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Host-specific plant signal and G-protein activator, mastoparan, trigger differentiation of zoospores of the phytopathogenic oomycete *Aphanomyces cochlioides*

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Abstract

We found that the gradient of a host-specific attractant, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone) isolated from the roots of spinach triggered encystment followed by germination of zoospores of *Aphanomyces cochlioides* at a concentration less than micromolar order. This compound did not affect the growth and reproduction of this phytopathogen up to 10^{-6} M concentration in the culture medium. We also observed that mastoparan, an activator of heterotrimeric G-protein could inhibit the motility of zoospores and then strikingly effect encystment followed by 60–80% germination of cysts. Concomitant application of cochliophilin A and mastoparan showed stronger encystment followed by 100% germination of cysts. In addition, we have observed that chemicals interfering with phospholipase C activity (neomycin) and Ca^{2+} influx/release (EGTA and loperamide) suppress cochliophilin A or mastoparan induced encystment and germination. These results suggest that G-protein mediated signal transduction mechanism may be involved in the differentiation of the *A. cochlioides* zoospores. This is the first report on the differentiation of oomycete zoospores initiated by a host-specific plant signal or a G-protein activator.

Introduction

Oomycetes that infect plants via zoospores have a multi-stage pre-infection sequence. The zoospores of those phytopathogens are specifically attracted to the potential infection sites of host roots by chemotaxis (Deacon and Donaldson, 1993; Zentmyer, 1961), and then orient, encyst, germinate and immediately initiate penetration directly or via appressoria (Bircher and Hohl, 1997; Islam et al., 2001). This sequence is extremely rapid, leading to infection within 30–40 min after the zoospore arriving at the host surface (Deacon and Donaldson, 1993). The sequence of pre-infection events has been characterized best for *Phytophthora, Pythium* and *Aphanomyces* spp., and some of the potential host-derived elicitors or triggers have been identified (Horio et al., 1992, 1993; Morris and Ward, 1992; Yokosawa and Kuninaga, 1979; Yokosawa et al., 1986) (Figure 1). For example, daidzein (**1**) and genistein (**2**) which are exuded from the roots of soybean into the rhizosphere are specific attractants of soybean pathogen *Phytophthora sojae* (Morris and Ward, 1992; Morris et al., 1998). The other host-derived compounds so far reported are indole 3-carbaldehyde (**3**) from cabbage seedlings (Yokosawa and Kuninaga, 1979), and prunetin (**4**) from pea seedlings (Yokosawa et al., 1986), which showed specific attraction to *A. raphani* and *A. euteiches*, respectively.

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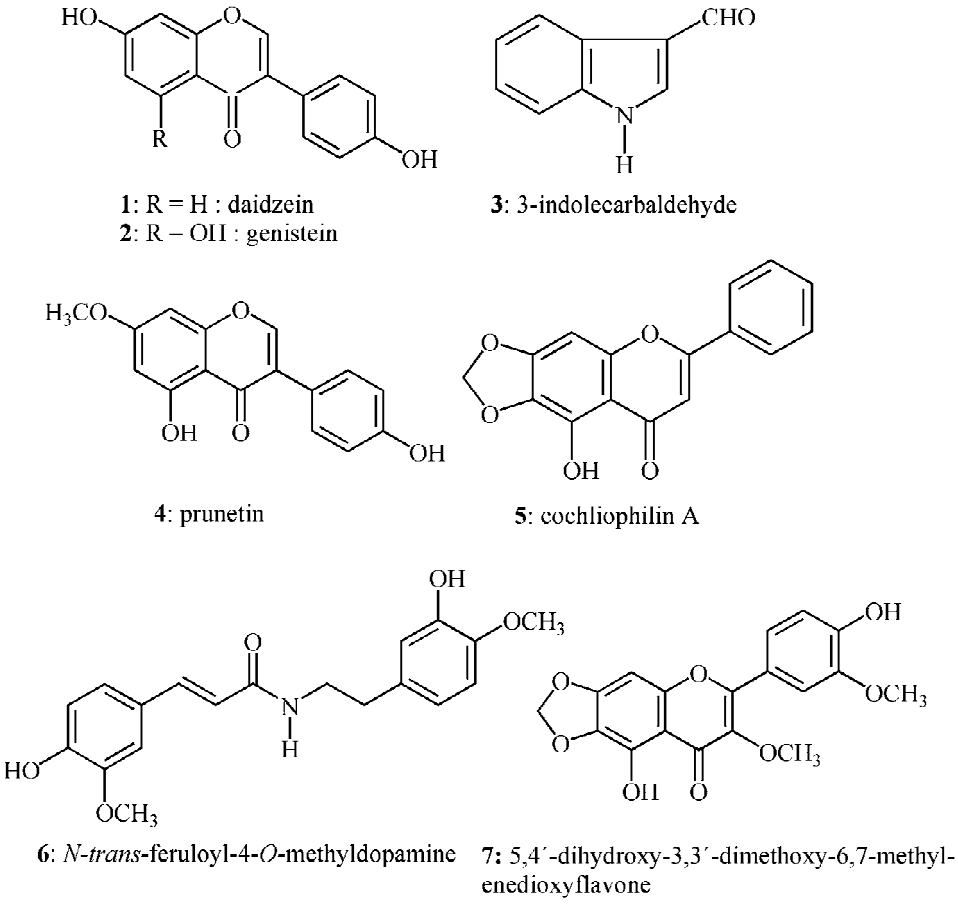

Figure 1. Some host-derived attractants for phytopathogenic oomycete zoospores.

All these compounds showed their attractant activity at a concentration as low as micromolar to nanomolar level.

