher attractant activity to zoospores. Tyler *et al.* (1996) carried out comparatively a detailed study to understand the chemotactic preference of the soybean pathogen *Phytophthora sojae* zoospores, which are specifically attracted by daidzein and genistein released from the roots of soybean (Morris and Ward, 1992; Tyler *et al.*, 1996). They found some variations among strains of *P. sojae* zoospores, but generally all showed preferential chemotaxis to the said host-specific isoflavonoids. However, surprisingly they observed that some synthetic flavones, such as 7-hydroxy-3-methylflavone, 7-hydroxy-5-methylflavone, 5,7-dimethoxyflavone, and 4',5,7-trimethoxyflavone mainly with a hydrophobic B-ring, elicited a potent repellent activity toward *P. sojae* zoospores. One of these potent repellants, 7-hydroxy-5-methylflavone, has been found to be a potent inhibitor of the nodulation response of several genotypes of *Bradyrhizobium japonicum* (Cunningham *et al.*, 1991). However, there is no information on the activity of these bioactive flavonoids to other soilborne microorganisms or soilborne phytopathogens. Nothing is known about the behaviour of other peronosporomycete zoospores toward these methylated flavones. On the other hand, mammalian estrogens, xenoestrogens or synthetic estrogenic compounds have been known to possess potent repellent activity toward zoospores of *A.*

cochlioides (Islam and Tahara, 2001a). Some plant flavonoids, such as equol, naringenin, and 8-prenylated naringenin, have been reported to exhibit potent estrogenic activity (Takamura-Enya *et al.*, 2003). To extend our understanding of the chemotactic behaviour of zoospores toward flavonoids and estrogenic compounds, this study aimed to test some synthetic methylated and methoxylated flavonoids including the reported repellants of *P. sojae* and some known flavonoidal phytoestrogens toward zoospores of *A. cochlioides*.

Material and Methods

General

Preparative thin layer chromatography and determination of the purity of the samples were done using Merck Kieselgel 60 F_{254} (0.2 mm thick) TLC plates. The spots were viewed under 254 and 365 nm UV light and the compounds were detected by spraying with 5% H_2SO_4 and EtOH. The mass spectra were recorded on a JEOL JMS-SX1D2A (FD) mass spectrometer, and a JEOL JNM-EX 270 instrument was used for recording the ^{1}H NMR spectra. TMS was used as the internal standard in ^{1}H NMR spectrometry.

Materials

All chemicals commercially available were of the highest purity and, unless otherwise stated, were used without further purification. Cochliophilin A (**1**) available in the laboratory was synthesized according to Horio *et al.* (1992). 6-Methyl-4'-methoxyflavone (**2**), 3-hydroxy-4'-methoxyflavone (**3**), 7-hydroxy-5-methyflavone (**4**), 3-hydroxy-4'-methoxy-6-methylflavone (**5**), 6-methylflavone (**6**), 8-methylflavone (**7**), 7-hydroxy-3-methylflavone (**8**), 5,6,7-trimethoxyflavone (**9**), and 3-hydroxy-6-methylflavone (**10**) were purchased from Indofine Chemical Company Inc., Somerville, NJ, USA. 17β-Estradiol (**11**) was purchased from Sigma-Aldrich, and equol (**12**), naringenin (**13**), 3'-, 8- and 6-prenylated naringenins (**14**–**16**) were available in the laboratory.

Production of zoospores and bioassay

The peronosporomycete *Aphanomyces cochlioides* (AC-5), a gift from Prof. R. Yokosawa, Health Science University of Hokkaido, Japan was cultured for 4–5 d on a corn meal agar (Difco) plate at 20 ºC (Islam and Tahara, 2001a). The production of zoospores and the particle bioassay were carried out as described previously (Islam and Tahara, 2001b; Islam *et al.*, 2003; Takayama *et al.*, 2004; Islam, 2008b).

Methylation of equol

Mono- and dimethylation of equol were done using a suitable ratio of dimethyl sulfate in a mixture of K_2CO_3 and acetone. After the methylation process, the reaction mixture was purified by preparative thin layer chromatography (PTLC) in *n*-hexane/EtOAc (3:1, v/v) (Islam and Tahara, 2001a), and the structures of the isolates were elucidated by spectroscopic methods including ^{1}H NMR spectroscopy.

