Secondary Metabolites from Nonhost Plants Affect the Motility and Viability of Phytopathogenic *Aphanomyces cochlioides* Zoospores

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The motile zoospores of the damping-off pathogen *Aphanomyces cochlioides* aggregate on host plants (*e.g.*, sugar beet, spinach) guided by the host-specific plant signal cochliophilin A before infection. To assess the potential role of secondary metabolites in nonhost resistance, acetone extracts of 200 nonhost traditional medicinal plants from Chinese and Bangladeshi origins were tested for the motility behaviour of *A. cochlioides* zoospores using a particle bioassay method. Nearly one third of the tested plant extracts exhibited diverse deleterious activities such as repellent, stimulant, motility halting and lysis against *A. cochlioides* zoospores. Among these active plants, an extract of the Chinese medicinal plant *Dalbergia odorifera* displayed potent repellent activity toward zoospores. Chromatographic separation of *D. odorifera* constituents revealed that the repellent activity was regulated by the cumulative effect of three motility-affecting isoflavonoids, *viz*. (±)-medicarpin (repellent at 150 μg/ml), (–)-claussequinone (stimulant at 100 μg/ml) and formononetin (stimulant and attractant at 50 μg/ml). A mixture (1:1:1, w/w/w) of these three compounds exhibited only repellent activity toward zoospores at a concentration lower than 50 μg/ml. These results suggest that nonhost plants might possess potential bioactive secondary metabolites to ward off zoosporic phytopathogens.

Key words: Chemotaxis of Zoospores, Repellent, Isoflavonoids

Introduction

Members of the Peronosporomycetes (Oömycetes in the old classification) (Dick, 2001) genera such as *Phytophthora, Pythium, Plasmopara, Aphanomyces* are among the most serious threats to agriculture and food production, causing devastating diseases in hundreds of plant hosts (Buczacki, 1983; Agrios, 1997; Islam and Tahara, 2001b). Some of them are also causing serious diseases in fishes, animals and even in humans (Mendoza *et al.*, 1996; Bruno and Wood, 1999). These fungus-like organisms fall within the kingdom Straminipila which also includes golden-brown algae, diatoms, and brown algae (Dick, 2001). Most of the Peronosporomycetes generate characteristic asexual motile zoospores which are propelled by two dissimilar flagella (heterokont). These motile spores are an important means of pathogen distribution and often the key infectious stage of the pathogenic species (Deacon and Donaldson, 1993; Islam and Tahara, 2001b; Judelson and Blanco, 2005). Our knowledge of the biology of Peronosporomycetes is limited, since their physiology differs from that of fungi; many fungicides are ineffective against them. New approaches are needed to identify novel targets and to develop biorational control measures to minimize the economic impact of these notorious phytopathogens.

Several species of the *Aphanomyces* genus are devastating pathogens of economically important crops and fishes (Papavizas and Ayers, 1974; Bruno and Wood, 1999). Among them, *Aphanomyces cochlioides* causes damping-off and root rot diseases in sugar beet, spinach and some other members of Chenopodiaceae and Amaranthaceae (Drechsler, 1929; Ui and Nakamura, 1963). Several lines of evidence support that the biflagellated motile zoospores of *A. cochlioides* locate and then aggregate on the host roots guided by the host-specific chemical signal cochliophilin A exuded from roots of the host plants (Horio *et al.*, 1992; Wen *et al.*, 2006). The attracted zoospores encyst and subsequently germinate on the root surface

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triggered by the same plant signal, and then penetrate the root tissue directly or *via* appresoria to initiate infection (Islam *et al.*, 2002a, 2003). In contrast to susceptible plants, it is hypothesized that the roots of nonhost plants may contain chemical signals responsible for their resistance (Islam and Tahara, 2001a). This hypothesis has been supported by the isolation of several zoospore-regulating compounds from some nonhost plants (Mizutani *et al.*, 1998; Begum *et al.*, 2002; Islam *et al.*, 2002b, 2004a). However, studies on screening of nonhost plants for identifying biologically active secondary metabolites toward phytopathogenic Peronosporomycete zoospores are very scant. Therefore, it may be important to survey more plant secondary metabolites from nonhost plants which interrupt the swimming behaviour and physiology of the zoospores for possible biorational control of the zoosporic phytopathogens.

