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The Third Naturally Occurring Attractant toward Zoospores of Phytopathogenic *Aphanomyces cochlioides* from the *Spinacia oleracea* Host Plant

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A bioassay-guided survey of spinach leaf constituents resulted in 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone being identified as the third naturally-occurring attractant in the host plant toward the zoospores of its pathogen, *Aphanomyces cochlioides*. The isolate showed attracting activity around Chromosorb W AW particles (60–80 mesh) coated with a 10^{-5} M solution in a zoospore suspension. However, this activity was 1/100–1/1000 less than that of cochliophilin A, an attractant in the roots of spinach. Bioassays with the present isolate and related compounds revealed that 5,3′,4′-trihydroxy-3-methoxy-6,7-methylenedioxyflavone did not possess attractant activity, but rather weak antagonistic activity toward the former two attractants from spinach.

Key words: *Aphanomyces cochlioides*; zoospore attractant; spinach; *Spinacia oleracea*; 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone

Aphanomyces cochlioides (Saprolegniaceae: Saprolegniales: Oomycota) Drechsler is a causal fungus of root rot disease in spinach (*Spinacia oleracea* L.) and infects some other species of Chenopodiaceae and Amaranthaceae.[1)] It is believed from some lines of evidence that when zoospores of the *Aphanomyces* spp. infest the host, they have to be initially attracted to an exudate from the roots of the host plant.[2-4)] Cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, **1**) was isolated by Horio *et al.* in 1992[5)] from spinach roots as a highly potent host-specific attractant toward the zoospores of *A. cochlioides*.

A second attractant, in addition to **1**, has been isolated from the roots of *Chenopodium album*, one of the host plants, and characterized as *N-trans*-feruloyl-4-*O*-methyldopamine (**2**).[6)]

Chemically characterized and host-specific attractants toward other phytopathogenic fungal zoospores have also been reported: for example, indole-3-carbaldehyde from the roots of cabbage for *A. raphani*,[7)] prunetin from the roots of pea for *A. euteiches*,[3)] and daidzein and genistein from soybean roots for *Phytophthora sojae*.[8)]

We report here the isolation and structural elucidation of a third attractant to zoospores of *A. cochlioides* from the aerial parts of spinach which was revealed to be 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (**3**) by spectroscopic and chemical methods. A glucuronic acid conjugate of **3** has already been reported as one of the constituents of spinach flavonoids,[9)] whereas the corresponding aglycone (**3**) was isolated for the first time as a natural product.

Methods and Materials

General procedures. The following analytical instruments were used: NMR spectra were recorded by a JEOL AX270 spectrometer, EIMS (70eV) and FDMS spectra were recorded with JEOL JMS-SX102A and JMS-AX500 spectrometers, and optical rotation was recorded with a JASCO DIP-370 polarimeter. Melting point (mp) data were determined with a Yanaco MP-30 micro-melting point apparatus and are uncorrected.

Fungus and zoospore suspension.[5)] *A. cochlioides* isolate AC-5 was presented by Prof. R. Yokosawa of Health Science University of Hokkaido. The strain was grown for 7–10 days on corn meal agar (Difco) in a Petri dish (9 cm i.d.) at 20°C. Half of the mycelium-covered agar was transferred to another Petri dish containing 80 ml of distilled water. To remove the nutrients from the agar, the water in the second Petri dish was changed three times (total of 240 ml) at intervals of 20 min. The Petri dish containing mycelia and agar, and a final 25 ml of distilled water, was then allowed to stand for 15–24 h at 20°C

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to promote the release of zoospores. The zoospore concentration was adjusted to *ca.* 1.2×10^5/ml with distilled water immediately before the bioassay was carried out.

