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Ecochemical Interactions between Plants and Phytopathogenic *Aphanomyces cochlioides* Zoospores[1]

Md. Tofazzal Islam*
Laboratory of Ecological Chemistry, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
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Contents

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*Permanent address: School of Agriculture and Rural Development, Bangladesh Open University, Gazipur-1705, Bangladesh.

I. GENERAL INTRODUCTION

Microorganisms that produce zoospores at a certain stage of their life cycle are often referred to as zoosporic microorganisms. Among the zoosporic group of microorganisms, the members of oomycetes (Peronosporomycetes in the new classification)[27] genera *Phytophthora*, *Pythium* and *Aphanomyces* are known as the most devastating pathogens of dicot plants[45,46]. Recent studies indicated that oomycetes are phylogenetic relatives of brown algae and diatoms. Based on their distinct structural and biochemical characters, oomycetes have been moved from the Kingdom Fungi to Stramenopila[27,92]. They can propagate both sexually and asexually. The sexual spores, oospores can survive independent of host plant for long time and can act as an extra inoculum source. Though some of the species of *Pythium* (*Py*.) and *Phytophthora* (*Ph*.) can spread by direct germination of air-borne sporangia, most parasitic varieties of oomycetes can orient to new host plants by targeted homing responses of aquatic zoospores[20]. Zoospores are liberated from a sporangium within an hour; disease caused by these oomycetes can be multi-cyclic, resulting in severe epidemics that can destroy whole crops within a single season.

Zoospores of phytopathogenic oomycetes accumulate at the potential infection sites of host roots by chemotaxis, after which they undergo a series of morphological changes before penetrating the root tissues[20,63,155]. Accumulated evidence suggests that zoospores have both general (low-affinity) and host-specific (high-affinity) receptor-based recognition systems for chemotaxis to root diffusates and for induction of encystment and germination by host surface components[19]. A few host-derived zoospore attractants have been identified[58,59,98,151,152]. However, there is no report clearly connecting the phenomenon of chemotaxis with the subsequent developmental stages of pre-infection[20]. Thus the significance of chemotaxis in host-specificity is still ambiguous. Scanty information is available on the modes of contact, attachment and differentiation of the oomycetes zoospores during interaction with a targeted host plant[19,63]. Very little is known about the characters of receptor and other components of the signal transduction pathways in oomycete zoospores[120].

Because of oomycetes' distinct physiology, many of the most effective fungicides targets are absent in them. For example, the azole fungicides, which are used extensively in agriculture and medicine, target ergosterol biosynthesis. However, oomycetes do not synthesize sterols but acquire them from their victims[30]. A further complication is that many oomycetes appear to have an extraordinary genetic flexibility that enables them to adapt rapidly to and overcome chemical control measures and genetic resistance bred into plant hosts[30]. Resistance to effective chemicals such as metalaxyl has arisen in several oomycete species against which they have been deployed, and careful management is required to preserve the usefulness of the chemicals[38]. Plants

bred with genetic resistance against *Ph. infestans* remained resistant for less than a year [35].

The destructiveness of oomycete diseases, and the difficulty of controlling them have led to a concerted effort to develop the new approaches to find novel targets[40]. Our knowledge in the biology of oomycetes is limited[40,45] and hence, it is needed to understand their biology for developing a biorational control measure against these notorious phytopathogens. Among the oomycetes, *Aphanomyces* species cause some of the destructive plant and fish diseases throughout the world[29,106,122,123,143]. Species of the phytopathogenic *Aphanomyces* exhibit high degree of specialization and can infect a limited number of plant species[142,148]. For example, most of the plants are resistant to the strains of *A. cochlioides* that infect sugar beet, spinach and a few members of Chenopodiaceae and Amaranthaceae. This phenomenon of nonhost resistance, the ability of a pathogen to cause a disease in particular species but not in others, has always intrigued plant pathologists but remains poorly understood especially in oomycetes[78].

Zoospores of the spinach and sugar beet pathogen *A. cochlioides* are highly sensitive to a rare flavone, cochliophilin A, which exuded from the roots of spinach into the rhizosphere[58,130]. Because this flavone found only in the hosts of *A. cochlioides*, Tahara and Ingham suggested that the specific attraction of zoospores to host flavone might be part of the mechanism that determines host range[130]. However, chemical signal involved in differentiation of zoospores on the spinach root surface is yet to be discovered.

The highly intriguing phenomenon in zoospore orienting towards the host surface provoke such a question as what sustains and what is the mechanism of zoospore motility, and how the chemotactic stimulus is perceived and translated into action. These questions of function prompted a more detailed study of the structure, physiology and biochemistry of the zoospore itself. Since the early stage of electron microscopy, the morphological diversity of many organisms including some oomycetes has been studied intensively, whereas reports concerning *Aphanomyces* spp. are very few[48]. Nor have there been studies conducted dealing with the electron microscopy of the morphological diversity of the zoospores of *Aphanomyces*, and on the dynamic morphological changes of zoospores during its interaction with the host root. The morphological features of zoospores and their flagella have been suggested to vary widely among the oomycetes, even within the same genus[25,26]. The role of flagella in motility and / or recognition of host have been proposed by many workers[19,26,45,46].

I postulate that zoospores of *A. cochlioides* may contact with host root by their posterior flagella and then change morphologically triggered by some host-specific chemical signals. The primary objective of my research is to morphological studies on zoospores of *A. cochlioides* and changes during interaction with host materials by scanning and transmission electron microscopy. A

time-course SEM investigation is also included to study the modes of contact, landing, attachment, differentiation and penetration of pathogen into root tissues. Another objective of research is to identify the host-specific plant signal(s), which triggers differentiation of zoospores on spinach root. Consequently, the structure of the third and fourth generations of zoospores and their sensitivity toward host signals are studied to verify the hypothesis of repeated zoospore emergence as a possible adaptation to parasitism in *Aphanomyces* species. Finally, some known pharmacological tools are used for understanding the signal transduction pathway in chemotaxis and differentiation of oomycete zoospores.

Preliminary studies indicated that some of the nonhost secondary metabolites and crude plant extracts could affect motility and viability of zoospores of *A. cochlioides*[31,97]. Based on these findings, I hypothesize that nonhost plants may contain "chemical weapons" to defend themselves against attack by oomycete phytopathogens. Other objectives of my research are to test this hypothesis by extensive screening extracts from nonhost plants, and isolate the active principles from some of the plant extracts.

II. LITERATURE REVIEW AND RESEARCH APPROACH

A. Economic importance and characteristic features of zoosporic microorganisms

The zoospores are naked unicellular propagules with one or two flagella and having limited power of mobility in aquatic media. Zoospores have fascinated biologists for over 150 years but the interest began with Benedict Prevost, who first set eyes upon a fungal zoospore and published his findings[11]. Parasitic interactions of zoospores with plants have been found in at least 400–million-year-old fossil records[140]. They are distributed in diverse ecosystems ranging from soil and water, aerial plant parts, animal hairs, and even in the extreme anaerobic conditions of the rumen[20]. They are economically problematic as they are parasites on plants, fishes, insects, amphibians, and other fungi, and some are vectors of phytopathogenic viruses[63]. Some of them, such as *Pythium insidiosum*, are also cause dangerous infections (pythiosis) of humans and animals[94].

Basically, all zoospores are motile, wall-less and flagellate cells but they differ in number and types of flagella, and in ultrastructural details in different groups of organisms[25]. Nevertheless, the zoospores share some common features: a) They are uninucleate, flagellate membrane-bound cells that lack a cell wall, and specialized for dispersal because they can not divide, nor absorb organic nutrients[45]; b) They swim for many hours, using endogenous food reserves, and then encyst by shedding or retracting their flagella and secreting a wall for encystment[20]; c) Zoospores have a limited but significant capacity for dispersal in addition to transport in moving water; d) They can respond to a range of

environmental signals, which they use to locate the sites where they will encyst[63].

B. Oomycete zoospores: taxonomic, morphological and pathogenic properties

The oomycetes are an important and distinct group of organisms. "Oomycota" means, "egg fungi" and refers to the large egg-shaped oogonia or structure containing the female gametes. The class contains species that cause many of the world's serious plant diseases[63], perhaps the most infamous being *Phytophthora infestans*, the late blight of potato. Late blight is a devastating disease of potato and was responsible for epidemics that led to the Irish potato famine in 1845[9]. *Phytophthora* species also cause root rots of a variety of crop, ornamental and forest plants. Species of *Pythium* cause seed rot and seedling damping off; species of *Bremia*, *Plasmopara, Perenospora, Pseudoperonospora* and *Sclerospora* cause downy mildew diseases of crops and ornamentals; species of *Albugo* cause white rusts of cruciferous plants[63]. One large order, the Saprolegniales, contains species in genera *Saprolegnia, Aphanomyces, Achlya* and *Dictyuchus*, which are responsible for diseases in crops, amphibians and fishes[81].

The oomycetes, however, differ from true fungi and are more plant-like in a range of structural and biochemical characters[25,66,78]. They have a diploid somatic thallus and have tubular rather than plate-like mitochondrial cristae[15]. Comparison of mtDNA and rRNA sequences indicate that oomycetes are more closely related to heterokont, chromatophyte algae (brown algae) than to uniflagellate chytridiomycetes or to the higher fungi[119]. Their unique complement of attributes has led Margulis and his co-workers[92] to place the oomycetes into a separate phylum, the Oomycota.

Oomycetes can propagate both sexually and asexually. The sexual spores, oospores can survive in soil independent of the host plant and can act as an extra inoculum source. Moreover, sexual recombination allows the pathogen to adapt even more easily to adverse conditions[40]. Both the taxonomy and pathogenicity of oomycetes depend greatly upon the features of motile zoospores that are produced in large numbers by most of these organisms[25]. Some species of oomycetes are dimorphic and produce two types of zoospore: primary zoospores are pear shaped with the two flagella emerging from the apex of the cell; secondary zoospores are roughly ellipsoidal, with a longitudinal groove along a slightly flattened face (the ventral surface) giving the zoospores a kidney-shaped profile in transverse section. The flagella, an anterior tinsel flagellum and a posterior whiplash flagellum, emerge from the center of the groove on the ventral surface. Monomorphic species may produce primary- or secondary-type zoospores, depending on the species[126].

The flagellar apparatus is also one of several morphological features that are critical for zoosporic pathogenicity. Flagellar action makes the zoospores motile, allowing them to swim towards potential hosts, to which they are chemotactically attracted. The elongated and grooved shape of the zoospores

probably contributes to hydrodynamic stability during motility[45–47]. The flagella appear to facilitate accurate docking and encystment of the zoospore at the host surface[45,63]. However, the exact role of flagella during orientation of zoospores on host surface is still unknown.