Zoospores of the phytopathogenic oomycete *Aphanomyces cochlioides* aggregate at the host root by chemotaxis and then undergo a sequence of physiological changes leading to the infection (Islam et al., 2001). This phytopathogen has a limited host range, namely, sugar beet, spinach, *Chenopodium album*, and a few other members of Chenopodiaceae and Amaranthaceae (Ui and Nakamura, 1963). We identified cochliophilin A (**5**) (active at 3×10^{-10} M, particle method), as a host-specific attractant of zoospores of the *A. cochlioides* from the roots of spinach (Horio et al., 1992). A phenolic amide (*N-trans*-feruloyl-4-*O*-methyldopamine, (**6**) was also isolated from the roots of another host plant *C. album* as a potent attractant of *A. cochlioides* zoospores (active at 10^{-8} m by the particle method) (Horio et al., 1993). The third attractant from the host, 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylene dioxyflavone (**7**) (active at 10^{-6} M, particle method) was isolated from the leaves of spinach (Tahara et al., 2001; Tahara and Ingham, 2000). Cochliophilin A (**5**) had also been isolated as a non-antifungal flavone from the roots of sugar beet inoculated with the fungus *Rhizoctonia solani* (Takahashi et al., 1987). We identified cochliophilin A (**5**) in the roots of sugar beet and *Chenopodium album* (our unpublished data). Moreover, we estimated that fresh spinach roots contained approximately 1.9×10^{-5} mol/kg cochliophilin A (**5**) and exude sufficient amount (34 ng/plant/day) of this compound for attracting the zoospores of *A. cochlioides* (Tahara and Ingham, 2000). So far, the distribution of compounds **5** and **6** are completely restricted in the hosts (Chenopodiaceae) of *A. cochlioides*. Thus, Tahara and Ingham (2000) suggested that chemotaxis might be a part of

the mechanism that determines the host range of this phytopathogen (Tahara and Ingham, 2000).

Despite of the discovery of some host-specific chemoattractants of zoospores, it is unclear whether the same signaling molecule induces encystment and germination or these following events are regulated by different host signals. Moreover, it is unknown whether the stages of pre-infection are necessarily under separate control or a part of the signaling cascade. This is important because a success of infection depends on the completion of sequential events. Deacon (1996) suggested that zoospores may be induced to encyst by the effect of specific root surface components. Evidence supporting the involvement of any host-specific plant signal in differentiation of pathogenic zoospores has been lacking. However, *in vitro* studies revealed that zoospores were encysted by root surface mucilage, fucosyl residues, pectin, alginate or specific polysaccharides, lectin or monoclonal antibodies specific for flagella, and some host root extracts (Deacon and Donaldson, 1993). Transmembrane Ca^{2+} fluxes were found to be associated with encystment and cyst germination of *Phytophthora* spp. indicating that Ca^{2+} might play a vital role in signal transduction pathways of oomycete zoospores (Connolly et al., 1999; Warburton and Deacon, 1998). All other components of the signal transduction pathway are yet to be known in oomycete zoospores.

Mastoparan is a cationic amphipathic tetradecapeptide isolated from wasp venom and acts as a generic activator of animal heterotrimeric GTP binding regulatory proteins (G-proteins) (Ross and Higashijima, 1994). As G-proteins are also believed to be key components of signal transduction pathways in many other motile cells (van Es and Devreotes, 1999), we asked whether mastoparan was capable of acting as an agonist by eliciting encystment followed by germination of zoospores in absence of the host-specific plant signal cochliophilin A. On the other hand, the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield inositol 1,4,5-triphosphate (IP_3) and diacyl-glycerol (DAG) is a common feature of signal transduction pathways in animal cells and most probably also in plants. Because this reaction is frequently catalyzed by G-protein-mediated activation of specific phospholipase C (PLC) isoenzymes, we also attempted to identify PLC inhibitors that are capable of blocking the differentiation of zoospores elicited by host-specific signal cochliophilin A. Neomycin is a positively charged aminoglycosides which is widely used eukaryotic PLC inhibitor (McDonald and Mamrack, 1995). We also tested the effect of this inhibitor in presence of cochliophilin A and mastoparan.

We investigated the factors responsible for encystment and germination of the zoospore of *A. cochlioides* and describe here the new findings that not only the host-specific attractant but also an activator of heterotrimeric G-protein, mastoparan, regulates signal transduction regarding the development of some steps in the life cycle of this oomycete phytopathogen.

Materials and methods

Chemicals

Cochliophilin A (**5**) and *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) used in this experiment were synthesized previously (Horio et al., 1992, 1993). 5,4′-Dihydroxy-3,3′-dimethoxy-6,7-methylene dioxyflavone (**7**) was isolated from the leaves of spinach (Tahara et al., 2001). Mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Ly s-Lys-Ile-Leu-amide, MP), Mastoparan 17 (Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Leu-Ala-Ly s-Lys-Leu-Leu-amide, Mas17), EGTA, loperamide and neomycin (fradiomycin sulfate) were purchased from reputed pharmaceutical companies.

Culture of phytopathogen and preparation of zoospores

A. cochlioides (AC-5) was a gift from Professor R. Yokosawa, which was isolated from the soil of sugar beet field. Culture of *A. cochlioides* and production of zoospores were conducted as described previously (Islam and Tahara, 2001). Briefly, the phytopathogen was grown for 4–6 days on a corn meal agar (CMA) (Difco) plate (9 cm i.d.) at 20 °C. About half of the agar layer on the plate covered with mycelia was divided into four parts and transferred to a petri dish containing about 70–80 mL of sterilized water. To wash out nutrients from the agar medium, the water in the petri dish was changed for three times with 80 ml of sterilized water at 20 min intervals. Finally, 5–6 mL of sterilized water containing 0.1 mM calcium chloride was added and allowed to stand for 16–18 h at 20 °C to promote the release of zoospores. Concentration of zoospores was adjusted to about 10^5 mL^{-1} with sterilized water before bioassay or microscopic preparations.

Induction of encystment and germination of zoospores by host-specific attractants or mastoparan

Taxis, encystment and germination of zoospores by host-specific compounds and mastoparan were carried out by "particle bioassay method" as reported previously (Horio et al., 1992; Islam and Tahara, 2001; Mizutani et al., 1998). Briefly, 5 μL of solution of each chemical dissolved in EtOAc or acetone, and adjusted to an appropriate concentration, was dropped onto a few particles of Chromosorb W AW (ca. 170–240 μm) on a watch glass. Excess solution was immediately absorbed by a tip of filter paper and the particles were allowed to evaporate the solvent. It was estimated that each particle holds the amount of compounds equivalent to ca. 4 nL of the test solution (Takayama, 1999). One or two of these particles were carefully dropped into 2 mL of a zoospore suspension (10^5 mL^{-1}) in a small petri dish (3 cm i.d.). Therefore, the particle exposed to high volume of water diffused coated compounds to the surrounding water and develops a gradient of test compound around it. So, the actual concentration of the solution around a particle during the bioassay must be far smaller than the concentration of test solution used to coat it. The motility of the zoospores around the particles was observed microscopically up to 60 min after addition of the particles. Control particles were treated with the solvent alone. Around particles treated with an inactive compound, the zoospores moved in an unvarying, regular manner and at a constant speed. In contrast, zoospores close to particle(s) treated with any active compound responded in one of the following ways. (1) Attractant activity: relatively large number of zoospores aggregated around the particles, moving with increased speed in a complex zigzag or circular manner. There was a clear gradient in zoospore density that decreased with increasing distance from the particle. (2) Encystment and germination activity: membrane bounded zoospores became immobile and gradually changed into spherical spores called cystospores surrounded by the cell wall. The cystospores were germinated forming a germ tube when specific signal substance(s) are present. To confirm the bioactivity, each treatment was replicated at least 3 times under equivalent conditions.