Bioassay.[5] Particles of Chromosorb W AW (60–80 mesh) were used as a carrier for the test compounds. The particles were placed on a watch glass, and on to them was dripped 5 μl of an ethyl acetate solution of the test compound. Any excess solution on the watch glass was immediately absorbed with a piece of filter paper, and the particles were then air-dried at room temperature. A few of the treated particles were carefully dropped into an aqueous suspension (2.5 ml) of zoospores in a small Petri dish (3 cm i.d.). The behavior of the zoospores around these particles was observed microscopically for 4 min after adding the particle(s). Control particles were treated with the solvent alone. Around the particles treated with an inactive compound, the zoospores moved in an unvarying, regular manner, and at a constant speed. In contrast, the zoospores close to particles that had been treated with any attractant responded in a different way. Relatively large numbers of zoospores aggregated around the particles, moving in a complex zigzag or circular manner, and at increasing speed. There was a clear gradient in the zoospore density which noticably decreased with increasing distance from the particles. The response to the positive control, a particle coated with a solution of cochliophilin A (**1**) at 10^{-8} M, was shown as a photograph in our previous paper[5] and evaluated as having (++) activity. Attracting to a degree less than that shown is denoted as (+). Cochliophilin A (**1**) used in the present study was prepared as previously described.[5]

Plant material and isolation of the zoospore attractant. Leaves of spinach cv. Solomon (141 kg) were frozen and crushed for extraction with MeOH. The MeOH soluble material was concentrated, and the residue miscible with diethyl ether (308 g) was charged at the top of a 3-kg silica gel column. The column was successively washed with 9 liter of $CHCl_3$ and 3 liter of $CHCl_3$-MeOH = 9:1, and subsequently eluted with a further 3 liter of $CHCl_3$-MeOH = 9:1 and then with 6 liter of $CHCl_3$-MeOH = 4:1 to give *ca.* 140 g of an active fraction (active at 30 ppm by the particle method). This fraction dissolved in 500 ml of EtOAc was washed with 5% aq. $NaHCO_3$ to make the active fraction free from carboxylic acids. The resulting crude attractant (SOL-A, 129 g) was used for further fractionation.

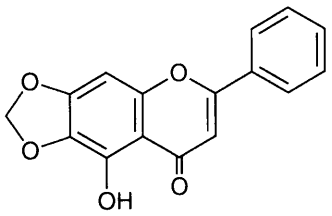
This crude attractant, SOL-A (1.46 g), was subjected to silica gel column chromatography (30 g of adsorbent) and subsequently eluted with 80 ml of toluene-acetone = 9:1 and then 60 ml of toluene-acetone = 4:1. The latter active eluate was concentrated to give 468 mg of a solute. Part of the concentrate (314 mg) was rechromatographed in a Cosmosil 75 C_{18}-OPN column (2.1 × 17 cm) which was successively washed with 120 ml of MeCN-H_2O = 3:7 and 60 ml of a 1:1 mixture. The active constituents were eluted in the subsequent 60 ml of MeCN-H_2O = 1:1 and 60 ml of the 3:2 mixture, which yielded 24.9 mg of solute. Final column chromatography was performed in a silica gel (3 g) column. The column charged with the former solute was washed with 9 ml of $CHCl_3$, and subsequently eluted with 6 ml of $CHCl_3$-acetone = 9:1. The concentrated eluate yielded 1.2 mg of needles (SOLAC) and 4.4 mg of constituents in the mother liquor (SOLAL). The crystallized compound (SOLAC) showed zoospore-attracting activity at 3 ppm (++), 1 ppm (+), and 0.3 ppm (−), whilst the constituents in the mother liquor (SOLAL) showed attraction at 1 ppm (++) and 0.3 ppm (−). Interestingly, the specific activity of SOLAL seemed to be greater than that of SOLAC. However, we could isolate neither any synergistic compound, nor a more active compound than SOLAC.

The active principle (SOLAC) was also detected by HPLC (Inertsil PREP-ODS column, 20 × 250 mm; detection, UV absorption at 254 nm) as an isolated peak at t_R32.2 min in an active fraction collected by Sephadex LH-20 (twice), silica gel, and Cosmosil (twice) column chromatographies applied to SOL-A.

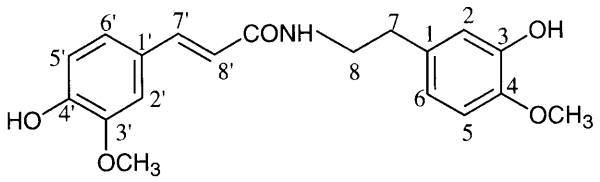
When 46 g of SOL-A was successively fractionated by Sephadex LH-20 (twice), silica gel and Cosmosil column chromatographies, the final active fraction (59.9 mg) yielded 43.0 mg of SOLAC as needles from 50% dioxane. Contrary to our expectation that the leaf extract may contain small amounts of highly active attractant(s), the isolate did not show high specific activity and its content was not particularly low.