Zoospores of the phytopathogenic oomycetes seem to have both general and host-specific receptor-based recognition systems for chemotaxis to root diffusates and for induction of encystment by host surface components[19]. The highly intriguing phenomena in zoospore's orienting towards the host surface or host-specific compounds provoke such questions as what sustains and what is the mechanism of zoospore motility, and how the chemotactic stimulus is perceived and translated into action. Many lines of evidence have been accumulated to unravel some of the biological myths and answer some of these questions[20,130]. However, there is no report clearly connecting the phenomenon of chemotaxis with the developmental stages of pre-infection.

C . Chemotaxis of zoospores

Chemotaxis may be defined as "a change in the direction of movement of a motile cell in response to a concentration gradient of a specific chemical". This change in the direction of movement may either cause the cell to move towards or away from the sensed chemical. Chemicals which are capable of eliciting a response from chemotactic cells are called 'Chemotactants' and may act as either attractants – in which case the chemotactic cell will move toward them, thereby exhibiting a positive chemotactic response – or as repellants – in which case the chemotactic cell will move away from them, therebay exhibiting a negative chemotactic response. Cells capable of chemotaxis include bacteria, protozoa, amoeba, cellular slime moulds, sperm, fibroblasts, phagocytes, oomycete zoospores etc.

Zoospores of phytopathogenic oomycetes exhibit positive chemotaxis to host-derived compounds[58,59,98,151,152] and negative chemotaxis to nonhost compounds[62,63,97]. Zoospores also show taxis towards plant root released electric currents known as electrotaxis[20]. Scanty information is available on the mechanism of zoospore chemotaxis.

The physiologically active compounds and their diverse activities toward various oomycete zoospores were summarized[63,66]. Among the general chemoattractants, amino acids have been found in general to be more powerful attractants than sugars or alcohols[20]. However, the sensitivity of zoospores to general attractants varied markedly among genera and even among the species of the same genus[20,141,155]. Therefore, the relationships between species of oomycete zoospores and general attractants are still ambiguous. The chemotactic thresholds (lowest effective concentrations) of most of the general attractants are usually more than the micromolar concentration, which may not actually exist in the root exudates of host plants. On the other hand, a few Host-derived

chemoattractants showed activity as low as micromolar to nanomolar concentration[58,59,98,152]. This indicates the possibility of host-specific compounds, which could specifically guide certain zoospores preferentially to the potential infection sites on the host surface. Therefore, it might be possible to consider that zoospores show chemotaxis toward general nutrients not for the purpose of parasitism, but for alternative preference for finding nutrient sources in the absence of host cues. However, the general attractants available in the root exudates of host plant may synergies the activity of host-specific compounds *in vivo* condition.

D. Host-specific chemotaxis

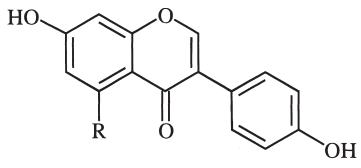
Zoospores of phytopathogens assemble at the potential infection sites of host by chemotaxis[64,74]. The accumulated zoospores adhere by exocytosis of a proteineous material[42,44,45,68] and encyst forming a thick cell wall by recognition of host surface components[19,64]. Preferential chemotaxis followed by encystment of zoospores toward susceptible hosts has been reported in several cases. Scanning electron microscopic observation showed that significantly higher numbers of zoospores of *Ph. cinnamomi* encysted on the surface of a susceptible cultivar of avocado compared to that of the tolerant cultivar. Fewer cysts were germinated on the roots of the tolerant cultivar than on the susceptible cultivar[2].

Kerwin *et al*. [80] found that *Py. marinum* encystment on the surfaces of red algae (its hosts) but not on green or brown algae (non-host). Galactose or anhydrogalactose contents in the surface of red algae were assumed to be responsible for such a specific response. Similarly, zoospores of downy mildews can precisely locate stomata[118] and those of the nematode parasite *Catenaria anguillulae* accumulate at the mouth of the host[20]. On the other hand, saprophytic species selectively colonize specific parts of the host depending on their food sources[20].

Continuous efforts to clarify the phenomenon of zoospore chemotaxis revealed that some host-specific compounds were shown to attract the zoospores at very low concentrations *in vitro* (Fig. 1). *Ph. sojae* showed taxis to the soybean isoflavonoids, diadzein (**1**) and genistein (**2**) at 10 nM (capillary method), but other Phytophthora spp. did not respond at all to these compounds[98,141]. Interestingly, these two isoflavones are also reported as the inducers of nodulation genes in *Bradyrhizobium japonicum*, the nitrogen fixing symbiont of soybean[3]. The other host-specific compounds so far reported are indole 3-carbaldehyde (**3**) (active at 1×10^{-9} M) from cabbage seedlings[151], and prunetin (**4**) (active at 5×10^{-7} M) from pea seedlings[152] which showed host-specific attraction to the zoospores of *A. raphani* and *A. euteiches*, respectively. Cochliophilin A (**5**) (active at 1×10^{-10} M) and *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) (active at 1×10^{-8} M) were also isolated from spinach and *Chenopodium album* roots, respectively, as the host specific attractants for the *A. cochlioides* zoospores[58,59].

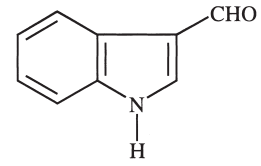
Similarly, zoospores of the fish pathogenic Oomycete *Saprolegnia diclina*

showed positive chemotaxis toward concentration gradients of chorionic membrane extract from live eggs of the brook trout, *Salvelinus fontinalis*[113]. A mosquito larval parasite, *Lagenidium giganteum*, selectively colonizes and encysts on culicid hosts[79,108]. It was found that certain conformations of chitin and chitosan could effectively cause encystment of the zoospores of this mosquito parasite[108].



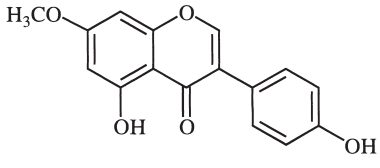
1: R = H : daidzein
2: R = OH : genistein

Host plant: *Glycine max*
Oomycete: *Phytophthora sojae*



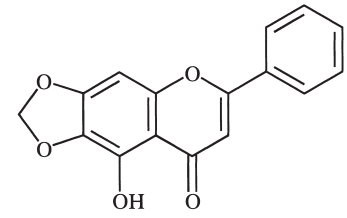
3: 3-indolecarbaldehyde

Host plant: *Brassica campestris* var. *capitata*
Oomycete: *Aphanomyces raphani*



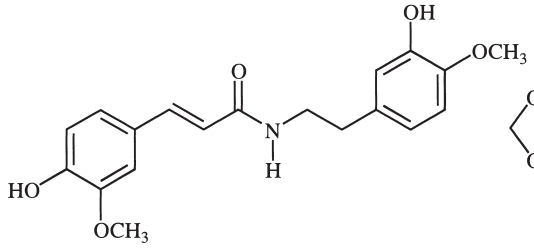
4: prunetin

Host plant: *Pisum sativum*
Oomycete: *Aphanomyces euteiches*



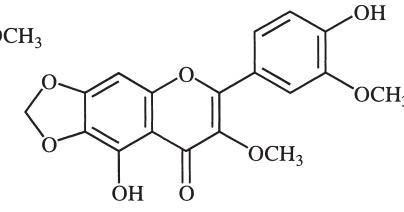
5: cochliophilin A

Host plant: *Spinacia oleracea*
Oomycete: *Aphanomyces cochlioides*



6: *N-trans*-feruloyl-4-*O*-methyldopamine

Host plant: *Chenopodium album*
Oomycete: *Aphanomyces cochlioides*



7: 5,4´-dihydroxy-3,3´-dimethoxy-6,7-methylenedioxyflavone

Host plant: *Spinacia oleracea*
Oomycete: *Aphanomyces cochlioides*

Fig. 1. Structures of some host-derived attractants for Oomycete zoospores.

E. Characteristics of *Aphanomyces cochlioides*

The genus *Aphanomyces* consists of 20 different species[123] including both parasitic and saprophytic representatives. It contains a wide range of species that are specialized to several different ecological niches, and therefore, offers

suitable models for comparative studies of specialization and differentiation[126].

Root diseases caused by soilborne pathogens within the genus *Aphanomyces* seriously limit production of several crop plants throughout the world. Severe damage is reported in sugar beet (*Beta vulgaris* L.)[106], spinach (*Spinacia oleracea* L.)[89], radish (*Raphanus sativus* L.)[29], cauliflower (*Brassica botrytis* L.), cabbage (*B. oleracea* L.), green bean (*Phaseolus vulgaris* L.), alfalfa (*Medicago sativa* L.) and pea (*Pisum sativum* L.)[123]. There are seven plant pathogenic species in the genus *Aphanomyces*[66,123]. The host range is of agronomic importance, as crop rotation is generally a means of controlling the diseases[106]. Some species of *Aphanomyces* are also known as serious pathogens of fishes[96,143].

A. cochlioides is a soilborne oomycete which infects its host through the biflagellated motile zoospores originated from oospores or zoosporangia formed in diseased plant tissues[29]. In a detailed study on the host-pathogen compatibility, Ui and Nakamura[142] found that the pathogenic Oomycete *A. cochlioides* was highly compatible with Chenopodiaceae (*Chenopodium album, Beta vulgaris* and *Spinacia oleracea*) and slightly less compatible with Amaranthaceae (*A. gangeticus, A. retroflexus*). It had low compatibility with the members of Portulacaceae (*Portulaca* sp.) and Caryophyllaceae, and was incompatible with members of the Cruciferae, Commelinaceae, Polygonaceae, Compositae, Cucurbitaceae, Solanaceae, Leguminosae, Linaceae, and Gramineae[142]. In another studies, Watanabe[151] found that *A. cochlioides* is a serious pathogen of *Celosia cristata* (Amaranthaceae), and was also compatible with *Amaranthus lividus*. The root extracts of 20 species belonging to 8 families were bioassayed by the particle bioassay method[130]. The crude root extracts from the plants belonging to Chenopodiaceae and Amaranthaceae showed strong attractant activity that reflected the positive correlations between plant-pathogen compatibility, and the zoospore attractant activity of root extracts[130]. Bioassay guided isolation of active constituents in plants of Chenopodiaceae (*Spinacia oleracea, Beta vulgaris, Chenopodium album*) resulted that all the hosts of *A. cochlioides* contained either cochliophilin A (**5**) or *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) or both[58,59]. However, the active constituents in nonhost *Amaranthus* spp. are still unknown and isolation of those nonhost constituents would be interesting for understanding their resistance to *A. cochlioides*.