Solution of host-specific compounds was freshly prepared in EtOAc. Mastoparan and mastoparan 17 were dissolved in MeOH (1 mg mL^{-1}) and stored at -20 °C. A series of dilution was done by EtOAc before use. Inhibitors were suspended in water and directly added to the zoospore suspension 5 min before the bioassay. In case of concomitant application, appropriate amount of solution of each compound was mixed well to make certain concentration of each compound in the mixture. The mixture of the compounds was used to coat Chromosorb particles.

Fixation and electron microscopy

Some Chromosorb particles treated with a solution of known concentration of the test compound were dipped into a 2–3 mL zoospore suspension (10^5 mL^{-1}) taken on a small petri dish (2 cm i.d.) and allowed to stand for certain periods (5, 10, 15, 20, 30, 40, 60 min) for elicitation of encystment and germination. Excised root tips (1–2 mm) of 6 day-old spinach seedlings grown on soaked paper towels were immersed into a zoospore suspension on small petri dish for 1 h (Islam et al., 2001). After certain intervals, the particles/root tips were taken out very carefully from the zoospore suspension by a Pasteur pipette to a thin cover glass and gently washed three times with sterilized water to remove the non-encysted zoospores. The specimens were then fixed on the cover glass for 2 h in 2% buffered glutaraldehyde (TAAB, UK) in distilled water at room temperature (about 23 °C). After dehydration in a graded acetone series (50%, 70%, 90%, 95% and 99.5%), they were critical-point dried using liquid CO_2. The specimens were mounted on a metallic stub and coated with 10 nm of platinum-palladium using a sputter coater. The coated specimen was observed under a scanning electron microscope (JEOL, JSM-6301F) with an accelerating voltage of 5 kV. Chemical induction of encystment and germination of zoospores was carried out on the SEMpore membrane by the addition of mastoparan or cochliophilin A through Chromosorb particles. After certain period, the specimens were fixed in buffered glutaraldehyde and rests of the microscopic preparations were similar as described above.

Influence of host-specific attractants on radial growth and oospore formation of Aphanomyces cochlioides *in solid culture*

The test compounds were suspended in water and then mixed with melted agar (about 50 °C) to make appropriate concentration in the agar medium. A mycelial disk (8 mm i.d.) of mycelia grown on CMA medium of isolate AC-5 was placed at the center of a petri dish (9.0 cm i.d.) containing either 0 or 10^{-12} to 10^{-6} M concentration of the host-specific attractant in the

CMA medium. Each treatment was replicated 4 times. Colonies were grown for 4 days after which the radial growth of the mycelium was measured and the mean value of each treatment was calculated. Data for host-specific compounds were calculated as relative value ± SE of control for standardization. The number of oospores formed (after 4 days) in at least 20 microscopic fields (25× magnification) was counted and the mean value of each treatment was compared to that of the control. The data were expressed as relative value ± standard error (SE) of control treatment.

Influence of host-specific attractants on the release of zoospores

The procedure for zoospore production was exactly same as described in the section above. Here, before incubation a certain amount of water suspended test compounds were mixed well to make appropriate concentration in the final solution in which zoospores are released. Each treatment was replicated 4 times. After 16 h of incubation, the number of zoospores per ml of zoospore suspension was counted microscopically. The mean value of each treatment was compared to that of the control. The data were expressed as the relative value ± SE of control.

Results

Taxis, encystment and germination of zoospores by host-specific attractants

The effects of three host-derived attractants, cochliophilin A (**5**), *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) and 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxy-flavone (**7**) have been assayed by particle bioassay method and the results are presented in Table 1. Cochliophilin A (**5**) induced encystment of the attracted zoospores at a range of 10^{-8} to 10^{-6} M concentration in a dose dependent manner (Table 1) and formed a mass of cystospores on and around the Chromosorb particles (Figure 2A–C). Initially the attracted zoospores became sluggish, moved in a circular fashion, halted and rapidly changed into round-shaped cystospores. Interestingly, the attracted zoospores landed and encysted on the surface of Chromosorb particle coated with 10^{-7} to 10^{-6} M solution of cochliophilin A (Figure 2A–C). All encysted zoospores germinated (100%) on and around the particles within 30–40 min (Figure 2B, C, E, F). The cystospores germinated close to the particles coated with host-specific attractants showed germ-tubes tropism toward the particles (Figure 2B, F). The particles coated with lower than 10^{-8} M concentration showed attractant activity but did not induce encystment of zoospores. On the other hand, the control particles treated with solvent alone neither affected the normal motility of zoospores in the aquatic medium nor resulted in encystment of any zoospore (Figure 2D). The other two host-specific attractants (**6** and **7**) also induce encystment and germination, albeit at higher concentrations.

The effect of cochliophilin A (**5**) on the encystment and germination of zoospore was evaluated by the direct application of **5** suspended in water at a range of 10^{-12} to 10^{-6} M concentration. The direct application of **5** into the zoospore suspension as a homogeneous solution at a range of 10^{-12} to 10^{-8} M just stimulated the motility of zoospores for 10–15 min without resulting any encystment and germination. However, at higher concentrations (10^{-7} to 10^{-6} M) of **5** in the above conditions, it showed no effect on the motility of zoospores. Interestingly, very slow release of 5×10^{-12} to 5×10^{-10} M cochliophilin A (**5**) solution to the zoospore suspension by a microsyringe showed strong stimulation of the motility of zoospores followed by encystment and germination (about 60–80%) (data not shown). In most cases, the stimulated zoospores formed the clumps of aggregated cells scatteredly at the bottom of glass petri dish, and then encysted and germinated (Figure 2G). The germ tubes of the spores germinated a little far from the aggregate center exhibited tropism toward the aggregate center (Figure 2G).