Spinach glucuronides. Spinach flavonoid glucuronides were isolated according to the procedure of Aritomi and Kawasaki[9] by using 2680 g of commercially available spinach leaves. In the present isolation, Amberlite XAD-2 as a glucuronide adsorbent and Toyopearl HW40C for column chromatography in MeOH-AcOH = 1000:1 were used to prepare the glucuronide fraction (890 mg). The constituent glucuronides were finally isolated by medium-pressure liquid chromatography over Lober LiChroprep DIOL with $CHCl_3$-MeOH = 12:1 as a mobile phase.

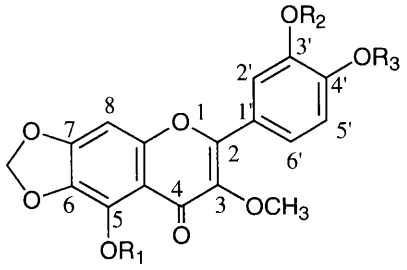
The fast-moving minor constituent, 32 mg from 530 mg of the Toyopearl glucuronide fraction, was identified by comparing its physicochemical properties (MS, NMR, UV and mp) with those reported for 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone 4′-*O*-β-D-glucuronide (**4**). Compound **4**: 18 mg recrystallized from 32 mg of crude crystals



1: cochliophilin A



2: *N-trans*-feruloyl-4'-*O*-methyldopamine



3: R_1=R_3=H, R_2=CH_3
4: R_1=H, R_2=CH_3, R_3=GlcU
5: R_1=R_2=H, R_3=GlcU
6: R_1=R_2=R_3=H
7: R_1=R_2=H, R_3=CH_3
8: R_1=H, R_2=R_3=CH_3
9: R_1=R_2=CH_3, R_3=H
10: R_1=R_2=R_3=CH_3

Fig. 1. Structures of Methylenedioxyflavones (**1**, **3**–**10**) Derived from Spinach and a Dopamine Derivative (**2**) from *Chenopodium album*.

from 50% dioxane. FDMS *m/z* (%): 534 (M^+, 10), 358 (M^+-176, 100); mp 167–170°C (lit.,[9] 165–168°).

The slow-moving major constituent, 430 mg from 530 mg of the Toyopearl glucuronide fraction was similarly identified as 5,3′,4′-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-*O*-β-D-glucuronide (**5**). Compound **5**: 280 mg recrystallized from 430 mg of crude crystals from 50% dioxane. FDMS *m/z* (%): 521 (M^++1, 78), 344 (M^+−176, 100); mp 200–203°C (lit.,[9] 197–198°).

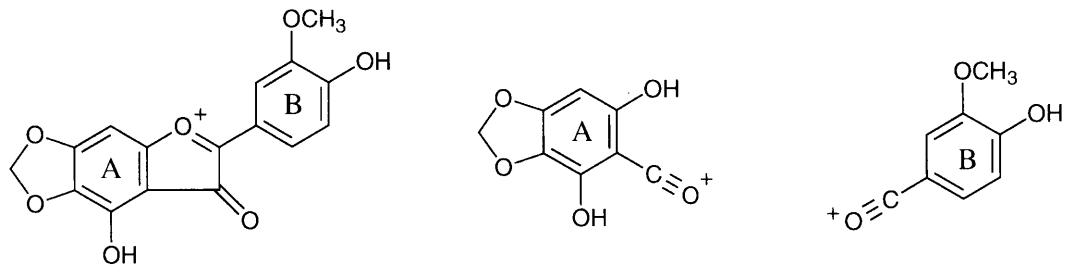
Conversion of the spinach flavonoids. Hydrolysis of the spinach glucuronides: Spinach glucuronide **5** (21 mg) was dissolved in 4 ml of water and combined with *Ampullaria* β-glucuronidase (Wako Pure Chem. Ind.) of 7920 units/0.36 ml of solution and 32 ml of a 0.07 M sodium acetate buffer (pH 5.0). The mixture was allowed to stand for 12 h at 60°C. The reaction product was worked up in the usual way to give the corresponding aglycone (**6**) in 79% yield. The other glucuronide (**4**) was similarly hydrolized like **5** to give 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (**3**), which was indistinguishable from the zoospore attractant (**3**) that had been isolated from the leaves of spinach by chromatographic and spectroscopic methods.