F. Attractants in Chenopodiaceae

It is believed that constituents in the root exudate from the host plant attract zoospores, and they aggregate as a hemispheric mass on the root surface before infection occurs. Interestingly, it was estimated that the spinach root exudes 27 ng/day/plant in hydroponics and 34 ng/day/plant cochliophilin A (**5**) in pot culture, which might be enough to attract the zoospores of *A. cochlioides*[130]. Compound **5** can attract only *A. cochlioides* but not other species of *Aphanomyces*[130]. Therefore, a rare flavone **5** is reasonably considered as the

host specific attractant for the *A. cochlioides* zoospores. The limited distribution of a 6,7-methylenedioxyflavone-structure characteristic of **5** among the plant kingdom (mainly in Chenopodiaceae) may correspond to the limited host range of *A. cochlioides*. However, it is still unknown whether cochliophilin A or any other host signal triggers successive differentiation (encystment, germination and penetration) of zoospores on spinach root surface.

Recently, a second attractant for zoospores of *A. cochlioides* has also been identified from the leaves of spinach as 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (**7**)[132]. Although this compound has already been found as a glucuronide derivative in the spinach leaves, the ecological significance of **7** in host-pathogen relationship is yet to be known.

G. Some roles of calcium and signal transduction in zoospore

Several *in vitro* studies suggest that the divalent calcium ion plays a vital role in adhesion, encystment, and germination of Oomycete zoospores[28]. Encystment of *Ph. parasitica* was found to be associated with large initial net Ca^{2+} influx, which was abolished by the channel blockers La^{3+} and verapamil[147]. The net influx was followed by a larger, progressive net Ca^{2+} efflux over 30 min of encystment induction, which was associated with cyst germination but inhibited by 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) (intracellular calcium antagonist), suggesting calcium release from intracellular stores. High (1 mM) exogenous Ca^{2+} enabling encystment and germination overcame the effects of inhibitors. X-ray microanalysis of zoospores showed that host-specific attractants, daidzein (**1**) and genistein (**2**) triggered a net influx of Ca^{2+} into the encysting zoospores of *Ph. sojae*[18]. This apparently central role of calcium in zoospore motility is consistent with many other studies that have implicated calcium in flagellar function and in motile responses of bacteria, sperm, algae, leukocytes, slime molds, and marine invertebrates[20].

Little is known about the characters of receptors or signaling components involved in zoospore chemotaxis. Specific chemotaxis of zoospores toward host materials or synthetic compounds suggests that respective surface receptors are involved in perception and subsequent translation of signals into cellular responses. However, the structure and characteristics of a surface receptor that involved in zoospore chemotaxis are yet to be discovered. Except the information of intracellular calcium fluxes, the components of signal transduction pathways are completely unknown. Although in other cells, for example, *Dictyostelium* and leukocites, heterotrimeric G-proteins were found to be involved in their chemotaxis[70].

H. Nonhost secondary metabolites affecting the behavior of zoospores

The molecular basis of nonhost resistance remains one of the major unknowns in the study of plant-microbe interactions. Performed barriers and

compounds such as saponins are ubiquitous in plants and play important roles in nonhost resistance to phytopathogens[103,104]. However, there is little evidence for a role for performed barriers in resistance to oomycetes. I hypothesized that root exudates or constituents of nonhost plants may contain some compounds that in some way may defend themselves from the attack of zoosporic phytopathogens. Considering this hypothesis, I screened 100 Chinese traditional medicinal plant extracts to see their effects on the behavior of *A. cochlioides* zoospores[60]. It appeared that about 40% of the crude drug extracts had diverse biological activities on the zoospores ranging from stimulant/attractant, repellent, motility inhibition (halting) and lysis of the cell membrane.

Bioassay-guided studies revealed that diverse nonhost secondary metabolites for example, anacardic acids (zoospore lytic factors) from the fruits of *Ginkgo biloba*[7] saponins from *Panax notogingeng*[91] *N-trans*-feruloyltyramine (stimulant) and 1-linoleoyl-2-lysophosphatidic acid monomethyl ester (repellent) from *Portulaca oleracea*[97] a flavone glycoside (anti-fouling) from the marine angiosperm *Thalassia testudinum*[73] a famous natural product, taxol (germination inhibitor)[153] exihibited diverse activity toward zoospores *in vitro*. These results suggest that nonhost plants may possess some secondary metabolites for protecting themselves from the attack of zoosporic phytopathogens. Research on nonhost plant constituents may yield some interesting novel lead compounds for designing biorational regulators against zoosporic phytopathogens. Morris and Ward[98] argued that chemoattractants of nonhost plants may give them a practical value for reducing or controlling the oomycete zoospore population, for example by using them as decoy crops.

I. Study approach

My study approach is to clarify the mechanisms of ecochemical interactions between plants (host and nonhost) and zoospores of the phytopathogenic oomycete *A. cochlioides*. The first step of this research is to study the structure (morphology) of zoospores and changes during interaction with the host roots. The second step is to investigate the mode of contact, attachment, differentiation and penetration of pathogen into the host tissues. Subsequently, the intention is to identify the host signal(s), which triggers attachment, encystment, germination and development of other pre-infection events on host surface. A screening of nearly 200 nonhost plants is undertaken to test their effects on the motility and viability of zoospores. Based on the screening results, three nonhost plants, *Dalbergia odorifera*, *Amaranthus gangeticus* and *Lannea coromandelica* are selected and their active principles are to be identified by bioassay-directed fractionations[66]. The ultimate aim is to clarify the mechanism of ecochemical interactions between both host and nonhost plants and oomycete zoospores. This fundamental knowledge of plant-oomycete interactions should be useful for developing a biorational low input sustainable agricultural (LISA) control

method for managing the obnoxious oomycete phytopathogens.

III. MORPHOLOGICAL STUDIES ON ZOOSPORES OF *APHANOMYCES COCHLIOIDES* AND CHANGES DURING INTERACTION WITH HOST MATERIALS

A. Introduction

The zoospores of phytopathogenic *Aphanomyces* spp. are believed to orient to the host surface by chemotaxis and then encyst, germinate and finally penetrate the host tissues[58,59,151,152]. Several efforts have been made to isolate the host-specific signal molecule(s) that guides the motile zoospores to their host roots[58,59,151,152]. Some host-specific chemoattractants have been reported, for example, indole-3-carbaldehyde (**3**) was isolated from cabbage for *A. raphani*[151], prunetin (5,4′-dihydroxy-7-methoxy-isoflavone, **4**) from garden pea for *A. euteiches*[152] and cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, **5**) from spinach and *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) from *Chenopodium album* for *A. cochlioides*[58,59]. Similarly, the zoospores of the soybean pathogen, *Phytophthora sojae*, were specifically attracted to its host isoflavones, daidzein (**1**) and genistein (**2**)[98].

Since the early days of electron microscopy, the morphological and cytological diversity of the zoospores of *Phytophthora* spp.[23,24,32] and *Pythium* spp.[90] have been studied intensively, whereas reports concerning *Aphanomyces* spp.[56] are very few. Nor have there been studies conducted dealing with the electron microscopy of the morphological diversity of the zoospores of *Aphanomyces*, and on the dynamic morphological changes of zoospores during its interaction with the host root. The morphological features of zoospores and their flagella have been suggested to vary widely among the zoosporic phytopathogens, even within the same genus[26,27]. The role of flagella in motility and / or recognition of host have been proposed by many workers[13]. Here I report detailed observations by electron microscopy of the zoospores of *A. cochlioides*, including its flagella, as well as the behavioral and morphological changes of the pathogen during the interaction with its host or in the presence of a host-specific attractant.

B. Materials and Methods

a. *A. cochlioides* strain, maintenance of phytopathogen and zoospore production

To induce zoospore production by *A. cochlioides* (AC-5, sugar beet field isolate by Yokosawa), four blocks of 4- to 6-day-old corn meal agar culture from nearly half of a plate culture (9-cm diameter) were transferred to 70 ml deionized sterilized water, washed three times to remove nutrients from the culture with 60 ml water in each treatment at 20-min intervals. Then 5-6 ml of sterilized water containing 0.01 mM $CaCl_2$ was added and the culture allowed standing for 15-18 hr at 20°C[58]. Zoospore concentration was adjusted to *ca* 1×10^5 ml^{-1} with

sterilized water before microscopic experiments. Zoospores were induced to encyst with the addition of Chromosorb W AW particles coated with a 1×10^{-6} M solution of cochliophilin A[58] into the zoospore suspension.

b. Germination of spinach seeds and preparations for microscopy

Spinach seeds (Daigaku's Hybrid Seeds, Daigaku-Noen, Tokyo) were soaked in running water overnight. The soaked seeds germinated on wetted filter paper in a petri dish at 20°C in a phytotron (16-hr light and 8-hr dark). When cotyledons emerged from the seed coat on day 6 of cultivation, the young seedlings were used in this experiment.

Zoospores of *A. cochlioides* were preferentially attracted to the roots just distal to the root cap or to the tip of cotyledons of germinated seedlings. Roots and cotyledon tips (*ca* 1-2 mm) were directly immersed in 2 ml of the zoospore suspension in a small petri dish (3-cm diameter) for a certain period (10 min or 60 min) to allow interaction between zoospores and host. The behavior of zoospores in presence of host materials was observed under a light microscope.

For scanning electron microscopy, the roots and cotyledon tips were taken from the zoospore suspension after varying durations, then gently washed with distilled water to remove unsettled zoospores. The washed tips were fixed on a cover glass for 2 hr in 2% (v/v) glutaraldehyde (TAAB, Berkshire, UK) in distilled water. The remaining procedure was similar to that for intact zoospores described in[64].

c. Transmission electron microscopy

Because of the fragility of flagellar hairs in scanning electron microscopy, detailed flagellar morphology was observed by a transmission electron microscopy as described previously[64]. Unbuffered glutaraldehyde solution prepared by using glutaraldehyde stored over barium carbonate was the preferred fixative for zoospores. Post fixation in 2% OsO_4[56] damaged the flagella of zoospores.

C. Results

a. Zoospores and their flagella

The zoospores of *A. cochlioides* (6.6-7.8 × 4.7-5.2 μm) are biflagellate, asymmetric reniform-ovate, spherical, or broadly ellipsoidal with a relatively pointed anterior, a blunt posterior end and a fairly deep, longitudinal groove region (Fig. 2A). Two heterokont flagella originated from near the center of the ventral groove (Fig. 2A).