Mastoparan, a peptide agonist toward heterotrimeric G-protein, elicits differentiation of zoospores

The effect of mastoparan on the differentiation of *A. cochlioides* zoospores are presented in Table 2 and illustrated in Figure 3. The swimming zoospores of *A. cochlioides* were halted almost immediately after dropping the mastoparan coated particles into the zoospore suspension at a range of 10–50 μM concentration in a dose dependent manner (Figure 3A, B). Some of the stimulated zoospores showed circular movement for few seconds prior to halt. When mastoparan coated Chromosorb particles dropped in a dense population of zoospore suspension, immobilized zoospores initially seemed to be connected each other by their posterior flagella (Figure 3E, F). All the aggregated zoospores (Figure 3F) settled down

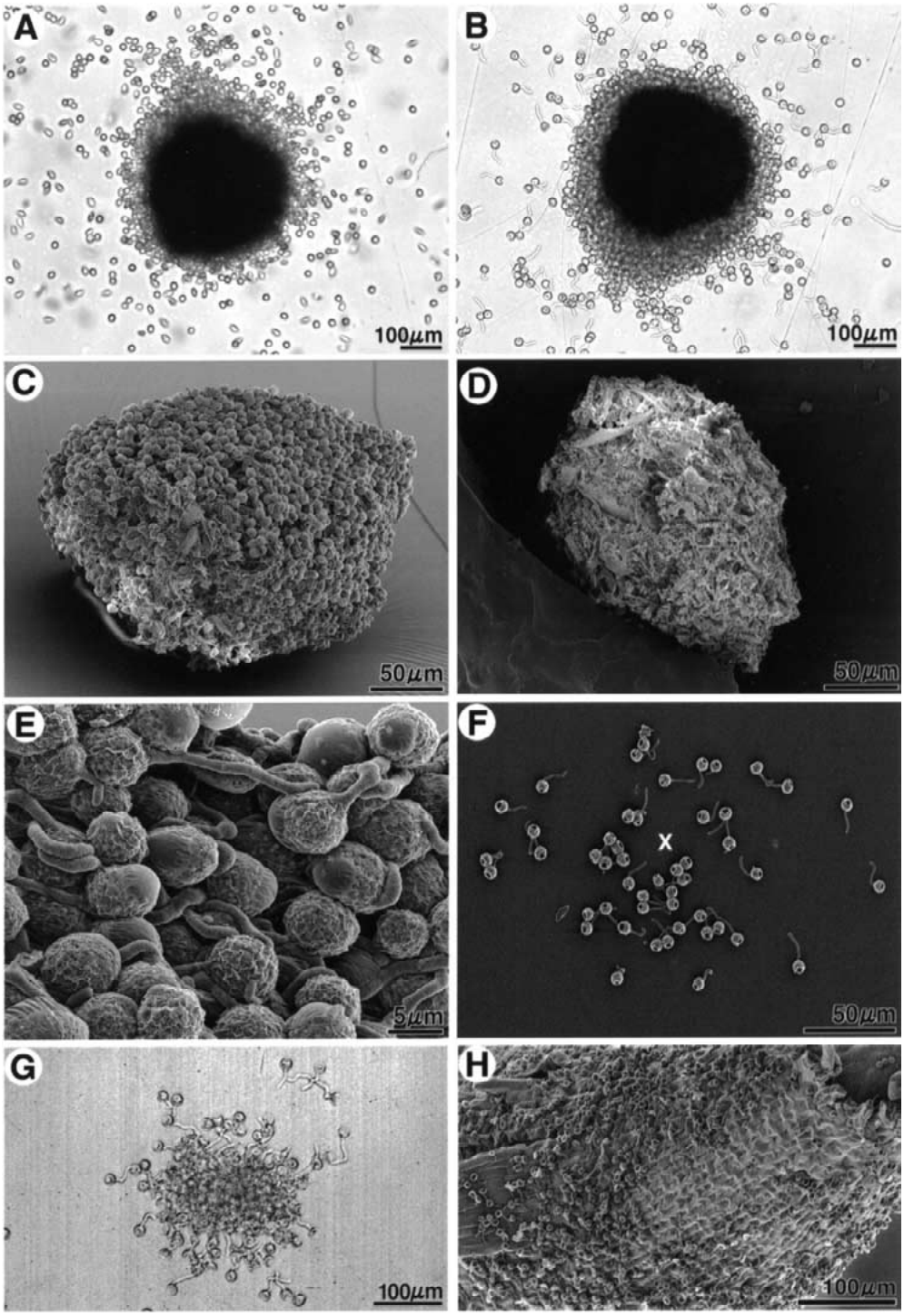

Figure 2. Light (A, B, G) and scanning electron micrographs (C-F, H) showing aggregation, encystment and germination activity of a host-specific zoospore attractant, cochliophilin A. (A) Zoospores aggregated around a Chromosorb particle coated with 10^{-7} M solution of cochliophilin A (after 10 min). The zoospores those are very closed to the particle become halted and changed into cystospores (round dots). (B) Accumulated zoospores formed mass of cystospores around a particle coated with 10^{-7} M cochliophilin A and germinated toward the center of that particle (after 20 min). (C) Accumulated and encysted zoospores germinated and covered on the surface of a Chromosorb particle coated with 10^{-6} M solution of cochliophilin A (after 60 min). (D) Control particle having no accumulation of zoospore on its surface. (E) An enlarged portion of the photomicrograph C. (F) Showing germ-tubes tropism toward the host-specific signal, cochliophilin A ('**X**' sign indicates the position of a Chromosorb particle which was removed prior to fixation of specimen by glutaraldehyde). (G) Showing tropism of germ-tubes toward the aggregate center (zoospores were induced to encystment by 10^{-11} M of cochliophilin A solution added very slowly to zoospore suspension using a microsyringe). (H) Accumulated and encysted zoospores germinated (penetrating) on the surface of a spinach root tip (60 min).