This study aimed to screen extracts of 200 nonhost plants from Chinese and Bangladeshi origins, which are known as traditional herbal medicines. The activities of crude acetone extracts were evaluated against the motility and viability of *A. cochlioides* zoospores using a simple bioassay method (Islam *et al.*, 2004b). Nearly one third of the crude plant extracts showed diverse deleterious activities such as repellent, stimulant, motility halting, and lysis against *A. cochlioides* zoospores. Based on the bioassay results, the repellent principles in a Chinese traditional medicinal plant extract of *Dalbergia odorifera* were isolated by bioassay-guided chromatographic separation. This paper describes activities of crude nonhost plant extracts along with some pure compounds isolated from *D. odorifera* toward the zoospores of *A. cochlioides*. The role of plant secondary metabolites in protecting nonhost plants from the attack of zoosporic microorganisms and their potential application in the biorational management of Peronosporomycete phytopathogens are also briefly discussed.

Materials and Methods

General

Instrumental analyses were conducted using a JEOL JMS-AX 500 (EI) and JEOL JMS-SX102A (FD) instrument for mass spectrometry, a HITACHI U-3210 spectrophotometer for UV spectroscopy, a JEOL JNM-EX 270 instrument for 1D (^{1}H and ^{13}C) NMR spectroscopy, a JASCO Model J-20 spectrometer for CD, a digital polarimeter JAS CO DIP-370 for measuring specific rotation and a Bruker AMX500 instrument for 2D (^{1}H-^{1}H COSY, NOESY, HMQC and HMBC) NMR spectroscopy. Tetramethylsilane (TMS) was used as the internal standard in NMR spectroscopy. Gibbs reagent was used to detect isoflavonoids on TLC plates. TLC separations were carried out on thin layer plates (Merck Kieselgel 60 F_{254}, 0.25 μm thickness) using $CHCl_3$/MeOH (25:1). Silica gel 60 (spherical, 100–210 mm) (SiO_2, Kanto Chemical) was used for open column chromatography.

Plant materials and extraction

Chinese herbal medicines (100, each 10 g) were purchased from a local Chinese drug shop (Kamimura Kanyaku-do) in Sapporo, Japan in November 1997. Bangladeshi traditional medicinal plant samples (100, each 50–100 g) were collected in and around Comilla, Dhaka and Mymensingh districts of Bangladesh. The air-dried plant samples were ground, extracted with acetone (1 g sample in 6 ml acetone) and the crude extracts were used for the subsequent bioassay.

On the basis of screening results, the extract of the Chinese herbal drug *Dalbergia odorifera,* which showed repellent activity toward *A. cochlioides* zoospores, was selected for isolating the active principles. The heartwood of *D. odorifera* T. Chen is used as a traditional medicine, known as Jiangxiang, in China. It has been used to treat blood disorder, ischemia, swelling, necrosis, and rheumatic pain (Chang, 1981). Ground heartwood of *D. odorifera* (1.0 kg) was successively extracted with acetone and 60% methanol. The chemical fractionation and chromatographic procedures of the bioassay-directed isolation of active compounds are presented in Fig. 1.

Production of zoospores and bioassay

Aphanomyces cochlioides AC-5, which was isolated from the soil of a sugar beet field, was a gift from R. Yokosawa. It was grown for 6 d on a corn meal agar (Difco) plate (9 cm i. d.) at 20 °C, and zoospores were produced as described previously (Horio *et al.*, 1992; Islam and Tahara, 2001b; Islam *et al.*, 2004b). The motility behaviour and viability of zoospores in the presence of crude extracts or pure compounds were tested by a "particle bioassay" as described earlier (Horio *et al.*, 1992; Islam and Tahara, 2001b; Islam *et al.*, 2004b). Briefly, one drop of solution of each extract or pure compound dissolved in EtOAc or acetone was dropped onto a few particles of Chromosorb W AW (60–80