The anterior flagellum was 17.0-19.0 μm long and 294-296 nm wide. The flagellum was ornamented throughout with stiff tubular tripartite flagellar hairs (TTHs) in two rows (straminipilous flagellum, formerly tinsel flagellum and flimmergeissel)[26] (Fig. 2E). The TTHs were distributed uniformly throughout the flagellum and the density of TTHs was recorded in 14-16 per micrometer. A

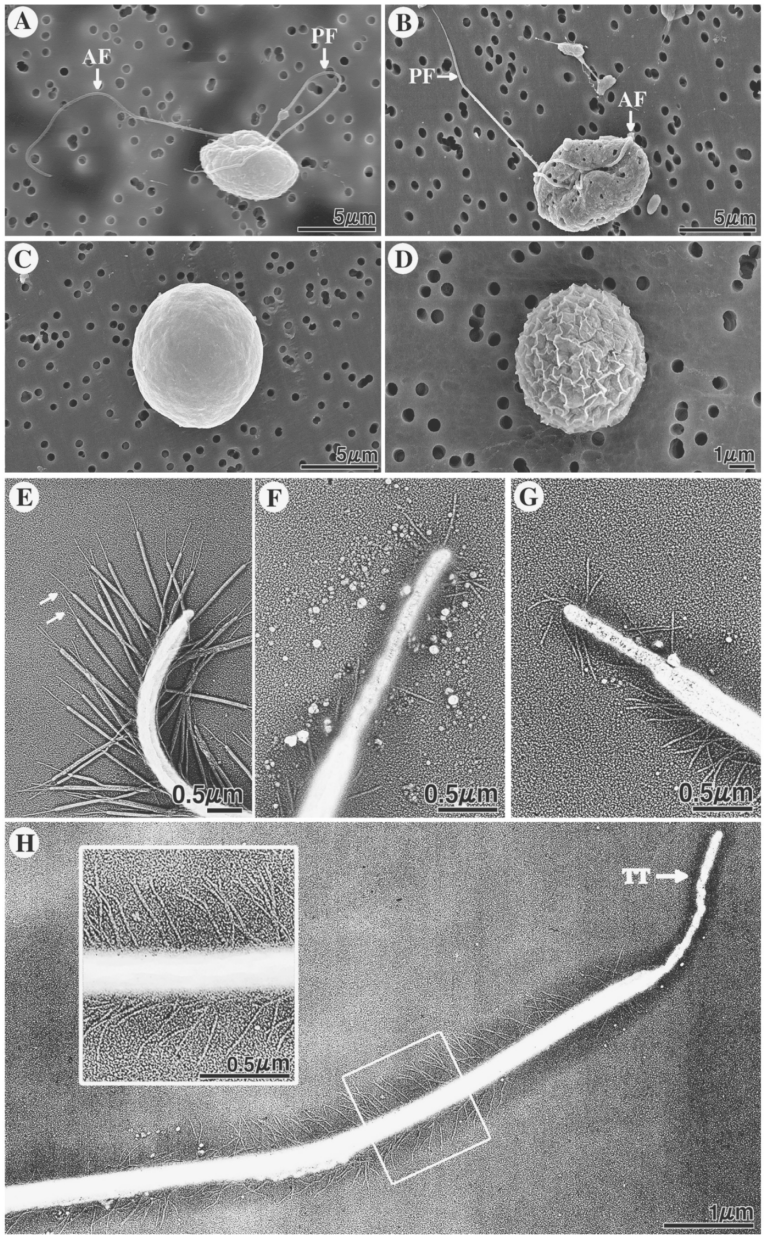


Fig. 2. Scanning (A-D) and transmission (E-H) electron micrographs of glutaraldehyde-fixed zoospore, flagella and cystospores of *A. cochlioides*.
A, A mature reniform-ovate zoospore with a shorter anterior (AF) and longer posterior (PF) flagella. B, An early stage of encystment of zoospore on SEMpore membrane after exposure to cochliophilin A. The anterior flagellum (AF) coils or winds around the zoospore and the posterior flagellum (PF) attaches to the membrane surface. C, An immature cystospore with a smooth surface. Flagella are not seen on the cystospore. D, A mature cystospore with a rough surface. E, Terminal part of an anterior flagellum possessing TTHs. The ends of the long and short terminal hairs (arrows) of a TTH on the anterior flagellum. F-G, Fine tubular hairs on the tip of posterior flagellum. H, A portion of posterior flagellum with its tapered tip (TT). Insert shows enlargment of the lateral hairs on the posterior flagellum.

TTH consisted of a long tube (1.58–1.60 μm long and 38–40 nm wide), which was divided into two unequal, fine tubes at its tip. The length of the longer tube was *ca.* 0.5 μm, the shorter one *ca.* 0.2 μm. The rounded tip of the anterior flagellum lacked TTHs (Fig. 2E).

The width of the posterior flagellum was abruptly tapered at its tip (Fig. 2F, H). The posterior flagellum extended 20.6–26.2 μm (including tip) beyond the soma. The flagellum was 260–266 nm wide throughout its length except at the tapered tip. The length and breadth of the tapered part was 1.1–1.8 μm and 130–134 nm, respectively. The posterior flagellum was also ornamented with two rows of dense, shorter, fine tubular hairs instead of TTHs (Fig. 2H). The hairs were *ca.* 0.5 μm long and about *ca.* 25 nm wide. In addition to lateral hairs, some of the specimens contained a bunch of tip hairs (Fig. 2F, G). The tip hairs were not found in the posterior flagellum of every zoospore observed. Because the number of tip hairs varied in different specimens, it was difficult to confirm the total number of tip hairs on the posterior flagellum. The length and diameter of tip hairs seemed to be equivalent to those of the lateral hairs. The density of the lateral hairs on the posterior flagellum was about 42–48 per micrometer.

b. Behavior and dynamic morphological changes of zoospores during interaction with host

A swarm of zoospores was found in the vicinity of the spinach root tips or cotyledon surface within 1 min of immersion of plant materials into the zoospore suspension. In both cases, most of the attracted zoospores became sluggish in their movement, turned frequently, moved in a circular fashion (spinning) or appeared to be probing the root surface. After only 3–5 min, they became immobile, then contracted their body frequently for a few minutes, rounded up and finally shed or retracted their flagella within 15–30 min (Fig. 2C, D). To examine the role of flagella in the precise docking of the zoospores, we fixed specimens 10 and 60 min after the immersion to allow interaction between root tips and zoospores. The posterior flagellum was attached to the root surface while the anterior flagellum was coiled around its own body during encystment (after 10 min). An almost similar phenomenon was observed when zoospores were induced to encyst by the host-specific attractant cochliophilin A on SEMpore membrane (Fig. 2B). There were no traces of detached flagella on the root surface after complete encystment of zoospores (Fig. 2C, D).

The encysted spores were oriented with their ventral grooves adhering to the root or Petri dish surface and were not detached by gentle washing with water. In some cases, the attracted zoospores formed masses on the root surface (micrograph not shown). A similar aggregation of cysts was also observed when zoospores encysted after exposure to the host-specific attractant cochliophilin A on the surface of Chromosorb W AW particles coated with 1×10^{-6} M solution of the attractant. All encysted spore (5.5 – 8.3 μm in diameter) on the root

surface or the cotyledon surface germinated forming a germ tube within 40–50 min (Fig. 3). The germ tubes were produced near the point of spore attachment to the root surface. An almost identical phenomenon was observed in the presence of cochliophilin A on SEMpore membranes (micrograph not shown). The initial diameter of a germ tube was about 1.1 μm. Light microscopic observation revealed that zoospores that encysted adjacent to the root tended to germinate and grow toward the root surface (germ tube tropism). Interestingly, the cystospores that germinated on the surface of host roots (spinach) were found to produce appressoria (Fig. 3C, D), which appeared to breach the root cuticle by mechanical force. The structure of the appressorium was variable in shape and size. Usually the germ tubes specifically formed appressoria on a ridge of the root surface. Otherwise, they grew along the surface until they reached a ridge (Fig. 3C, D).

Zoospores encysted on the cotyledon surface germinated and grew along the surface rarely forming appressorium (Fig. 3A). When the cotyledon surface was injured with a pin, germinated cystospores were found to enter the tissues through the injury (Fig. 3B). In all cases, the round cystospores were proportionally

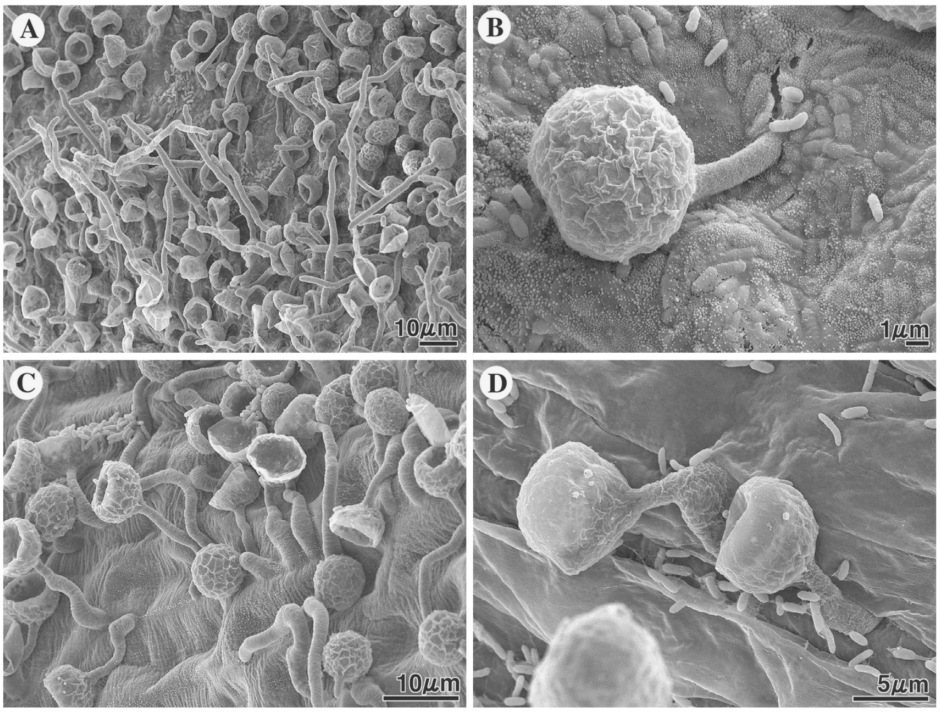


Fig. 3. Scanning electron micrographs of zoospores or cystospores of *A. cochlioides* on cotyledons or roots of spinach fixed with glutaraldehyde.
A, Germinated spores with hyphae extending along the cotyledon surface (almost no appressoria are present). B, Hypha from germinated spore penetrating the cotyledon tissue through a wound caused by pin. No appressorium formed. C, Germinated spores on the root surface. Most penetrations are by way of appressoria. D, Two germinated cystospores and tongue-like appressoria penetrating root tissue.

compressed during the elongation of germ tubes from release or transfer of cellular contents to the growing mycelium (Fig. 3A, C, D).

D. Discussion

Oomycete zoospores are biflagellate, with an anterior tinsel flagellum and posterior whiplash flagellum extending from the ventral groove. The shape of *A. cochlioides* zoospores discussed here is similar to but smaller than that of *Phytophthora* spp. zoospores[23].

Zoospores of *A. cochlioides* have two heterokont flagella ornamented with characteristic hairs, as does the tip of the posterior flagellum. The ornamentation on the flagellar shaft was also seen in *A. euteiches* but the details of flagellar hairs were not investigated[56]. Although lateral hairs on the posterior flagellum have been reported in some species of *Phytophthora*[23,24] others do not have these lateral hairs[32]. The arrangement and uniform distribution of lateral hairs on the tinsel flagellum of *A. cochlioides* were also similar to that of *Phytophthora* spp.[23,32], but different from the arrangement in fascicles or tufts reported for *Ph. cinnamomi*[20]. The role of the TTHs on the anterior flagellum in forward motion has recently been established in *Ph. cinnamomi* by using TTHs (mastigonemes)-specific monoclonal antibodies[13]. The role of fine hairs on the posterior flagellum is still unknown.