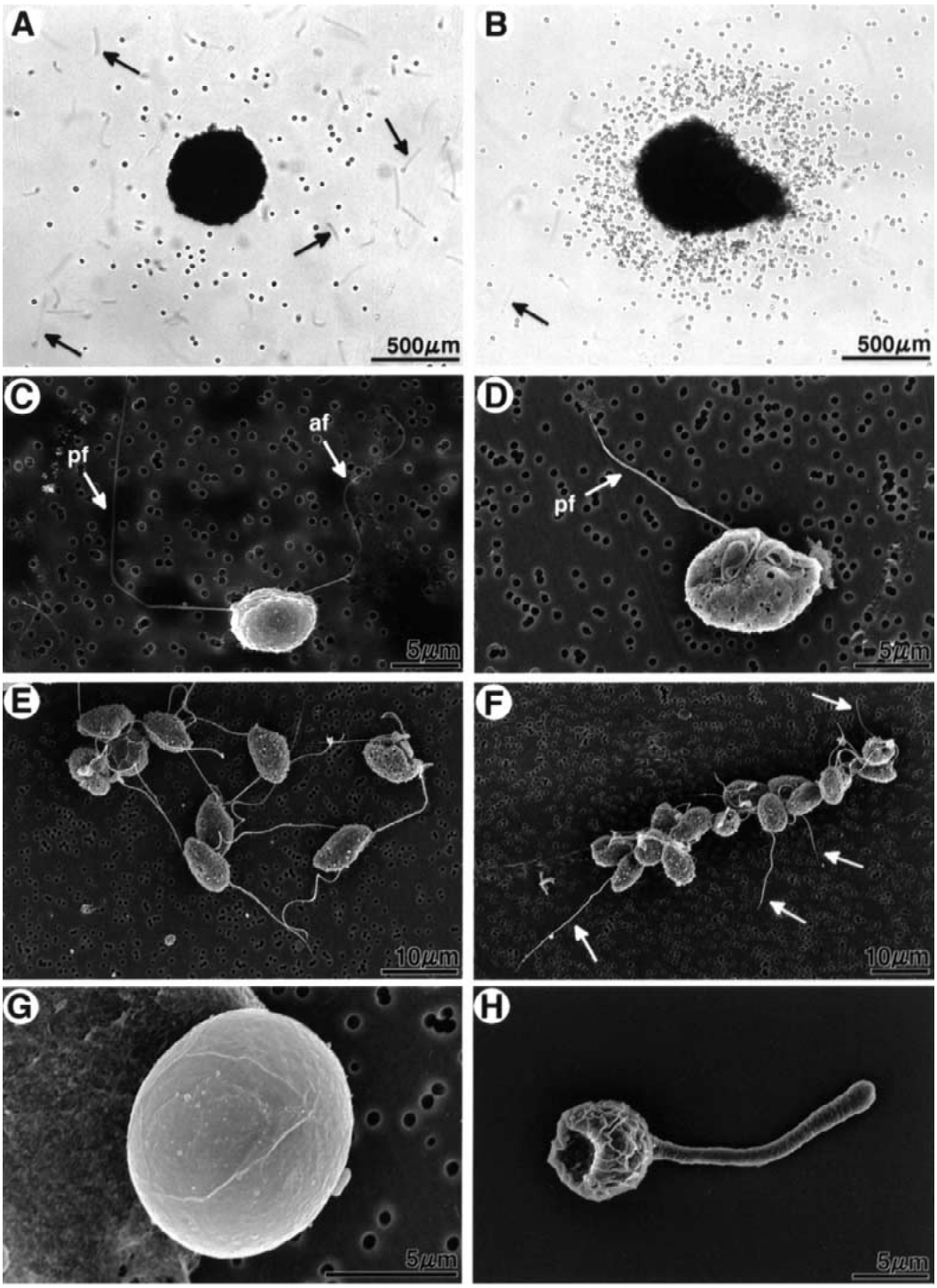

Figure 3. Light (A and B) and scanning electron photomicrographs (C–H) showing the halting activity and subsequent morphological changes of *A. cochlioides* zoospores exposed to mastoparan. (A) Halted zoospores (dots) around a Chromosorb particle coated with 10^{-5} M solution of mastoparan (after 5 min treatment). Arrows (A and B) indicate the traces (lines) of swimming zoospores (0.5 s exposure). (B) High number of zoospores halted within 10 min around the particle coated with 10^{-4} M mastoparan (C) A biflagellate zoospore (af: anterior flagellum, pf: posterior flagellum). (D) A mastoparan stimulated zoospore on SEMpore membrane (after 5 min). (E) Zoospores connected with each other by their flagella during stimulation (after 10 min) by mastoparan. (F) Mastoparan stimulated zoospores (arrows: tips of posterior flagella) aggregated at the bottom of petri dish (after 15 min). (G) Stimulated spores encysted after 20 min. This smooth surfaced enlarged cell is an immature cystospore. (H) A mature cystospore germinated forming a long germ-tube (60 min).

Table 1. Effects of host-specific attractants on the taxis and differentiation of zoospores of the *Aphanomyces cochlioides*

Treatment	Concentration (M)	Effects of treatments on taxis and differentiation of the *Aphanomyces cochlioides* zoospores*		
		Taxis	Encystment	Germination
Cochliophilin A (**5**)	10^{-9}	+	0	0
	10^{-8}	+++	+	+
	10^{-7}	+++	+++	+++
	10^{-6}	+++	+++	+++
N-trans-feruloyl-4-*O*-	10^{-8}	+	0	0
methyldopamine (**6**)	10^{-7}	+++	0	0
	10^{-6}	+++	0	0
	10^{-5}	+++	+	+
5,4′ –dihydroxy-3,3′ -	10^{-6}	+	0	0
dimethoxy-6,7-methylene-	10^{-5}	++	0	0
dioxyflavone (**7**)	10^{-4}	+++	+	+
5 + neomycin (10^{-5} M)	10^{-8}	++	0	0
5 + neomycin (10^{-5} M)	10^{-7}	+++	+	+

*Particle bioassay method; '+' sign indicates clear positive bioactivity over control; Number of '+' sign indicates the degree bioactivity (attractant/encystment/germination); + = weak, ++ = medium, +++ = strong response equivalent to the photomicrograph Figure 2A and 2B; 0 = no response.