Diazomethane methylation of 6: Compound **6** was dissolved in a mixture of 1.5 ml of methanol and 2 ml of isopropanol to which had been added an excess amount of TMS diazomethane/hexane (*ca.* 3 eq.) at ambient temperature, and the mixture stood for 3 h. The reaction mixture was concentrated and subjected to PTLC and then to medium-pressure liquid column chromatography in a Lobar B LiChroprep DIOL column to give minor amounts of various methylated products, 5,3′-dihydroxy-3,4′-dimethoxy-6,7-methylenedioxyflavone (**7**), 5-hydroxy-3,3′,4′-trimethoxy-6,7-methylenedioxyflavone (**8**), and 3,5,3′,4′-tetramethoxy-6,7-methylenedioxyflavone (**10**).

4′-Hydroxy-3,5,3′-trimethoxy-6,7-methylenedioxyflavone (9): This compound was prepared according to the method of Aritomi and Kawasaki.[9] Major spinach glucuronide **5** was permethylated, and the product was then hydrolyzed under alkaline conditions. Finally, the methylated glucuronic acid conjugate was hydrolized by β-glucuronidase to yield **9**.

Physicochemical properties of the flavonoids used in the bioassay. 5,4′-Dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (**3**): Pale yellow needles, mp 199–202°C (uncor.). TLC: RP-18 F_{254S} 0.25 mm plates (Merck) in MeCN-H_2O=4:1, *Rf* 0.51; and silica gel 60 F_{254} 0.25 mm plates (Merck) in $CHCl_3$-acetone=5:1, *Rf* 0.46. HR EIMS: *m/z* M^+ 358.0706, $C_{18}H_{14}O_8$ (calcd., 358.0688). EIMS *m/z* (%): 358 (M^+, 100), 357 (51), 343 (46), 315 (38), 181 (9), 180 (7), 165 (11), 151 (16), 123 (5). UV λ_{max} (MeOH) nm (log ε): 242 (4.13), 278 (4.10), 349 (4.33); + NaOMe, 252, 270, 387; + $AlCl_3$ + HCl, 244, 266, 293, 376; + NaOAc, 277, 350, 405 (br. sh.). ^{1}H-NMR δ (270 MHz, $CDCl_3$): 3.86 (3H, s, 3-OCH_3), 3.98 (3H, s, 3′-OCH_3), 5.99 (1H, s, 4′-OH), 6.10 (2H, s, -OCH_2O-), 6.55 (1H, s, H-8), 7.04 (1H, d, J=8.3 Hz, H-5′), 7.64–7.69 (2H, m, H-2′ and H-6′), 12.61 (1H, s, 5-OH).

5,3′-Dihydroxy-3,4′-dimethoxy-6,7-methylenedioxyflavone (**7**): Powder, mp 247–250°C. HR EIMS: *m/z* M^+ 358.0713, $C_{18}H_{14}O_8$ (calcd., 358.0688). EIMS *m/z* (%): 359 (M^++1, 21), 358 (M^+, 100), 357 (71), 343 (9), 341 (7), 340 (7), 339 (6), 315 (16), 181 (4), 180 (4), 151 (6). UV λmax (MeOH) nm (log ε): 257 (4.11), 278 (4.08), 348 (4.29); + NaOMe, 274, 290sh, 369; + $AlCl_3$ + HCl, 265, 293, 374; + NaOAc, unchanged. ^{1}H-NMR δ (270 MHz,



a) *m/z* 315 (M^+ - 43, 38 %) **b)** *m/z* 181 (9 %) **c)** *m/z* 151 (16 %)

Fig. 2. Mass Fragments of the Spinach Leaf Attractant toward *Aphanomyces cochlioides* Zoospores (SOLAC, **1**).
a) Mass fragment characteristics of 3-methoxyflavone; **b)** retro-Diels-Alder fission product from the A-ring; **c)** retro-Diels-Alder fission product from the B-ring.