When the zoospores of *A. cochlioides* came very close to the spinach root surface, they became immobilized by shedding or retracting their flagella, and then transformed into cystospores. During encystment, the zoospores adhered precisely (at the ventral groove) to the root surface and finally germinated at one side of the cystospore (close to the point of adherence) forming a germ tube (Fig. 3). *Phytophthora* and *Pythium* zoospores also encysted on the host surface after adherence along their ventral grooves[47] but the germ tube formed just next to the point of adherence[22,47,74]. Thus the location of germ tubes formation in *Aphanomyces* and *Pythium* or *Phytophthora* cystospores seemed to be different. Encystment may be induced by some kind of elicitor, such as cochliophilin A, at higher concentrations available near the root surface[20,98] because I observed that *A. cochlioides* zoospores were induced to encyst when they encountered a gradient of cochliophilin A at higher concentration than that required for chemotaxis.

The differential response of germ tube growth and appressorium formation of the cystospores of *A. cochlioides* on the surfaces of the host root and cotyledon observed in this experiment reflects the involvement of specific signal/surface cue(s) for the induction of appressoria. Surface topography may be another regulatory factor for appressorium formation; appressoria formed only when the germ tube reached a ridge on the root surface. It is not clear, however, whether the host-specific attractant cochliophilin A influences germ tube tropism and / or appressorium formation, because appressoria merely formed on the cotyledon surface. At present, I have no information whether spinach cotyledons contain

cochliophilin A or not. In *Phytophthora sojae*, host-specific chemoattractants were found to regulate germ tube tropism[99]. The formation and structural type of appressoria were regulated by surface topography in *Ph. Sojae*[99] while surface topography apparently did not affect appressorium formation in *Lagenidium giganteum*[108].

The precise docking at the ventral groove of zoospores to the host surface seemed to be regulated by flagellar activity. The posterior flagellum may have a certain role in contact with the host surface by way of the flagellar tip, then shed or retract during encystment. No traces of detached flagella were found on the root surface when zoospores completely encysted on the root surface, indicating that flagella may be retracted during encystment. However, Peterson *et al*. used scanning electron microscopy to document the presence of detached flagella of *Lagenidium giganteum* (Oomycetes: Lagenidiales) during zoospore encystment.

There is very little information on the orientation of encystment in other zoosporic phytopathogens. The results presented here indicated that, before encystment, the posterior flagellum of *A. cochlioides* zoospores might be involved in successful landing on the host root through contact with the tip followed by retraction/shedding. Time-course electron microscopic observation may yield sufficient evidence for the involvement of posterior flagellum in precise docking of zoospores on the host surface[27].

E. Conclusion

The detailed morphology of *A. cochlioides* zoospore itself and its dynamic changes during interacting with host materials illustrated in this thesis have not been reported in other phytopathogenic zoosporic fungi. Moreover, this study clearly visualized the pre-infectional events of a zoosporic plant pathogen, *A. cochlioides*. The precise homing responses of *A. cochlioides* zoospores described here raise questions about the number of host-mediated signaling factors that lead zoopores to invasion of the host tissues. How do zoospores perceive signals and translate them into action? Further study is needed to clarify the signaling and communication between spinach roots and zoospore of *A. cochlioides*. Such information should be useful for biorational control of zoosporic plant pathogens.

IV. MICROSCOPIC STUDIES ON ATTACHMENT AND DIFFERENTIATIONOF ZOOSPORES OF THE PHYTOPATHOGENIC OOMYCETE *APHANOMYCES COCHLIOIDES*

A. Introduction

Zoospores of the spinach pathogen, *Aphanomyces cochlioides* are highly sensitive to the host-specific flavone, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, **5**) which exudes from the roots of spinach (*Spinacia oleracea*) into the rhizosphere[58,130]. Host-specific cochliophilin A (3×10^{-10} M) has also been

shown to attract zoospores of *A. cochlioides*[58]. Mastoparan (Val-Asp-Trp-Lys-Lys-Ile-Gly-Gln-His-Ile-Leu-Ser-Val-Leu-NH_2, FW = 1635) is a cationic amphipathic tetradecapeptide isolated from wasp venom and acts as a generic activator of animal heterotrimeric GTP- binding regulatory proteins (G-proteins). Mastoparan can be used to test whether G-protein-coupled-receptor exists in the membrane of zoospores.

Oomycete zoospores possess two heterokont flagella, the anterior flagellum (shorter) and the posterior flagellum (longer). Both flagella are ornamented with characteristic hairs[27,64]. The tripartite tubular hairs (TTHs) on the anterior flagellum in *Phytophthora cinnamomi* have been found to be responsible for forward motion of zoospores in aqueous media[13]. The posterior flagellum of oomycete zoospores is believed to be involved in steering the swimming zoospores with the anterior flagellum[20]. Although in phytopathogenic zoosporic phytopathogens, the role of the posterior flagellum in contacting the host before encystment has been proposed by several researchers[20,26,27] evidence supporting this view has been lacking.

The sequence of pre-infection events in zoosporic phytopathogen is extremely rapid, and the success of infection depends on the completion of these events[19,20]. Indeed, our understanding of recognition phenomena between plants and their zoosporic fungal pathogens is still very limited[20]. Hence, it is important to get precise information about the pre-infection events between the zoosporic phytopathogens and their hosts for the biorational control of those soilborne pests.

The zoospores of *A. cochlioides* rapidly aggregate at the spinach root and encyst to yield cystospores, which subsequently germinate. The resulting germ tubes finally penetrate into the root tissues[64]. To get precise information, I microscopically investigated the mode of contact, docking, adhesion and subsequent morphological changes of zoospores while interacting with the host. In addition, I recorded the time-course morphological changes of the zoospores into cystospores when elicited by cochliophilin A (**5**) and mastoparan. Here I present possible involvement of the posterior flagellum of *A. cochlioides* zoospore in precise docking on the host, and the sequence of morphological events when a zoospore develops into a cystospore upon interaction with the host or chemical elicitors. The possible role of the posterior flagellum in the aggregation of encysting spores is also illustrated.

B. Materials and Methods

a. Chemicals

Mastoparan (Val-Asp-Trp-Lys-Lys-Ile-Gly-Gln-His-Ile-Leu-Ser-Val-Leu-NH_2, FW = 1635) (*Polistes jadwagae*; synthetic), was purchased from Sigma and glutaraldehyde from TAAB, UK. Cochliophilin A (**5**) used in this experiment was synthesized by Horio *et al.*[58].

b. *Aphanomyces cochlioides* strain, culture and zoospore production

A. cochlioides (AC-5) was a gift from Professor Yokosawa. *A. cochlioides* was grown for 4–6 days on a corn meal agar (Difco) plate (9-cm diameter) at 20°C, and zoospores were produced as described in previously[72].

c. Germination of spinach seeds and electron microscopy

Seeds of spinach cv. Solomon from a local shop were surface sterilized with sodium hypochlorite, soaked overnight in running water and germinated on wetted filter paper in a petri dish at 20°C in a phytotron (16-hr light, 8-hr dark). When cotyledons emerged from the seed coat on day 6 of cultivation, the young seedlings were used in this experiment.

Some root tips (2–4 mm) were directly submerged in 2 ml of the zoospore suspension (1×10^5 ml^{-1}) in a small petri dish (3-cm diameter) for varying periods of time (5, 10, 20, 30, 40, 60 min) to allow interaction between zoospore and host. After a set time, the root tips were transferred very carefully from the zoospore suspension with a Pasteur pipette to a thin cover glass and gently washed three times with sterilized and deionized water to remove unsettled zoospores. Specimen preparation and scanning electron microscopy were carried out as described in previous section (Section III).

In the study without the host, cochliophilin A or mastoparan was added to 200 μl of a zoospore suspension on a SEMpore membrane by using two or three particles of Chromosorb W AW (60–80 mesh) coated with a solution of 1×10^{-6} M cochliophilin A (**5**) or that of 2×10^{-5} M mastoparan. The zoospores were fixed with 2% buffered glutaraldehyde after varying lengths (5, 10, 20, 30, 40, 60 min) of interaction. Further preparations for microscopy were similar to those described in earlier chapter of this thesis[64].

Each experiment was repeated at least three times, and more than 50 cells were observed in each preparation.

C. Results

a. Attachment of zoospores on the host root

Light microscopic observation revealed that the zoospores quickly aggregated around the root tip, and nearly 100% of the aggregated spores halted within 3–5 min (Fig. 4A). We also observed the time-course changes of individual zoospores with a light microscope connected to a TV monitor. Before their motility halted, the attracted zoospores became sluggish in their movement, and they appeared to be probing the root surface, probably with the posterior flagellum. Because of the low magnification (X 20) it was not clear which flagellum was involved in probing. The attracted zoospores sometimes formed a mass of encysted spores at a certain site on the root (Fig. 4B). Within 5 min, the anterior flagellum of the halted zoospores (more than 80%) was coiled on their bodies, while 100% of the posterior flagella remained undistorted and attached to the