Table 2. Effects of G-protein activator on the halting motility and differentiation of zoospores of the *Aphanomyces cochlioides*

Treatment	Concentration (μM)	Effects of treatments on the halting motility and differentiation of zoospores*		
		Halting motility	Encystment	Germination
Mastoparan	1	0	0	0
	10	+	+	+
	25	++	++	+
	50	+++	+++	++
	100	+++	+++	++
Mastoparan 17	10	0	0	0
	25	0	0	0
	50	0	0	0
	100	0	0	0
Mastoparan + 5 (10^{-9} M)	10	++	++	++
Mastoparan + 5 (10^{-8} M)	10	+++	+++	+++
Mastoparan + neomycin (10 μm)	10	0	0	0
Mastoparan + neomycin (10 μm)	25	+	+	0

* Particle bioassay method; '+' sign indicates clear positive bioactivity over control; Number of '+' sign indicates the degree bioactivity (halting motility/encystment/germination); + = weak (Figure 3A), ++ = medium, +++ = strong response (Figure 3B) 0 = no response.

and then became round shape and encysted at the bottom of petri dish. Most of the cystospores (60–80%) germinated within 40–60 min (Figure 3H). To provide further evidence that mastoparan is acting as a G-protein agonist, we tested an analog of mastoparan, known as Mas17, which is unable to activate G-proteins (Higashijima et al., 1990). This peptide contains a charged residue (replacement of Leu-6 by Lys; 'Materials and methods') located in the middle of the hydrophobic stretch required for the α-helical membrane conformation apparently essential for G-protein agonist activity. Significantly, Mas17 (1 – 100 μM) (Table 2) totally lacked the capacity to elicit encystment and germination of zoospores. On the other hand, concomitant application of mastoparan and cochliophilin A showed stronger encystment

activity followed by 100% germination of cysts within 30–40 min (Table 2). However, the presence of mastoparan could not increase the attractant activity of cochliophilin A (**5**).

Morphological changes of zoospores exposed to cochliophilin A or mastoparan

To see the mechanism of halting response of the zoospores to cochliophilin A or mastoparan followed by encystment and germination, we undertook a time-course observation of changes by scanning electron microscopy. Time-course scanning electron microscopic observation revealed that zoospores stimulated with cochliophilin A or mastoparan underwent a similar sequence of morphological changes up to germination of cystospores (Figure 3D–H). During the first 5–10 min of stimulation, zoospores were found to coil or wind up their anterior flagellum on their own bodies (Figure 3D). At this stage, the posterior flagellum was attached to the body of other zoospores (in case of dense population) or remained unchanged (Figure 3D). The stimulated zoospores became almost round shape by shedding their flagella within 20 min of stimulation and soon became the enlarged cystospores (8.5–10.5 μm i.d.) bounded by a smooth cyst-coat (Figure 3G). The detached flagella were found to lose their fine structures immediately after detachment from the zoospore (photomicrograph not shown). The initial cystospore coated with a smooth cyst-coat rapidly changed into the mature cystospore (5.7–7.1 μm i.d.) coated with rough cell wall within 20–30 min and finally germinated within 40–60 min (Figure 3H). Interestingly, the sequence of morphological changes of zoospores by cochliophilin A or mastoparan was completely similar to those occurred during interaction with spinach roots (Figure 2H).

Effect of host-specific attractants on the growth and reproduction of Aphanomyces cochlioides

The effects of host-specific attractants on the vegetative growth and the sporulation of *A. cochlioides* were evaluated using a series of concentrations (10^{-11} to 10^{-6} M). The radial growth and oospore production of this phytopathogen on a corn meal agar medium was unaffected in the presence of any host-derived compounds (Compounds **5**–**7**) at a range from 10^{-12} to 10^{-6} M (data not shown). The release of zoospores from the mycelia was also not at all affected up to 10^{-6} M concentration of host-specific compounds in the culture media.

Phospholipase C or Ca^{2+} release/influx antagonists inhibit cochliophilin A or mastoparan elicited zoospore differentiation

Application of PLC inhibitor, neomycin at 10^{-5} M to the zoospore suspension 5 min before the addition of mastoparan or host-specific attractants partially reduced the encystment and germination of cystospores (Table 2). Addition of Ca^{2+} channel blocker loperamide (20 m*M*) or Ca^{2+} chelator EGTA (10 m*M*) decreased (approximately 70%) the encystment and germination activity of mastoparan or host-specific attractant, cochliophilin A.

Discussion

Zoospores of soil-borne oomycete phytopathogens are believed to locate their host by utilizing chemical signals released from the roots of host plant. The zoospores attracted to the host root, adhere to its surface by exocytosis of a proteineous material (Hardham, 1992), encyst and germinate by the recognition of host-surface components (Deacon, 1996; Islam et al., 2001), and finally penetrate into the host directly or via appressoria (Bircher and Hohl, 1997; Islam et al., 2001). In this experiment, a gradient of a host-specific attractant, cochliophilin A (**5**) triggered encystment and germination of *A. cochlioides* zoospores at a concentration approximately ten times higher than that observed to elicit chemotaxis (Figure 2A–C). The behavior of zoospores on around Chromosorb particles coated with cochliophilin A (Figure 2C, E, H) was similar to that of zoospores toward spinach roots. The amount of cochliophilin A (**5**) in the spinach root (ca. 1.9×10^{-5} mol/kg fresh root) seems to be enough to initiate encystment of zoospores followed by germination which are regenerated by Chromosorb W AW particles coated with a 10^{-8} M solution of **5** (Tahara and Ingham, 2000). In addition, the time-course morphological changes of zoospores by cochliophilin A on Chromosorb particles were identical with the changes of zoospores interacting with spinach roots (Figure 2C, H). These observations suggest that cochliophilin A (**5**) is indeed a host-specific plant signal which may play essential roles in both locating host roots and initiating encystment and germination. Encystment and germination, two important

pre-infectional events, which are prerequisites for invasion of the zoosporic phytopathogen into the host. Only a germinated cystospore can penetrates into root tissues directly or via appressorium (Bircher and Hohl, 1997; Islam et al., 2001). Interestingly, an almost similar phenomenon was observed in bacteria. As signal for chemotaxis of rhizobia a concentration as low as 10^{-9} M luteolin is sufficient, and at 10^{-6} M concentration luteolin stimulates *nod* gene expression (Bauer and Caetano-Anolles, 1990; Fisher and Long, 1992).