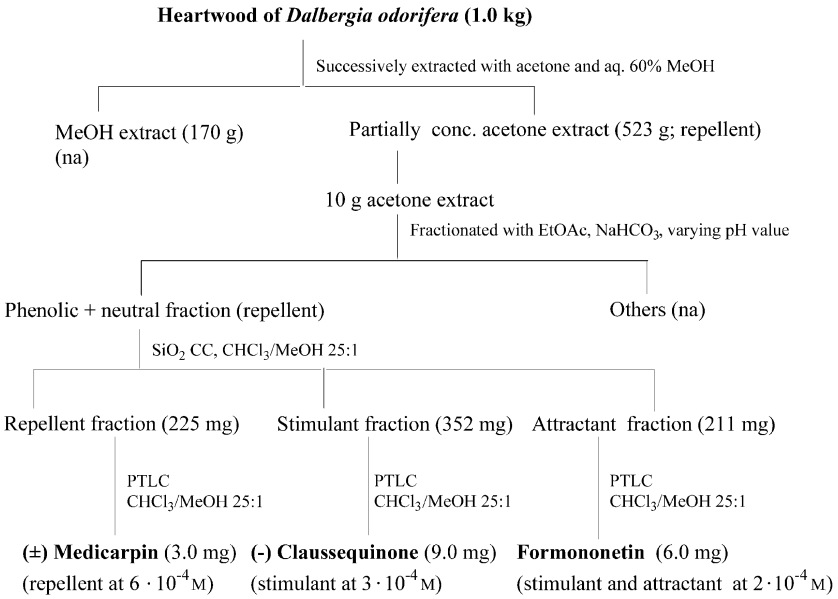

Fig. 1. Isolation procedure for compounds in a *Dalbergia odorifera* extract exhibiting repellent activity to zoospores of *Aphanomyces cochlioides*. SiO_2 CC, silica gel column chromatography; PTLC, preparative thin layer chromatography; na, not active.

mesh) on a watch glass. Excess solution was immediately absorbed by a tip of filter paper and the solvent was allowed to evaporate; each particle absorbed *ca.* 4 nl of solution (Takayama *et al.*, 2004). After evaporation of the solvent, one to two of the particles were carefully dropped into 2 ml of a zoospore suspension (*ca.* 10^5/ml) in a Petri dish (3 cm i. d.), and the motility behaviour of the zoospores around the particle(s) was observed microscopically up to 30 min after addition of the particle(s). Particles treated with solvent alone were used as control. Around the particles treated with an inactive extract or compound, the zoospores swam in an unvarying, regular manner and at a constant speed. In contrast, zoospores close to particle(s) coated with any active substance showed attractant, repellent, stimulant, halting or halting and bursting activity (Islam *et al.*, 2004b).

Cochliophilin A (**5**), a host-specific attractant of *A. cochlioides* zoospores, was synthesized (Horio *et al.*, 1992) and used in the bioassay as standard compound.

Results and Discussion

Activities of traditional medicinal plants toward zoospores

Screening tests are considered as promising tools to find out novel bioactive secondary metabolites from natural sources. Screening of 100 Chinese herbal medicines and 100 Bangladeshi traditional medicinal plant extracts revealed that some of the nonhost plants possess potential secondary metabolites having motility and viability-regulating activities towards *A. cochlioides* zoospores. Out of 100 Chinese herbal medicines, 18 showed attractant or repellent activities toward *A. cochlioides* zoospores (Table I). Zoospores of *A. cochlioides* were attracted by nine crude extracts whereas repelled by nine extracts of Chinese herbal medicines at 200–1000 μg/ml concentration. Crude extracts of *Paeonia suffruticosa* and *Dalbergia odorifera* showed potent repellent activity against *A. cochlioides* zoospores at 200 μg/ml. On the other hand, *Sinomenium acutum, Akebia* sp., and *Achyranthes* sp. extracts showed attractant activity at 200 μg/ml.