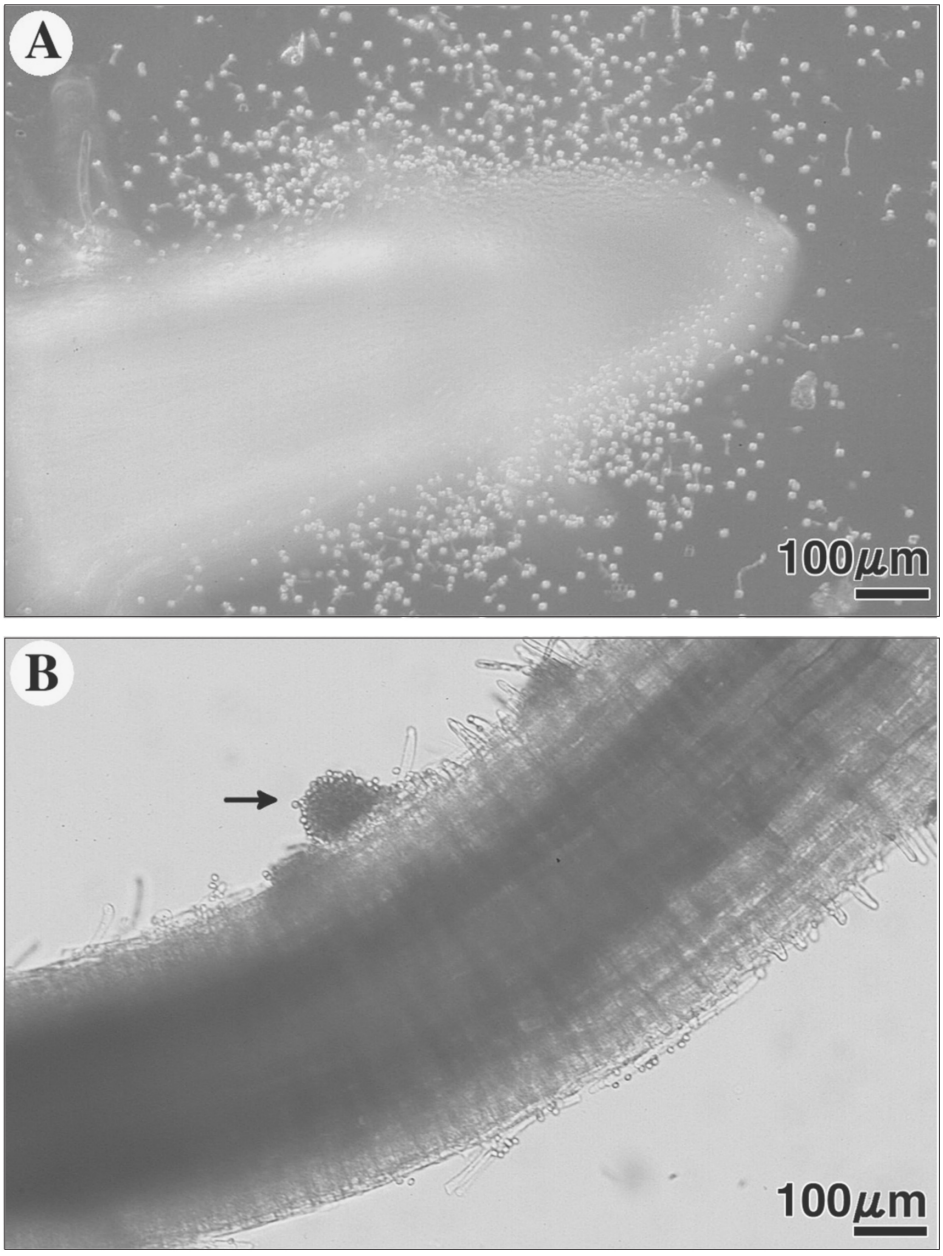


Fig. 4. Response of *Aphanomyces cochlioides* zoospores toward spinach roots.
A, Photomicrograph (dark field) of aggregated zoospores (dots close to root surface) just behind root cap of spinach root tip. B, A mass of cystospores (arrow) on spinach root.

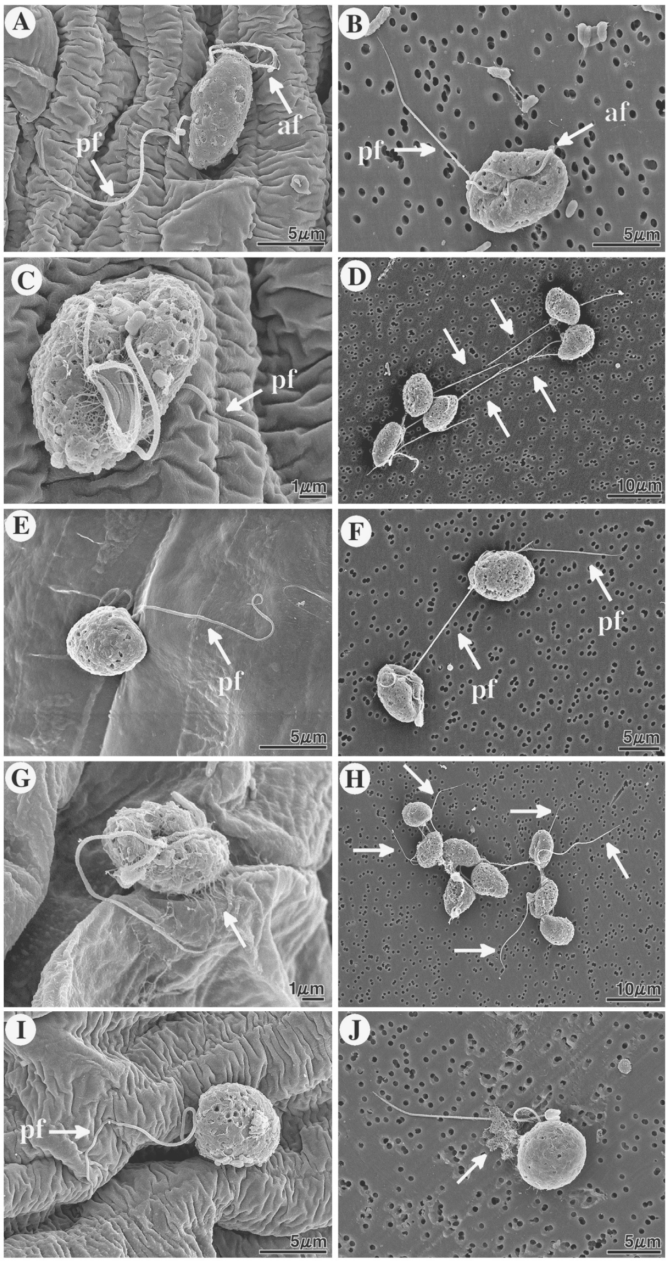


Fig. 5. Scanning electron photomicrographs of zoospores of *A. cochlioides* interacting with its host root (A, C, E, G, I), and different stages of mastoparan-stimulated spores on SEMpore membrane (B, D, F, H, J).
A, The tip of posterior flagellum (pf) attaches to the root surface and seems to pull the zoospore onto the host surface by partially coiling at the base, and anterior flagellum (af) coils around zoospore body. B, The pf attaches to membrane surface, while af winds around zoospore. C, A zoospore attaches to root surface by its dorsal surface, af coils around body, and pf attaches to root surface. D, The pfs of some mastoparan-induced spores have a tendency to trap the surrounding spores (arrows). E, A nearly spherical spore attaches to root surface at its ventral groove, and pf coils at the distal end (no trace of af). F, A mastoparan-induced spore hooks to another by the tip of the pf, while tip of the other zoospore attaches to membrane surface. G, A partial turn of zoospore from its original placement exposes traces of adhesive material. H, Aggregated spores stimulated by mastoparan connect to each other by their pf on membrane surface, and tips of pf of some spores are seen away from their body (arrow). I, An almost round encysting spore with its pf. J, A round, encysting spore with pf on the membrane. The residue of detached af (arrow) is near the spore

root surface (Fig. 5A-C). Almost all zoospores retained their original shape. Zoospores at this stage were found to attach to the root surface or to the surface of the SEMpore (pore size 0.6 μm, JEOL) membrane, usually at their dorsal surface (Fig. 5B, C).

Most of the zoospores changed their position of attachment by coiling the posterior flagellum within 10 min of interaction (Fig. 5E, I). Many zoospores attached to the root surface with their ventral groove and the posterior flagellum was partly coiled, whereas the tip of the flagellum seemed to closely adhere to the root surface (Fig. 5E, I). The zoospore became nearly a round shape (Fig. 5I, J) and finally shed its flagella and gradually changed into a cystospore.

b. Encystment and germination of zoospores

No trace of flagella (even the posterior flagellum) was observed in specimens fixed after 20 min of interaction (Fig. 6A, B). Interestingly, most spores became round and significantly enlarged (8.5–10.5 μm), and they were bounded by a smooth cyst coat (Fig. 6A, B). This appeared to be an intermediate stage of cell wall formation because a portion of the spores changed partially or completely into the shape of a mature cystospore with wrinkled walls (Fig. 6C, D). Before this stage, the encysting spore was well anchored to the host surface by adhesive material, probably released from inside the spore. Plate 5–I-G shows a trace of adhesive material, which became visible accidentally when an adhered spore was moved from its original state during specimen preparation. Those zoospores induced to encyst by cochliophilin A or mastoparan on a glass petri dish or slide glass adhered in a similar way to the substrate and was not removed by gentle water flow (data not shown).

All zoospores fixed 30 min after interaction with the host roots had encysted on the root surface (Fig. 6E). The surface of the cystospores was wrinkled, and they were almost similar in size (5.7–7.1 μm). Cystospores adhered to the root surface either scattered or in groups mostly behind the root cap.

All cystospores germinated after 40 min of interaction and the germ tubes started to penetrate the host tissue after 60 min (Fig. 6F). Germination of all cystospores occurred at one side of the cystospores forming cylindrical germ tubes. The tip of the germ tubes changed into appressorial structures only when they reached a ridge on the root surface (Fig. 6F). Finally, all cellular materials of a cystospore seemed to move to the growing hypha leaving an empty thin cyst coat (Fig. 6F).

Mechanical stimulation of the zoospores with a vortex mixer for 25–30 sec resulted in encystment. These cysts usually remain the initial stage of cystospores and regenerated a new generation of zoospores after 12–14 hr instead of germinating. I examined the morphology of mechanically stimulated cysts (45 min after vortexing) with scanning electron microscopy and found that they possess mainly smooth cyst coats. Interestingly, when the new generation of

zoospores emerged from these vortex-induced cysts, they left their smooth cyst coats (Fig. 6G).