Direct application of host-specific attractants homogeneously to the zoospore suspension did not show any encystment and germination activity, however, very slow release of highly diluted solution of **5** (10^{-11} – 10^{-10} M) by a micro-syringe into the zoospore suspension stimulated the formation of clumps of encysted spores at the bottom of petri dish (Figure 2G). All aggregated spores were encysted and then germinated. It clearly indicates that a gradient of host signal is necessary for taxis and differentiation of zoospores. Thus, our particle bioassay appears to be a suitable method for studying chemotaxis and subsequent differentiation of zoospores where a gradient of chemical is essential for the response of cells (Figure 2A–E) (Parent and Devreotes, 1999).

The germ-tubes from encysted spores close to the particles coated with **5** or those from autoaggregated spores showed tropism toward the aggregate center (Figure 2B, F). Tropic responses of hyphal germlings to host-specific signals have also been observed in *Phytophthora sojae* (Morris et al., 1998) and autoaggregation of zoospores in the absence of a host appears to be characteristic of many other Oomycetes (Reid et al., 1995). Thus, it appeared that similar aggregation phenomenon may be also induced by the host-specific compounds. Aggregation of inoculated zoospores on a certain point of the host root might increase the vigor of the inoculum for successful infection.

The growth and sporulation of *A. cochlioides* on a corm meal agar medium were unaffected up to 10^{-6} M concentration of cochliophilin A (**5**) in our preliminary experiments. This information supports that this phytopathogen can grow well and produces zoospores for further dissemination of pathogens to spread the disease through surrounding healthy plants. All these interesting features of host-pathogen interactions might have ecological significance, and may find useful application in the investigation of biochemical and molecular mechanism in pathogenicity where it is definitely desired to synchronize the development of pathogen with that of the host.

Mastoparan is commonly used as diagnostics for the participation of G-proteins in both animal and plant signal transduction pathways (Munnik et al., 1998; Pingret et al., 1998). The heterotrimeric G-protein activator, mastoparan promoted both encystment and germination of zoospores at micromolar concentration (Table 2 and Figure 3). The real concentration of mastoparan around the particles was far lower than the concentration of solution used to coat the Chromosorb particles (please see 'Materials and methods'). Furthermore, the synthetic peptide analog Mas17, predicted not to form an amphipathic helix at the lipid interface because of the replacement of Leu-6 by Lys, is totally devoid of agonist activity. The concomitant application of mastoparan and the host-specific attractant cochliophilin A (**5**) appeared to further enhance encystment of zoospores and rapid germination of cysts (Table 2). In contrast, inhibitors of PLC or cytoplasmic Ca^{2+}fluxes markedly decreased activity of both mastoparan and cochliophilin A. Transient rises in cytoplasmic Ca^{2+} concentration have been implicated in the differentiation of zoospores by external signals (Connolly et al., 1999; Warburton and Deacon, 1998). We have examined whether cochliophilin A induced differentiation of zoospores requires Ca^{2+} fluxes across the plasma membrane. Our preliminary experiments suggest that the zoospore differentiation by host-specific cochliophilin A may be mediated by G-protein-coupled receptors to activate both phosphoinositide and Ca^{2+}second messenger pathways. The role of calcium in zoospore differentiation is now well established (Connolly et al., 1998; Donaldson and Deacon, 1993; von Broemsen and Deacon, 1996; Xu and Morris, 1998).

To our knowledge, this is the first indication that G-protein mediated signaling mechanism is involved in oomycete zoospores. Our observations on the promoting effects of a G-protein agonism may indicate that chemotaxis and subsequent differentiation in Oomycetes are regulated by pathways similar to those already characterized for *Dictyostelium* and leukocytes (Caterina and Devreotes, 1991; Parent and Devreotes, 1999; van Es and Devreotes, 1999). Similar striking effect of G-proteins activator was observed in unicellular green alga, *Chlamydomonas,* where mastoparan caused rapid deflagellation of algal spores by the way of G-protein mediated signaling pathways (Munnik et al., 1998; van Himbergen et al., 1999).

We previously found that the interaction of two non-host natural compounds, *N-trans*-feruloyltyramine

(a zoospore stimulant) and 1-linoleoyl-2-lysophosphatidic acid monomethyl ester (a zoospore repellent) caused motility inhibition followed by encystment and germination of zoospores of the *A. cochlioides* (Mizutani et al., 1998). Recent identification and cloning of lysophosphatidic acid-specific receptor has led to the elucidation of G-protein and signaling pathways through which lysophosphatidic acid functions (Swarthout and Walling, 2000). Rapid increase of phosphatidic acid (PA) was observed in the differentiating zoospores of *Phytophthora palmivora* by Tris-pectate (Zhang et al., 1992). Zhang et al. (1992) suggested that the kinetics of PA production during differentiation of zoospores may arise via a stimulus-activated phospholipase D. However, they emphasized that their data do not totally exclude the possibility of a phospholipase C-generated signal coupled with a very rapid kinase reaction which eventually supports our present findings.

In summary, we have shown that at elevated concentrations, host-specific attractant or G-protein activator triggers encystment followed by germination of *A. cochlioides* zoospores. Our results suggest that chemotaxis and subsequent differentiation of zoospores may be initiated by a G-protein-coupled receptor, which activates phosphoinositide and Ca^{2+} second messenger pathways.

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References

Bauer W D and Caetano-Anolles G 1990 Chemotaxis, induced gene expression and competitiveness in the rhizosphere. Plant Soil 129, 45–52.

Bircher U and Hohl H R 1997 Environmental signaling during induction of appressorium formation in *Phytophthora*. Mycol. Res. 101, 395–402.

Caterina M J and Devreotes P N 1991 Molecular insights into eukaryotic chemotaxis. FASEB J. 5, 3078–3085.

Connolly M S, Williams N, Heckman C A and Morris P F 1999 Soybean isoflavones trigger a calcium influx in *Phytophthora sojae*. Fungal Genet. Biol. 28, 6–11.

Deacon J W and Donaldson S P 1993 Molecular recognition in the homing responses of zoosporic fungi, with special reference to *Pythium* and *Phytophthora.* Mycol. Res. 97, 1153–1157.

Deacon J W 1996 Ecological implications of recognition events in the pre-infection stages of root rot pathogens. New Phytol. 133, 135–145.