Amongst the Bangladeshi plant samples, 34 showed different levels and kinds of bioactivities on the motility behaviour (attractant, repellent, and stimulant) and viability (halting and bursting) of *A. cochlioides* zoospores (Table I). The extracts of *Amaranthus gangeticus, Acacia nilotica, Basella alba*, *B. rubra, Papaver somniferum, Aegle marmelos*, and *Capsicum annuum* (whole plant) showed potent attractant activity at 100 μg/ml, while *Ampelygonum chinense, Curcuma longa,* and *Azadirachta indica* displayed repellent activity at the

Table I. Motility and behaviour of *Aphanomyces cochlioides* zoospores toward Chinese and Bangladeshi medicinal plant extracts[a].

Plant species	Family	Organ	Zoospore bioassay results [μg/ml]			
			1000	500	200	100
Chinese plant samples						
Achyranthes sp.	Amaranthaceae	Root	+++	++	+	na
Akebia quinata	Lardizabalaceae	Aerial parts	+++	++	+	na
Allium chinensis	Liliaceae	Bulb	++	+	na	na
Angelica dahurica	Umbelliferae	Root	–	±	na	na
Atractylodes lancea	Compositae	Rhizome	–	na	na	na
Aucklandis lappa	Compositae	Root	–	na	na	na
Cyperus rotundus	Cyperaceae	Rhizome	–	±	na	na
Copotis sp.	Ranunculaceae	Rhizome	+	±	na	na
Dalbergia odorifera	Leguminosae	Heartwood	– – –	– – –	–	na
Foeniculum vulgare	Umbelliferae	Fruit	– –	–	na	na
Glycyrrhiza glabra var. *glandulifera*	Leguminosae	Root	–	±	na	na
Leonurus heterophyllus	Labiatae	Aerial parts	++	+	na	na
Paeonia suffruticosa	Ranunculaceae	Root bark	– – –	– –	–	na
Phellodendron chinense	Rutaceae	Stem bark	++	+	na	na
Puerraria lobata var. *chinensis*	Leguminosae	Root	++	+	na	na
Rehmania glutinosa var. *hueichingensis*	Scrophulariaceae	Root tuber	++	+	na	na
Scutellaria baicalensis	Labiatae	Root	–	na	na	na
Sinomenium acutum	Menispermaceae	Stem	+++	++	+	na
Bangladeshi plant samples						
Abroma augusta	Sterculiaceae	Aerial parts	ss	s	na	na
Acacia catechu	Leguminosae	Aerial parts	h & b	h & b	h & b	na
Acacia nilotica	Leguminosae	Aerial parts	+++	++	++	+
Aegle marmelos	Rutaceae	Aerial parts	+++	+++	++	+
Allium cepa	Liliaceae	Bulb	–	±	na	na
Allium sativum	Liliaceae	Bulb	+	±	na	na
Amaranthus gangeticus	Amaranthaceae	Whole plant	+++ & h	+++ & h	++ & h	+[b]
Amaranthus tricolor	Amaranthaceae	Root	+++ & h	++ & h	+	na
Amaranthus caudatus	Amaranthaceae	Root	++	+	na	na
Amaranthus magnostanus	Amaranthaceae	Root	++	+	na	na
Ampelygonum chinense	Polygonaceae	Whole plant	– – –	– –	– –	–
Azadirachta indica	Meliaceae	Leaves	– – –	– –	– –	–
Basella alba	Basellaceae	Whole plant	+++	+++	++	+[b]
Basella rubra	Basellaceae	Whole plant	+++	++	++	+
Capsicum annuum	Solanaceae	Whole plant	+++	+++	+++	+[b]
Capsicum annuum	Solanaceae	Ripe fruit	s/+	s	na	na
Catharanthus roseus	Apocyanaceae	Whole plant	sss/+++	ss/++	s/+	s
Cuminum cyminum	Umbelliferae	Seed	+++	++	+	na
Curcuma longa	Zingiberaceae	Leaves and rhizome	– – –	– –	– –	–
Elettaria cardamomum	Zingiberaceae	Fruit	–	–	na	na
Eucalyptus sp.	Myrtaceae	Aerial parts	– – –	– –	–	na
Hibiscus rosa-sinensis	Malvaceae	Aerial parts	+++	+	na	na
Lannea coromandelica	Anacardiaceae	Stem bark	h & b	h & b	h & b	na
Leucas zeylanica	Labiatae	Whole plant	++	+	na	na
Mangifera indica	Anacardiaceae	Leaves	– –	–	na	na
Nigella sativa	Ranunculaceae	Seed	ss	s	na	na
Papaver somniferum	Papaveraceae	Whole plant	+++	+++	++	+
Phyllanthus emblica	Euphorbiaceae	Aerial parts	– – –	– –	–	na
Terminalia arjuna	Combritaceae	Aerial parts	– –	–	na	na
Terminalia bellirica	Combritaceae	Aerial parts	– –	–	na	na
Terminalia chebula	Combritaceae	Aerial parts	– –	–	na	na
Trigonella foenum-graceum	Leguminosae	Seed	ss	ss	na	na
Vitex negundo	Verbenaceae	Leaves	ss	s	na	na
Zingiber officinale	Zingiberaceae	Rhizome	s/+	s	na	na