Results and Discussion

Effects of synthetic flavonoids toward motility of zoospores

The bioassay results of flavonoidal substances toward the zoospores of *Aphanomyces cochlioides* are presented in Table I. Chemical structures of all tested flavonoids and estrogen are illustrated in Fig. 1. Surprisingly, the synthetic flavonoids, which have been reported as repellants of *Phytophthora sojae* zoospores, exhibited potent attractant activity toward zoospores of *A. cochlioides*. Among the tested compounds, 6-methyl-4'-methoxyflavone (**2**), 3-hydroxy-4'-methoxyflavone (**3**), 7-hydroxy-5-methylflavone (**4**) and 3-hydroxy-4'-methoxy-6-methylflavone (**5**) displayed clear attractant activity at concentration as low as 0.01 nM which was 100-fold lower than the threshold concentration of the host-specific attractant cochliophilin A (**1**) (Table I). Other tested flavonoids, such as 6-methylflavone (**6**), 8-methylflavone (**7**), 7-hydroxy-3-methylflavone (**8**), 5,6,7-trimethoxyflavone (**9**), 3-hydroxy-6-methylflavone (**10**), also exhibited attractant activity at a range of 0.1–10 nM, *i.e.* they were equivalent or even stronger active than the host-specific attractant cochliophilin A (**1**). Furthermore, the synthetic flavonoids induced encystment of the attracted zoospores at ca. 100-fold higher concentrations than their threshold concentrations. However, these synthetic flavonoids neither induced formation of a mass of cystospores around the treated particles, nor the newly formed cystospores adhered to the particle or glass surfaces indicating that they were incapable of releasing adhesive materials during

Table I. Chemotaxis of *Aphanomyces cochlioides* zoospores to natural and synthetic flavonoids and estrogenic compounds.

Compound	Chemotaxis						
	10000 nM	1000 nM	100 nM	10 nM	1 nM	0.1 nM	0.01 nM
Cochliophilin A (**1**)	nt	nt	+++	++	+	±	na
6-Methyl-4'-methoxyflavone (**2**)	nt	nt	nt	nt	+++	++	+
3-Hydroxy-4'-methoxyflavone (**3**)	nt	nt	nt	nt	+++	++	+
7-Hydroxy-5-methylflavone (**4**)	nt	nt	nt	nt	+++	++	+
3-Hydroxy-4'-methoxy-6-methylflavone (**5**)	nt	nt	nt	nt	+++	++	+
6-Methylflavone (**6**)	nt	nt	nt	+++	++	+	na
8-Methylflavone (**7**)	nt	nt	nt	+++	++	+	na
7-Hydroxy-3-methylflavone (**8**)	nt	nt	+++	++	+	na	nt
5,6,7-Trimethoxyflavone (**9**)	nt	+++	+++	++	+	na	nt
3-Hydroxy-6-methylflavone (**10**)	nt	+++	++	+	na	nt	nt
17β-Estradiol (**11**)	– –	±	na	nt	nt	nt	nt
Equol (**12**)	– – –	– –	±	na	nt	nt	nt
Naringenin (**13**)	–	na	nt	nt	nt	nt	nt
3'-Prenylated naringenin (**14**)	– –	–	na	nt	nt	nt	nt
8-Prenylated naringenin (**15**)	– –	–	na	nt	nt	nt	nt
6-Prenylated naringenin (**16**)	+	na	nt	nt	nt	nt	nt

Each individual compound was dissolved in EtOAc and the solution was used for coating Chromosorb W AW particles before the bioassay by the particle method (Horio *et al.*, 1992; Islam and Tahara, 2001a, b; Takayama *et al.*, 2004). The host-specific attractant cochliophilin A was used as standard. The mono- or dimethylether of equol did not show any activity at concentrations up to 100000 nM in this bioassay system.
+, attractant; –, repellant; na, non-active; nt, not tested.
Each treatment was repeated three times.