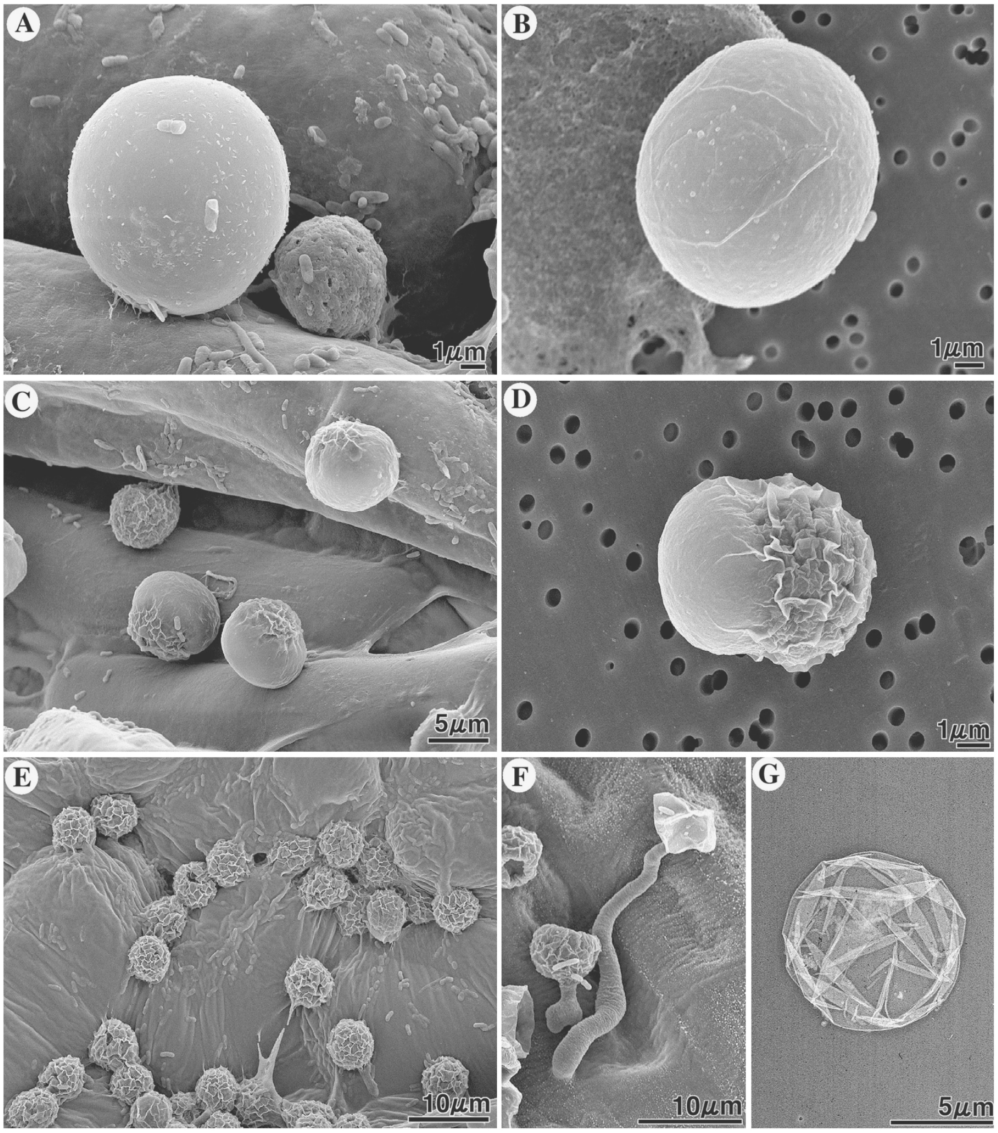


Fig. 6. Glutaraldehyde-fixed scanning electron micrographs of differentiating zoospores of *A. cochlioides* on spinach (host) root surface (A, C, E, F) and on SEMpore membrane stimulated to encyst by mastoparan (B, D).
A, An enlarged spherical spore covered by a smooth cyst coat and spore with a relatively rough surface at an earlier stage of encystment. B, Similar enlarged spore on membrane surface. C, Formation of thick cell wall on the surface of cystospores. D, An immature cystospore showing the formation of wrinkled cell wall. E, Mature cystospores on the host root. F, A hypha formed from a cystospore leaving an empty cyst coat. G, Empty cyst coat (ghost) after regeneration of zoospore from a vortex-induced cyst.

c. Differentiation of zoospores induced by host-specific attractant or G-protein activator, mastoparan

I found that the gradient of host-specific compound, cochliophilin A- or the G-protein activator, mastoparan-treated encystment of zoospores followed by germination (particle method) (Section V). When I observed the morphological changes of zoospores with scanning electron microscopy, almost similar phenomena were found both in cochliophilin A (**5**)- and mastoparan-induced zoospores on the SEMpore membrane. In both cases, zoospores initially stopped (instantly with mastoparan or within 3–5 min with cochliophilin A) and coiled the anterior flagellum on their bodies (Fig. 5B). The posterior flagellum was undistorted and attached to the membrane surface (if there is no other zoospore) (Fig. 5B) or to the body of a nearby zoospore (Fig. 5F, H). At an early stage of induction, the posterior flagellum had a high tendency to trap other zoospores (Fig. 5D). Interestingly, the tip of the posterior flagellum also consistently caught other encysting spores on the SEMpore membrane (Fig. 5F), resulting in aggregation of encysted spores on the membrane surface (Fig. 5H). Although some zoospores (30–40%) encysted singly, a high density of spores in the zoospore suspension favored the aggregation of encysting spores. The rest of the changes were exactly the same as those on the root surface. No appressorial structures formed in the germinated cystospores on the membrane surface (micrograph not shown).

D. Discussion

The time-course observations of the interactions between *A. cochlioides* zoospores and its host roots (spinach roots) revealed that the tip of the posterior flagellum may be involved in homing of the halted zoospores by cochliophilin A on the root surface (Fig. 5A, C, E, I). The fine hairs on the shaft and tip of the posterior flagellum may be involved in tightly adhering the flagellum to the root surface[64]. The immobile spores may also use tip-contact to hook up to their target host to prevent water flow in the soil from washing them off their host (Fig. 5A, C). After landing by this tip-contact mechanism, the flagellum seemed coil and drew the spore into the right position (Fig. 5E, I). Adhesive material was probably then released through the ventral groove (Fig. 5G)[20]. Release of adhesive material for anchoring spores on the host surface during encystment is common to all oomycete zoospores[20,47,108]. Contact with the host by the tips of posterior flagella has also been claimed for some other zoosporic fungi[52,53]. In contrast, fine hairy structures, called fimbriae or pili, surrounding the bacterial cells, play vital roles in adhesion of bacteria to the host surfac[86].

Zoospores of *Pythium* and *Phytophthora* have a fixed site of germination, located near the point of flagellar insertion[19]. Hence, the zoospore orients itself precisely during encystment so that it does not germinate away from the host[47]. Precise docking has been reported in several organisms, suggesting that it is a common or even universal feature of fungal zoospores. Considering the precise

orientation observed in encystment of *Pythium* and *Phytophthora*, Deacon[19] suggested that the flagella of zoospores are involved in a recognition event. My scanning microscopic observation suggests that *A. cochlioides* zoospores use their posterior flagellum for precise docking on the host surface.

After successful docking on the host surface and release of adhesive materials from inside the spore, the role of posterior flagellum was apparently complete. Consequently, the adhered spore shed its posterior flagellum before changing into a cystospore. Flagellar detachment during differentiation of zoospores has been reported in many cases, and it appears to be a common feature in oomycetes[56,108].

The time-course of microscopic observation revealed that a zoospore changed into a cystospore *via* a distinct transitional stage, *i.e.*, enlarged, smooth-surfaced cysts. Figures 6C and D clearly show the progression of changes of a smooth-surfaced cyst into a mature cystospore, which indicates that the wrinkled surfaces of mature cystospores are not an artifact caused by dehydration. My scanning electron microscopic investigation is the first to illustrate this intermediate stage as a pre-infection event of a phytopathogenic zoospore. In a zoosporic phytopathogen, the success of infection depends on the successful completion of all pre-infection events in a sequence[19]. At present, I have no information about the composition of smooth coats of the immature cystospores. Both vortex- and cochliophilin A-induced cysts appeared to shed their smooth cyst coats during their regeneration into zoospores, whilst germination to produce new hyphae, the cystospore coat remained at one end, respectively (Fig. 6F, G). These results indicate that the outer, smooth cyst coat formed at an early stage of encystment, may serve only as a protective shield around the cysts; they are not reused in the new generation of zoospores. Another behavioral characteristic of *A. cochlioides* is that the posterior flagellum may be involved in aggregation of encysting zoospores near the host surface or in the absence of the host when zoospores are induced to encyst by the effect of a chemical (here by mastoparan, Fig. 5D, F, H) or mechanical stimulus. In the former case, the aggregation of zoospores on a specific area of the root surface might be effective in increasing the inoculum density for successful infection (Fig. 4B). On the other hand, in the absence of the host, the aggregation of encysted spores by way of their posterior flagella and the regeneration of zoospores under adverse environmental conditions may be an adaptive mechanism for the survival of potential fungal spores. These adaptive mechanisms may exist in other biflagellated oomycete zoospores in a natural ecosystem.

E. Conclusion

In conclusion, the results from the present work demonstrate that zoospores of *A. cochlioides* pass through a series of distinct pre-infection events before penetrating host tissues by the aid of chemical signals. The posterior flagella of

zoospores may be involved in contact and docking of zoospores on the host surface, as well as in the aggregation of encysting spores in the absence of host.

V. HOST-SPECIFIC PLANT SIGNAL AND G-PROTEIN ACTIVATOR, MASTOPARAN, TRIGGER DIFFERENTIATION OF ZOOSPORES OF THE PHYTOPATHOGENIC OOMYCETE *APHANOMYCES COCHLIOIDES*

A. Introduction

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[63]. This fungus has a limited host range, namely, sugar beet, spinach, *Chenopodium album*, and a few other members of Chenopodiaceae and Amaranthaceae[142]. Cochliophilin A (**5**) (active at 3×10^{-10} M, particle method), was identified as a host-specific attractant of zoospores of the *A. cochlioides* from the roots of spinach[58]. A phenolic amide (*N-trans*-feruloyl-4-*O*-methyldopamine, **6**) was also isolated from the roots of another host plant *Chenopodium album* as a potent attractant of *A. cochlioides* zoospores (active at 1×10^{-8} M by the particle method)[59]. The third attractant from the host, 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (**7**) (active at 1×10^{-6} M, particle method) was isolated from the leaves of spinach[130–132]. Cochliophilin A (**5**) had also been isolated as a non-antifungal flavone from the roots of sugar beet inoculated with the fungus *Rhizoctonia solani*[133]. Compound **5** was also found in the roots of sugar beet and *Chenopodium album*[59]. Moreover, it was estimated that fresh spinach roots contained approximately 2×10^{-8} M cochliophilin A (**5**) and exude sufficient amount (34 ng/plant/day) of this compound for attracting the zoospores of *A. cochlioides*[130]. So far, the distribution of compounds **5** and **6** are completely restricted in the hosts (Chenopodiaceae) of *A. cochlioides*. Thus, Tahara and Ingham[130] suggested that chemotaxis might be a part of the mechanism that determines the host range of this phytopathogen[130].

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[19] suggested that zoospores might 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, polysaccharide with fucosyl residues, pectin, alginate or specific polysaccharides, lectin or monoclonal antibodies specific for flagella, and some

host root extracts[20]. 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 in the development of phytopathogenic zoospores[18,147]. All other components of the signal transduction pathway are yet to be known in oomycete zoospores.

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

B. Materials and Methods

a. Chemicals

Cochliophilin A (**5**) and *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) used in this experiment were synthesized previously[58,59]. 5,4′-Dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (**7**) was isolated from the leaves of spinach (Tahara *et al*., 2001a). Mastoparan (Val-Asp-Trp-Lys-Lys-Ile-Gly-Gln-His-Ile-Leu-Ser-Val-Leu-NH_2, MP FW, 1635.0) Mastoparan 17 (Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Leu-Ala-Lys-Lys-Leu-Leu-amide, Mas17) EGTA, loperamide and neomycin (fradiomycin sulfate) were purchased from the reputed pharmaceutical companies.

b. Culture of *Aphanomyces cochlioides* and preparation of zoospores

Culture of *A. cochlioides* (AC-5) and production of zoospores were conducted as described previously[72]. Concentration of zoospores was adjusted to about 1×10^5 ml^{-1} with sterilized water before bioassay or microscopic preparations.

c. 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 described in section IV. 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*. 200–225 μm) on a watch glass. A tip of filter paper immediately absorbed excess solution 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[138]. One or two of these particles were carefully dropped into 2 ml of a zoospore suspension (1×10^5 ml^{-1}) in a small petri dish (3 cm i.d.). Therefore, when the particle exposed to high volume of water, the coated compounds diffused to the surrounding water and develop 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. Otherwise, the mixture of the compounds was used to coat Chromosorb particles.

d. 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 (1×10^5 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 in small petri dish for one hour[64]. 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 encyst-

ment and germination of zoospores were 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.

e. 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 corn meal agar (CMA) medium of isolate AC-5 was placed at the center of a petri dish (9.0 cm i.d.) containing either 0 or 1×10^{-12} to 1×10^{-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 (X 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.

f. 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 certain amount of water suspended test compounds was 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.