[a] Particle bioassay method: na, not active; s, stimulant; +, attractant; –, repellent; h & b, halting motility and bursting zoospore; h, halting motility.

[b] Active up to 30 μg/ml. 1 g of ground plant material was extracted with 6 ml of acetone. The acetone-soluble part was directly used for screening in the zoospore bioassay. Only the active extracts were subjected to the quantitative bioassay.

same level of concentrations. In addition, all attracted zoospores were immediately halted and became round cystospores in case of *A. gangeticus*. However, the presence of *Lannea coromandelica* and *Acacia catechu* extracts instantly stopped the motility and subsequently burst *A. cochlioides* zoospores when dosed at 200 μg/ml. Just after the drop of Chromosorb W AW particles containing a *Lannea* extract, most of the zoospores around the particles were halted immediately and continued to spread their activity over the whole Petri dish within 2–3 min. Some of the affected zoospores acquired a round shape, expanded losing their flagella. The inner cell organelles seemed to be reorganized and became dark granular precipitates moving like Brownian movement, and finally dispersed into the zoospore suspension by rupturing the cell membrane.

It is noteworthy to mention that the nature of attracting behaviours of zoospores toward some nonhost extracts and host-specific cochliophilin A (**5**), respectively, was quite different. In case of **5**, the zoospores were attracted and developed a mass of zoospores around the treated particle and then became round-shaped cystospores. All cystospores were germinated to form germ tubes within 30 min after encystment (Islam *et al.*, 2003), while they were only attracted by nonhost plant extracts without developing any mass of zoospores around the treated particle and did not encyst by shedding flagella. This difference might be due to the lack of some releasing factors associated with the nonhost extract responsible for the encystment process of zoospores. In this study, number and degree of zoospore-regulating activities by Bangladeshi medicinal plant extracts were much higher than those of Chinese herbal medicines. The possible reasons behind this difference might be related to the long storage and preservation of the Chinese herbal drugs in the shops compared to the fresh materials in the case of Bangladeshi plant samples.

After the screening, the active principles of two Bangladeshi traditional medicinal plant extracts, *Lannea coromandelica* (motility halting and bursting) and *Amaranthus gangeticus* (attractant and halting), have been characterized (Islam *et al.*, 2002b, 2004a). The interesting factor that caused motility inhibition and subsequent lysis of the zoospores in the *L. coromandelica* extract was characterized by MALDI-TOF-MS as polyflavonoid tannins (Islam *et al.*, 2002b). On the other hand, bioassay-guided chromatographic separation of the *A. gangeticus* constituents revealed that the cumulative effects of two chemically distinct secondary metabolites regulated taxis and subsequent motility inhibition of the zoospores (Islam *et al.*, 2004a). The attractant was identified as the rare *N-trans*-feruloyl-4-*O*-methyldopamine and the motility inhibitor was nicotinamide. The first compound had no inhibitory effect on zoospore motility whereas nicotinamide immediately halted the motility and caused encystment in a dose-dependent manner (Shimai *et al.*, 2002; Islam *et al.*, 2004a). Interestingly, the cysts induced by nicotinamide regenerated zoospores (*ca.* 80%) instead of germinating.