encystment. A further bioassay revealed that all flavonoid attractants appeared to act through a common and specific receptor because the activity of one flavonoid in the particle bioassay was blocked by another flavonoid when added homogenously to the zoospore suspension. These results suggest that the mode of action of the synthetic flavonoid attractants is slightly different from that of the host-specific cochliophilin A (**1**). Islam *et al.* reported that an approx. 10-fold higher concentration than the threshold concentration of the host-specific attractant cochliophilin A triggers the encystment of all attracted zoopores followed by germination of the cystospores (Islam *et al.*, 2003; Islam, 2008b).

The results of the particle bioassay revealed that the synthetic flavonoids having a hydrophobic B-ring with an alkylated (methylated) A-ring or a methoxylated B-ring with an unsubstituted A-ring in the flavonoid skeleton play important roles in higher attractant activity. It further revealed that the behaviour of peronosporomycete zoospores toward flavonoids is species-specific because repellants of *P. sojae* zoospores attracted the *A. cochlioides* zoospores (Tyler *et al.*, 1996). A further bioassay using different species of peronosporomycete zoospores is needed for a better understanding of the chemotactic behaviour of zoospores toward flavonoids.

Phytoestrogens show repellent activity toward A. cochlioides zoospores

Several flavonoids have exhibited potent estrogenic activities in mammalian systems. Some known estrogenic flavonoids such as equol (**12**), naringenin (**13**), 3'-, 8-, and 6-prenylated naringenins (**14**–**16**) (Takamura-Enya *et al.*, 2003) were tested to see whether estrogenic activity is correlated to the repellent activity of *A. cochlioides* zoospores or not. As expected, the powerful estrogenic compounds equol (**12**) and 8-prenylated naringenin (**15**) displayed potent repellent activity toward zoospores. Comparatively weak estrogenic compounds such as naringenin (**13**) and 3'-prenylated naringenin (**14**) also exhibited repellent activity toward zoospores of *A. cochlioides* at 0.1 μM and 0.01 μM, respectively. The mammalian estrogen 17β-estradiol (**11**) showed strong repellent activity at 0.1 μM in the same bioassay system

(Islam and Tahara, 2001a). However, 6-prenylated naringenin (**16**), which lacks estrogenic activity, displayed no activity toward zoospores at concentrations up to 0.01 μM. However, this compound showed attractant activity at 0.1 μM. These results further confirm that compounds having the estrogenic principle also possess repellent activity against *A. cochlioides* zoospores. The estrogenic and repellent activities of known phytoestrogens revealed to be correlated.