$CDCl_3$): 3.87 (3H, s, 3-OCH_3), 3.97 (3H, s, 4′-OCH_3), 5.70 (1H, s, 3′-OH), 6.09 (2H, s, -OCH_2O-), 6.53 (1H, s, H-8), 6.96 (1H, d, J=8.3 Hz, H-5′), 7.67–7.72 (2H, m, H-2′ and H-6′), 12.61 (1H, s, 5-OH).

5-Hydroxy-3,3′,4′-trimethoxy-6,7-methylenedioxyflavone (**8**): Pale yellow needles, mp 254–257°C. HR EIMS: *m/z* M^+ 372.0879, $C_{19}H_{16}O_8$ (calcd., 372.0845). EIMS *m/z* (%): 372 (M^+, 100), 371 (51), 357 (41), 341 (12), 329 (30), 186 (3), 175 (8), 165 (7). UV λmax (MeOH) nm (log ε): 248 (3.78), 279 (3.79), 346 (4.02); +NaOMe, 250sh, 295, 325; +$AlCl_3$+HCl, 242, 264, 293, 375; +NaOAc, unchanged. ^{1}H-NMR δ (270 MHz, $CDCl_3$): 3.87 (3H, s, 3-OCH_3), 3.96 and 3.97 (two 3H, both s, 3′- and 4′-OCH_3), 6.10 (2H, s, -OCH_2O-), 6.55 (1H, s, H-8), 6.99 (1H, d, J=8.3 Hz, H-5′), 7.67–7.72 (2H, m, H-2′ and H-6′), 12.61 (1H, br. s, 5-OH).

The physicochemical properties of the following compounds have been reported in ref. 9: 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone 4′-*O*-β-D-glucuronide (**4**), 5,3′,4′-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-*O*-β-D-glucuronide (**5**), 5,3′,4′-trihydroxy-3-methoxy-6,7-methylenedioxyflavone (**6**), 4′-hydroxy-3,5,3′-trimethoxy-6,7-methylenedioxyflavone (**9**), and 3,5,3′,4′-tetramethoxy-6,7-methylenedioxyflavone (**10**).

Results and Discussion

The flavone skeleton of the spinach leaf attractant, SOLAC, with the molecular formula $C_{18}H_{14}O_8$ (HR-MS, M^+ 358.0688) was deduced from the positive result of the Shinoda test,[10] which gave an anthocyanidin product with a destinctive reddish color. The part structures of SOLAC were feasible from the ^{1}H-NMR (in $CDCl_3$) spectral data: two methoxy groups (δ 3.86 and 3.98), a methylenedioxy at δ 6.10 (2H, s) (the corresponding methylene resonated at δ 6.10[5] in **1**), an aromatic proton for the A-ring at δ 6.55 (1H, s) (H-8 resonated at δ 6.60 in **1**), and 1,2,4-trisubstituted B-ring protons at δ 7.04 (1H, *ortho*-coupled doublet, J=8.3) and 2H (δ 7.64–7.69, overlapped *meta*-coupled doublet and *ortho-meta*-coupled double doublets). The presence of C-5-OH was confirmed by the ^{1}H-NMR detection of hydrogen-bonding OH at δ 12.61[5] and the observation of a UV bathochromic shift of methanolic band I at 349 nm to 376 nm caused by the addition of aluminium chloride.[11] The large bathochromic shift (38 nm) induced by the sodium methoxide reagent was indicative of the presence of 4′-OH in the B-ring of SOLAC.[11]

The mass spectrum of SOLAC exhibited the following fragments: **a** (M^+-43, 38%) characteristic of 3-methoxyflavones, and **b** and **c** (in Fig. 2) both arising from retro-Diels-Alder fission, respectively from the A- and B-rings.[12] Thus, the complete structure of spinach leaf attractant SOLAC was evaluated on the basis of its spectroscopic properties to be 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (**3**) which corresponds to the aglycone of one of the spinach flavone glucuronides reported by Aritomi and Kawasaki.[9] The evaluation was verified by directly comparing the physicochemical properties of SOLAC (**3**) with those of an authentic compound prepared by the hydrolysis of spinach flavone glucuronide (**4**).