Repellent principles of Dalbergia odorifera

Three isoflavonoids were isolated from the extract of *D. odorifera* by a detailed chromatographic separation procedure (Fig. 1) as active principles. Their chemical structures were assigned on the basis of physicochemical data including 1D and 2D NMR (Fig. 2).

The first isolate gave an intense molecular ion peak at *m/z* 270 ([M]$^+$, 100%) in the FD-MS spectrum and HR-EI-MS analysis established the molecular formula of **1** as $C_{16}H_{14}O_4$. The UV, EI-MS, ^{1}H and ^{13}C NMR data were found to match with those reported for medicarpin (Goda *et al.*, 1992; Herath *et al.*, 1998). Thus the structure of **1** was confirmed as (±)-medicarpin (3-hydoxy-9-methoxypterocarpan, **1**) ($[\alpha]_D^{28}$ 0° in MeOH, c = 0.045).

The HR-EI mass spectrum of compound **2** exhibited the exact molecular mass (calcd. 286.0841, found 286.0834) corresponding to the molecular formula $C_{16}H_{14}O_5$. The UV, EI-MS, ^{1}H and ^{13}C NMR data agreed with those of the reported (–)-claussequinone (**2**) (Gottlieb *et al.*, 1975). The optical data recorded for **2** was $[\alpha]_D^{28}$ –31.5° in MeOH (c = 0.0069).

The HR-EI-MS and the ^{1}H NMR data of compound **3** estimated its molecular formula as $C_{16}H_{12}O_4$. The ^{1}H and ^{13}C NMR assignments were compared with those in the literature and all chemical shifts and coupling patterns were found to be identical with those of formononetin (Bezuidenhoudt *et al.*, 1987; Goda *et al.*, 1992). Thus **3** was confirmed as formononetin (7-hydroxy-4′-methoxyisoflavone). Compound **3** was acetylated and the bioactivity of the acetylated formononetin **4** was also evaluated. Previous studies on this plant have resulted in the isolation of a number of dif-

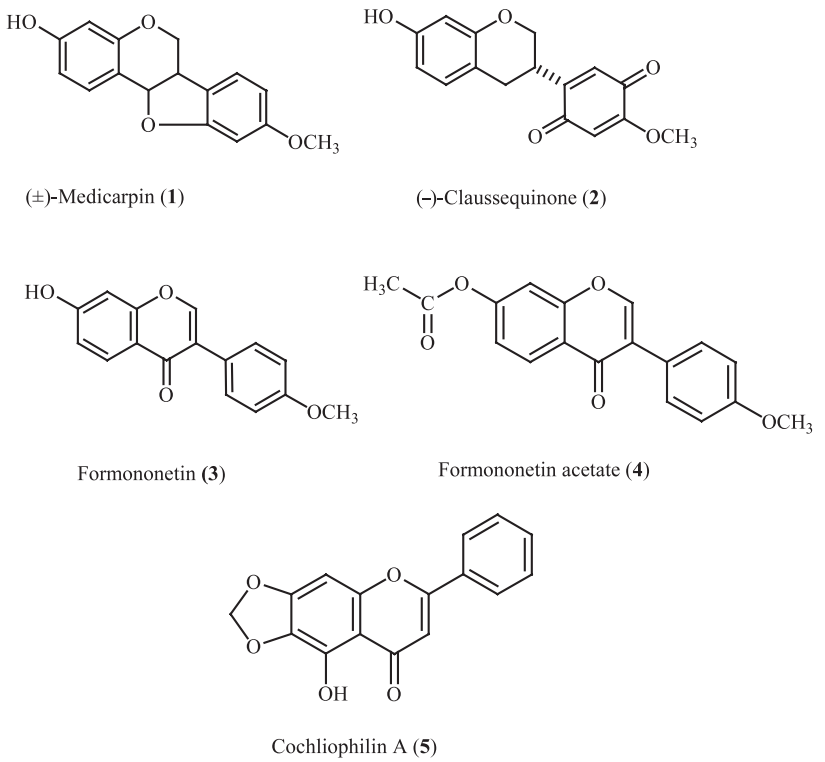

Fig. 2. Structures of *Dalbergia odorifera* constituents **1**–**3**, an acetylated derivative of formononetin and the host-specific attractant cochliophilin A.

ferent types of flavonoids and other phenolic constituents (Goda *et al.*, 1992; Miller *et al.*, 1989; Ogata *et al.*, 1990; Chan *et al.*, 1998).