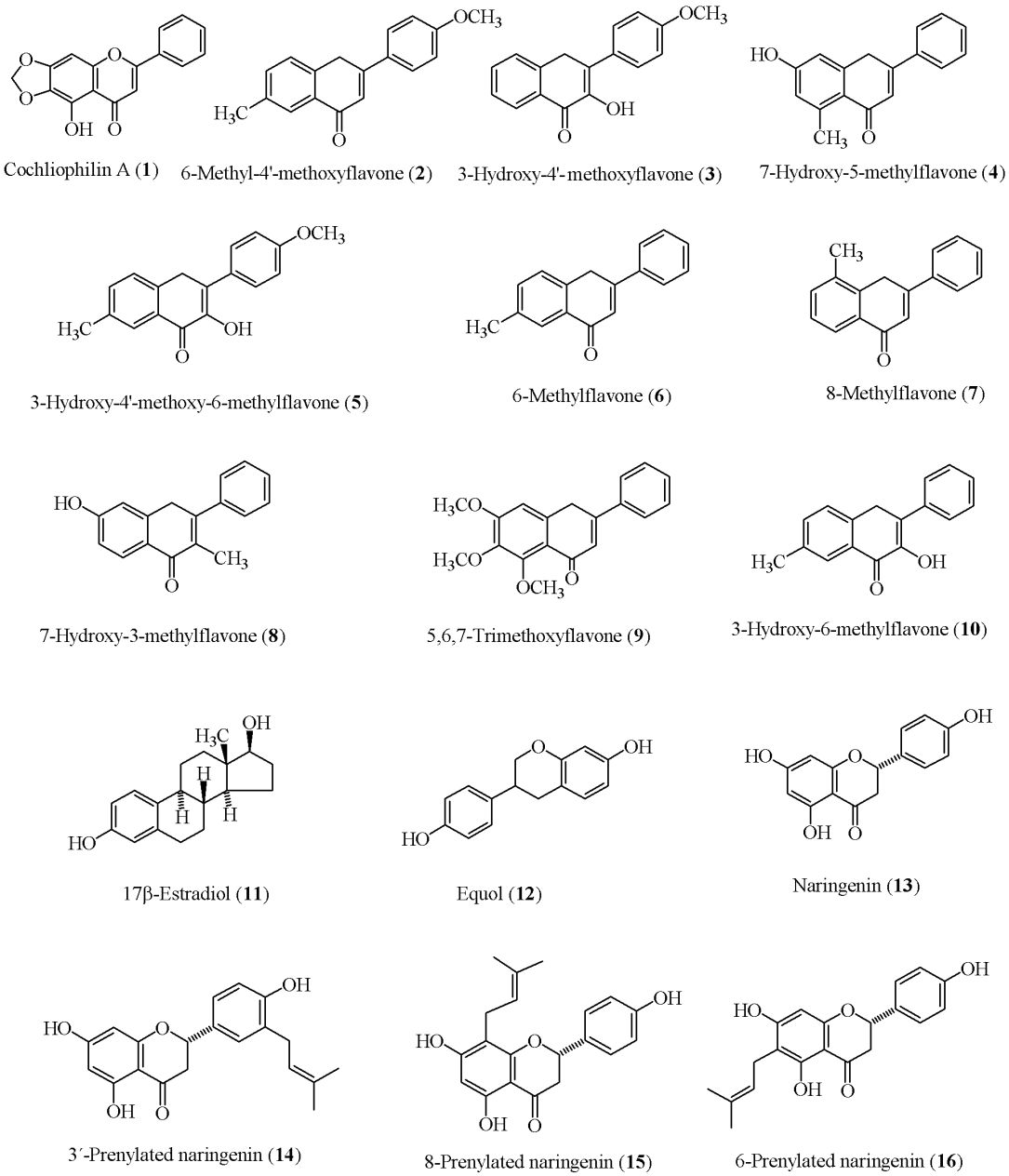

Fig. 1. Chemical structure of some compounds which regulate the motility behaviour (chemotaxis) of *Aphanomyces cochlioides* zoospores.

Phytoestrogens, particularly flavonoids, are sporadically reported in legumes and many other plants. The negative chemotaxis of zoospores toward phytoestrogens shown in the present paper raises the question whether the occurrence of this phenomenon is beneficial for plants from the viewpoint of ecology, particularly in host-parasite interactions (Islam and Tahara, 2001a; Islam, 2008b). In a previous study, Islam and Tahara (2001a) reported that methylated products of a synthetic estrogen, diethylstilbestrol (DES), exhibited attractant activity instead of repelling zoospores. However, unexpectedly the mono- and dimethylether of equol did not show any activity toward zoospores at concentrations up to 1 μM. These results suggest that repellent activity shown by the phytoestrogen equol (**12**) and the xenoestrogen DES might be executed by different signal transduction pathways.

The mechanism of very strong attractant activity of synthetic flavonoids to *A. cochlioides* zoospores shown in this report is difficult to explain by our current knowledge (Islam and Tahara, 2001b). Recently, it has been reported that silencing of a gene (*PsGPA1*) encoding the G-protein α subunit in the soybean pathogen *Phytophthora sojae* resulted in mutants having impaired chemotaxis toward the host-specific attractants daidzein and genistein and the mutants were incapable of infecting soybean seedlings (Hua *et al.*, 2008). Therefore, a further understanding of signal transduction pathways of positive (attractant) and negative (repellent) chemotaxis of *A. cochlioides* zoospores shown in the present report would help us to develop novel disease control strategies against peronosporomycete phytopathogens.

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