To identify the structural requirements of spinach methylenedioxyflavone as the zoospore attractant, spinach glucuronides (**4** and **5**), and different derivatives (**5–10**) by diazomethane methylation of **3** were subjected to the zoospore bioassay. As shown in Table 1, only **3** from compounds **3–10** showed clear attractant activity by using Chromosorb W AW particles coated with 10 ppm solution, whilst **4–10** were inactive up to 1000 ppm (particle method). We were therefore unable to deduce any structure-activity relationship for **3**, in the present study.

Both **1** and **3** contained a 6,7-methylenedioxy group as a common part-structure. From a chemotaxonomical point of view, the rare 6,7-methylenedioxy-substituted flavonoids have been found in Rutaceae, Chenopodiaceae and Amaranthaceae, and sporadically in some other families (Iridaceae, Polygonaceae and Solanaceae).[13,14] This

Table 1. Attractant Activity of Spinach Flavonoids and Related Compounds toward *Aphanomyces cochlioides* Zoospores

Compound No. (see Fig. 1.)	Zoospore response[b]			
Dose (ppm)[a]	1000	100	10	1
3	++	++	++	+
4	−	−	nt	nt
5	−	−	nt	nt
6	−	−	nt	nt
7	−	−	nt	nt
8	−	−	nt	nt
9	−	−	nt	nt
10	−	−	nt	nt

[a] Concentration (ppm) of dose: Chromosorb W AW particles (60–80 mesh) were coated with the test solution at the described concentration (particle method) as described in the methods and materials section. The positive controls, cochliophilin A (**1**) and amide **2**, showed + + activity at $1/10^8$ M (0.0028 ppm) and $1/10^5$ M (3.4 ppm), respectively.
[b] ++ strong attraction; + weak but clear attraction; − no attraction; nt, not tested

Table 2. Effect of Non-attractant Flavones on the Flavonoidal Attractants (**1** and **3**) toward *Aphanomyces cochlioides* Zoospores

Non-attractant flavone (1000 ppm)[a]	Zoospore response[b]	
	Cochliophilin A (**1**) 0.028 ppm ($1/10^7$ M)	Spinach leaf flavone (**3**) 100 ppm
6	−	+
7	++	++
8	++	++
9	++	++
10	++	++
None[c]	++	++

[a] Chromosorb W AW particles (60–80 mesh) were coated with a mixture of a non-attractant flavone (**6–10**) and one of the attractants (**1** and **3**).
[b] See footnote to Table 1.
[c] attractant alone (**1** or **3**)

must be indispensable for an ecologically effective host attractant to have limited natural distribution. However, in the case of spinach attractant cochliophilin A (**1**), the methylenedioxy part-structure seems not to have been essential to the potent activity, because the attractant activity of the 6,7-dimethoxy derivative of **1** was apparently stronger than that of **1** as described in our previous paper.[15]

On the other hand, the substitution pattern of **3** in the B-ring was different from that of the phenethylamine moiety in the second host-specific attractant, *N-trans*-feruloyl-4′-*O*-methyldopamine (**2**). 3′-Hydroxy-4′-methoxy substitution at the dopamine moiety has been revealed to be significant for the attractant activity of **2**.[15] However, the methylenedioxyflavone (**7**) substituted by 3′-hydroxy and 4′-methoxy groups like **2** displayed no activity.

Although the 3′,4′-dihydroxy isomer (**6**) by itself did not show any activity toward zoospores, a large excess of **6** suppressed or partly inhibited the attractant activity of **1** and **3** (Table 2). However, such a kind of antagonistic activity of **6** was not apparent against amide attractant **2**. This finding concerning the antagonistic activity of **6** against host-specific flavonoidal zoospore attractants (**1** and **3**) seems to be significant for making further studies on characterization of the receptor and signal transduction in the zoospore attraction to the roots of the host plant, and for developing regulators which would disturb motile zoospores to orient the host plants in soil water.

It has already been confirmed that host specific attractant **1** is secreted from spinach roots.[16] The significant role of host-specific attractants for pathogenic zoospores has been reviewed.[17] However, the practical role of spinach leaf flavonoids in the interaction between spinach and its pathogen, *Aphanomyces cochlioides*, is not clear at present. It may be concerned with the life-cycle of *A. cochlioides* in field grown spinach; for example, mycelial growth stimulation and oospore germination.