The bioactivity of the isolated compounds **1**–**3** and the derivative **4** were evaluated by a particle method. The possible combinations of **1**–**4** were also tested. All the compounds showed different motility activities of *A. cochlioides* zoospores (Table II). (±)-Medicarpin (**1**) showed repellent activity at 150 μg/ml, while (–)-claussequinone (**2**) and formononetin (**3**) showed stimulating, and attracting and stimulating activity at 100 and 50 μg/ml, respectively. A mixture of the three isoflavonoids (1:1:1, w/w/w) exhibited repellent activity at 50 μg/ml. The repellent activity of (±)-medicarpin (**1**) was enhanced in the presence of **2** and **3**. Compounds **1**–**3** are known to be antimicrobial as well as bioregulating in human physiology (Hamburger *et al.*, 1987; Miller *et al.*, 1989; Goda *et al.*, 1992; Chan *et al.*, 1998). In a previous study, a mixture of two different compounds from nonhost *Portulaca oleracea* inhibited the motility of *A. cochlioides* zoospores, whereas when applied individually, they exhibited stimulant and repellent activities, respectively (Mizutani *et al.*, 1998).

Screening results and the negative chemotaxis of *D. odorifera* isoflavonoids to zoospores shown in this paper suggest that nonhost plants might ward off zoosporic phytopathogens by secondary metabolites. Medicarpin has been found as phytoalexin in many legumes. Antimicrobial activities of the three *D. odorifera* isoflavonoids **1**–**3** have been reported but the repellent activity against zoospores by these compounds shown in this report has not been claimed. Negative chemotaxis by isoflavonoids which are found in many plants, raises questions on the occurrence of this phenomenon particularly during host/parasite interactions. In a previous study mammalian estrogenic compounds including phytoestrogens have been shown to possess potent repellent activity toward zoospores of *A. cochlioides* (Islam and Tahara, 2001a).

The screening results and subsequent isolation of repellant factors from *D. odorifera* suggest that nonhost plants might possess potential secondary metabolites, which may be useful for the biora-

Table II. Chemotactic and motility behaviour of *Aphanomyces cochlioides* zoospores to *Dalbergia odorifera* isoflavonoids and their mixture in the particle bioassay.

Compound(s)/extract	Responses of *Aphanomyces cochlioides* zoospores toward particles coated with chemical compound(s)/extract [μg/ml][a]			
	150	100	50	25
(±)-Medicarpin (**1**)	–	na	na	na
(–)-Claussequinone (**2**)	ss	s	na	na
Formononetin (**3**)	+++ & sss	++ & ss	+ & s	na
Formononetin acetate(**4**)	s/+	na	na	na
1 + **2**	– –	–	na	na
1 + **3**	– –	–	na	na
1 + **4**	–	na	na	na
1 + **2** + **3**	– – –	– –	–	±
1 + **2** + **3** + **4**	– –	–	na	na
2 + **3**	sss	ss	s	na
2 + **4**	+ & s	na	na	na
2 + **3** + **4**	s	na	na	na
3 + **4**	ss	s	na	na
Crude acetone extracts[b]	na	na	na	na
Cochliophilin A (**5**)	nt	nt	nt	+++

[a] +, Attractant; –, repellant; s, stimulant; na, not active; nt, not tested. Each treatment was repeated three times.
[b] Crude acetone extracts of *Dalbergia odorifera* showed clear repellent activity at 200 μg/ml. Each individual compound or their mixture (equal ratios in weight basis) or crude extract 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, 2001b). The host-specific attractant cochliophilin A was used as standard, which showed attractant activity as low as 10^{-9} M in this bioassay system.

tional control of phytopathogenic Peronosporomycetes. Further studies on the isolation of more zoospore regulating principles from other nonhost plants may result in interesting compounds or lead compounds for developing an effective control strategy to combat the notorious Perenosporomycete plant parasites.

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