Donaldson S P and Deacon J W 1993 Changes in motility of *Pythium* zoospores induced by calcium and calcium-modulating drugs. Mycol. Res. 97, 877–883.

Fisher R F and Long S R 1992 *Rhizobium*-plant signal exchange. Nature 357, 655–659.

Hardham A R 1992 Cell biology of pathogenesis. Ann. Rev. Plant Physiol. Plant Mol. Biol. 43, 491–526.

Higashijima T, Burnier J and Ross E M 1990 Regulation of g_{q_i} and g_o by mastoparan, related amphiphilic peptides, and hydrophobic amines. J. Biol. Chem. 265, 14176–14186.

Horio T, Kawabata Y, Takayama T, Tahara S, Kawabata J, Fukushi Y, Nishimura H and Mizutani J 1992 A potent attractant of zoospores of *Aphanomyces cochlioides* isolated from its host, *Spinacia oleracea.* Experientia 48, 410–414.

Horio T, Yoshida K, Kikuchi H, Kawabata J and Mizutani J 1993 A phenolic amide from roots of *Chenopodium album*. Phytochemistry 33, 807–808.

Islam M T, Ito T and Tahara S 2001 Morphological studies on the *Aphanomyces cochlioides* zoospore and its changes during the interaction with host materials. J. Gen. Plant Pathol. 67, 255–261.

Islam M T and Tahara S 2001 Repellent activity of estrogenic compounds toward zoospores of the phytopathogenic fungus *Aphanomyces cochlioides.* Z. Naturforsch. 56c, 253–261.

McDonald L J and Mamrack M D 1995 Phosphoinositide hydrolysis by phospholipase C modulated by multivalent cations La^{3+}, Al^{3+}, neomycin, polyamines, and melitin. J. Lipid Mediat. Cell Signal. 11, 81–91.

Mizutani M, Hashidoko Y and Tahara S 1998 Factors responsible for inhibiting the motility of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides* isolated from the non-host plant *Portulaca oleracea.* FEBS Lett. 438, 236–240.

Morris P F, Bone E and Tyler B M 1998 Chemotropic and contact response of *Phytophthora sojae* hyphae to soybean isoflavonoids and artificial substrates. Plant Physiol. 117, 1171–1178.

Morris P F and Ward E W B 1992 Chemoattraction of the zoospores of the soybean pathogen, *Phytophthora sojae*, by isoflavones. Physiol. Mol. Plant Pathol. 40, 17–22.

Munnik T, van Himbergen J A J, ter Riet B, Braun F-J, Irvine R F, van den Ende H and Musgrave A 1998 Detailed analysis on the turnover of polyphosphoinositides and phosphatidic acid upon activation of phospholipase C and D in *Chlamydomonas* cells treated with non-permeabilizing concentration of mastoparan. Planta 207, 133–145.

Parent C A and Devreotes P N 1999 A cell's sense of direction. Science 284, 765–770.

Reid B, Morris B M and Gow N A R 1995 Calcium-dependent genus specific autoaggregation of zoospores of phytopathogenic fungi. Exp. Mycol. 19, 202–213.

Ross E M and Higashijima T 1994 Regulation of G-protein activation by mastoparan and other cationic peptides. Methods Enzymol. 237, 26–37.

Swarthout J T and Walling H W 2000 Lysophosphatidic acid: receptors, signaling and survival. Cell. Mol. Life Sci. 57, 1978–1985.

Tahara S and Ingham J L 2000 Simple flavones possessing complex biological activity. *In* Studies in Natural Products Chemistry, Vol

22. Ed. Atta-ur-Rahman. pp. 457–505. Elsevier Science Pub., Amsterdam.

Tahara S, Ohkawa K, Takayama T and Ogawa Y 2001 The third naturally-occurring attractant toward zoospores of phyotopathogenic *Aphanomyces cochlioides* from the host plant *Spinacia oleracea*. Biosci. Biotechnol. Biochem. 65, 1755–1760.

Takahashi H, Sasaki T and Ito M 1987 New flavonoids isolated from infected sugar beet roots. Bull. Chem. Soc. Jpn. 60, 2261–2262.

Takayama T 1999 Ecochemical Studies on Chemotaxis of Spinach Root Rot Pathogen, *Aphanomyces cochlioides* Zoospores. Ph.D. Thesis, Grad. Sch. Agric., Hokkaido University, Sapporo, Japan (in Japanese). 27–40 pp.

Ui T and Nakamura S 1963 Sugar-beet black root and its pathogen *Aphanomyces cochlioides*: pathogenicity and host specificity. Tensai-Kenkyukai-Hokoku 3, 78–95 (in Japanese).

van Es S and Devreotes P N 1999 Molecular basis of localized responses during chemotaxis in amoeba and leukocytes. Cell. Mol. Life Sci. 55, 1341–1351.

van Himbergen J A J, ter Riet B, Meijer H J G, van den Ende H Musgrave A and Munnik T 1999 Mastoparan analogues stimulate phospholipase C- and phospholipase D-activity in *Chlamydomonas*: a comparative study. J. Exp. Bot. 50, 1735–1742.

von Broembsen S and Deacon J W 1996 Effects of calcium on germination and further zoospore release from zoospore cysts of *Phytophthora parasitica*. Mycol. Res. 100, 1498–1504.

Warburton A J and Deacon J W 1998 Transmembrane Ca^{2+} fluxes associated with zoospore encystment and cyst germination by the phytopathogen *Phytophthora parasitica*. Fungal Genet. Biol. 25, 54–62.

Xu C and Morris P F 1998 External calcium controls the developmental strategy of *Phytophthora sojae* cysts. Mycologia 90, 269–275.

Yokosawa R and Kuninaga S 1979 *Aphanomyces raphani* zoospore attractant isolated from cabbage: Indole-3-aldehyde. Ann. Phytopathol. Soc. Jpn. 45, 339–343.

Yokosawa R, Kuninaga S and Sekizaki H 1986 *Aphanomyces euteiches* zoospore attractant isolated from pea root: Prunetin. Ann. Phytopathol. Soc. Jpn. 52, 809–816.

Zentmyer G A 1961 Chemotaxis of zoospores for root exudates. Science 133, 1595–1596.

Zhang Q, Griffith J M and Grant B R 1992 Role of phosphatidic acid during differentiation of *Phytophthora palmivora* zoospores. J. Gen. Microbiol. 138, 451–459.