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References

1) Ui, T. and Nakamura, S., Black root disease of sugar beet: Pathogenicity and host-specificity of the causal fungus *Aphanomyces cochlioides* Drechsler. *Tensai-Kenkyukai-Hokoku* (Japanese), **3**, 78–95 (1963).
2) Yokosawa, R., Ogoshi, A., and Sakai, R., Taxis of *Aphanomyces raphani* Kendrick to hypocotyl of host plant and role of exudate from hypocotyl. *Ann. Phytopath. Soc. Japan*, **40**, 46–51 (1974).
3) Yokosawa, R., Kuninaga, S., and Sekizaki, H., *Aphanomyces euteiches* zoospore attractant isolated from pea root; Prunetin. *Ann. Phytopath. Soc. Japan*, **52**, 809–816 (1986).
4) Rai, P. V. and Strobel, G. A., Chemotaxis of zoospores of *Aphanomyces cochlioides*. *Phytopathology*, **56**, 1365–1369 (1966).
5) Horio, T., Kawabata, Y., Takayama, T., Tahara, S., Kawabata, J., Nishimura, H., and Mizutani, J., A highly potent attractant of *Aphanomyces cochlioides* zoospores from its host, *Spinacia oleracea*. *Experientia*, **48**, 410–414 (1992).
6) Horio, T., Yoshida, K., Kikuchi, H., Kawabata, J., and Mizutani, J., A phenolic amide from roots of *Chenopodium album*. *Phytochemistry*, **33**, 807–808 (1993).
7) Yokosawa, R. and Kuninaga, S., *Aphanomyces raphani* zoospore attractant isolated from cabbage: Indole-3-aldehyde. *Ann. Phytopath. Soc. Japan*

(Japanese), **45**, 339–343 (1979).
8) Morris, P. F. and Ward, E. W. B., Chemoattraction of zoospores of the soybean pathogen, *Phytophthora sojae*, by isoflavones. *Physiol. Mol. Plant Pathol.*, **40**, 17–22 (1992).
9) Aritomi, M. and Kawasaki, T., Three highly oxygenated flavone glucuronides in leaves of *Spinacia oleracea*. *Phytochemistry*, **23**, 2043–2047 (1984).
10) Ingham, J. L., Tahara, S., and Dziedzic, S. Z., New 3-hydroxyflavanone (dihydroflavonol) phytoalexins from the papilionate legume *Shuteria vestita*. *J. Nat. Prod.*, **49**, 631–638 (1986).
11) Markham, K. R. and Mabry, T. J., Ultraviolet-visible and proton magnetic resonance spectroscopy of flavonoids. In "The Flavonoids," ed. by Harborne, J. B., Mabry, T. J., and Mabry, H., Capman & Hall, London, pp. 45–77 (1975).
12) Mabry, T. J. and Markham, K. R., Mass spectrometry of flavonoids. In "The Flavonoids", ed. by J. B. Harborne, T. J. Mabry, and H. Mabry, Chapman & Hall, London, pp. 78–126 (1975).
13) Sanderson, S. C., Ge-Ling, C., McArther, E. D., and Stutz, H. C., Evolutionary loss of flavonoids and other chemical characters in the Chenopodiaceae. *Biochem. Sys. Ecol.*, **16**, 143–149 (1988).
14) Wollenweber, E., Flavones and flavonols. In "The Flavonoids: Advances in Research since 1986," ed. by Harborne, J. B., Chapman & Hall, London, pp. 259–335 (1994).
15) Kikuchi, H., Horio, T., Kawabata, J., Koyama, N., Fukushi, Y. Mizutani, J., and Tahara, S., Activity of host-derived attractants and their related compounds toward the zoospores of phytopathogenic *Aphanomyces cochlioides*. *Biosci. Biotechnol. Biochem.*, **59**, 2033–2035 (1995).
16) Tahara, S. and Ingham, J. L. Simple flavones possessing complex biological activity. In "Studies in Natural Product Chemistry," Vol. 22, ed. by Atta-ur-Rahman, Elsevier, Amsterdam, pp. 457–505 (2000).
17) Deacon, J. W., Ecological implications of recognition events in the pre-infection stages of root pathogens. *New Phytologist*, **133**, 135–145 (1996).