C. Results

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

The effects of three host-specific attractants, cochliophilin A (**5**) *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) and 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone (**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 1×10^{-8} to 1×10^{-6} M concentration in a dose dependent manner (Table 1) and formed a mass of cystospores on and around the Chromosorb W AW particles (Fig. 7A-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 1×10^{-7} to 1×10^{-6} M solution of cochliophilin A (Fig. 7A-C). All encysted zoospores germinated (100%) on and around the particles within 30–40 min (Fig. 7B, C, E, F). The cystospores germinated close to the particles coated with host-specific attractants showed germ-tubes tropism toward the particles (Fig. 7B, F). The particles coated with lower than 1×10^{-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 (Fig. 7D). The other two host-specific attractants (**6** and **7**) also induce encystment and germination, albeit at higher concentrations.

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}	+	na	na
	10^{-8}	+++	+	+
	10^{-7}	+++	+++	+++
	10^{-6}	+++	+++	+++
N-trans-feruloyl-4-*O*-	10^{-8}	+	na	na
methyldopamine (**6**)	10^{-7}	+++	na	na
	10^{-6}	+++	na	na
	10^{-5}	+++	+	+
5,4´-dihydroxy-3,3´-	10^{-6}	+	na	na
dimethoxy-6,7-methylene-	10^{-5}	++	na	na
dioxyflavone (**7**)	10^{-4}	+++	+	+
5 + neomycin (10^{-5} M)	10^{-8}	++	na	na
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 7A and 7B; na = no response.

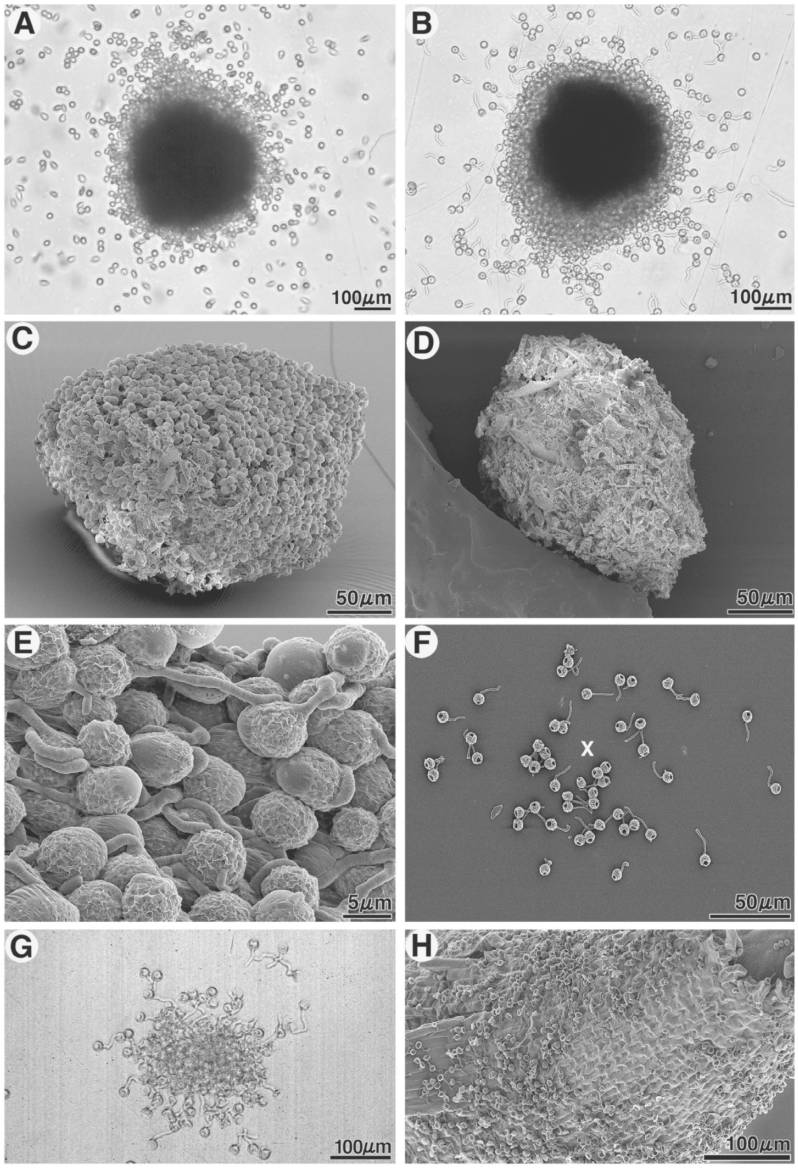


Fig. 7. Light (A, B, G) and scanning electron micrographs (C-F, H) showing aggregation, encystment and germination of *Aphanomyces cochlioides* by activity by a host-specific zoospore attractant, cochliophilin A. A, Zoospores aggregated around a Chromosorb particle coated with 10^{-7} M solution of cochliophilin 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 W AW 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).

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 1×10^{-12} to 1×10^{-6} M concentration. The direct application of **5** into the zoospore suspension as a homogeneous solution at a range of 1×10^{-12} to 1×10^{-8} M just stimulated the motility of zoospores for 10–15 minutes without resulting any encystment and germination. However, at higher concentrations (1×10^{-7} to 1×10^{-6} M) of **5** in the above conditions, 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 (Fig. 7G). The germ tubes of the spores germinated a little far from the aggregate center exhibited tropism toward the aggregate center (Fig. 7G).

b. Mastoparan, a peptide agonist toward heterotrimeric G-protein, elicits differentiation of 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)[117]. As G-proteins are also believed to be key components of signal transduction pathways in many other motile cells[144] 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. The effect of mastoparan on the differentiation of *A. cochlioides* zoospores are presented in Table 2 and illustrated in Figure 8. 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 to 50 μM concentration in a dose dependent manner (Fig. 8A, 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 (Fig. 8E, F). All the aggregated zoospores (Fig. 8F) settled down and then became round shape and encysted at the bottom of petri dish. Most of the cystospores (60–80%) germinated within 40–60 min (Fig. 8H). 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[54]. 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 to 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**).

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	na	na	na
	10	+	+	+
	25	++	++	+
	50	+++	+++	++
	100	+++	+++	++
Mastoparan 17	10	na	na	na
	25	na	na	na
	50	na	na	na
	100	na	na	na
Mastoparan + **5** (10^{-9} M)	10	++	++	++
Mastoparan + **5** (10^{-8} M)	10	+++	+++	+++
Mastoparan + neomycin (10 μM)	10	na	na	na
Mastoparan + neomycin (10 μM)	25	+	+	na

*Particle bioassay method; '+' sign indicates clear positive bioactivity over control; Number of '+' sign indicates the degree bioactivity (halting motility/encystment/germination); + = weak (Figure 8A), ++ = medium, +++ = strong response (Figure 8B) ; na = no response.

c. 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, I 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 mor-

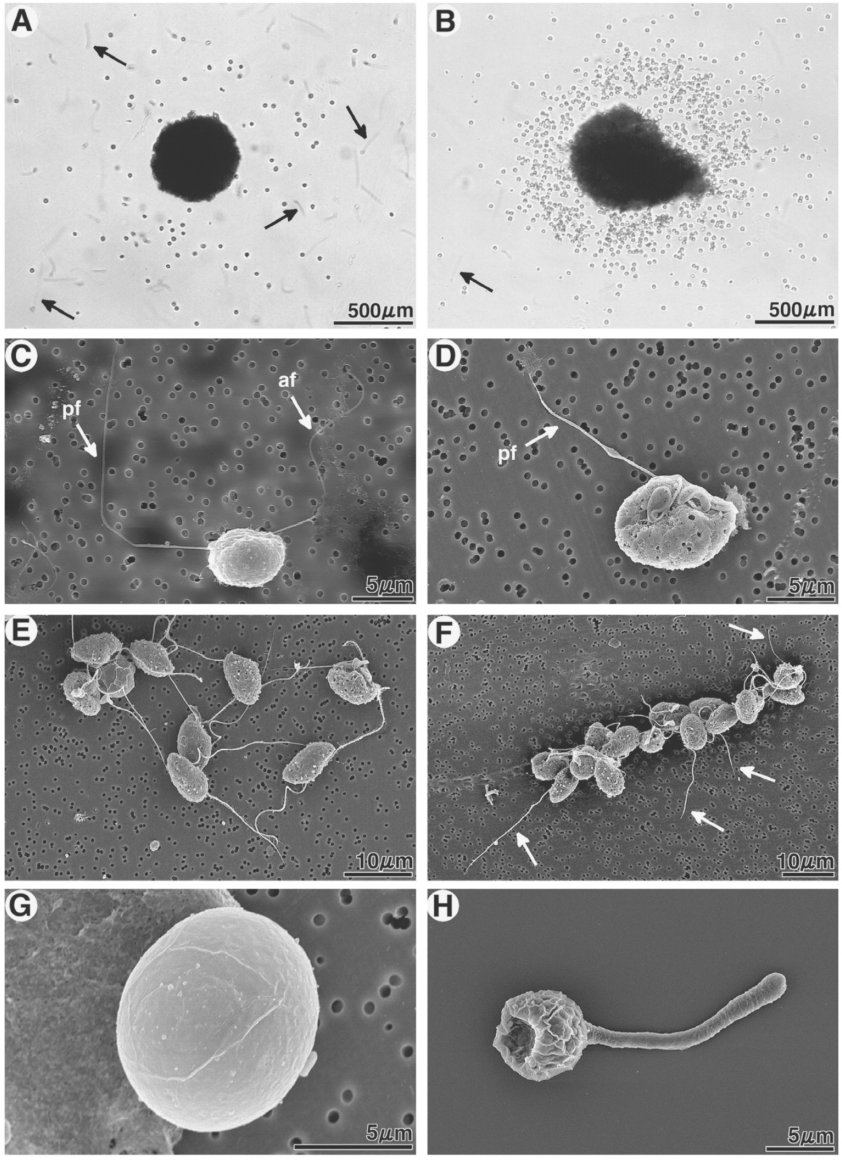


Fig. 8. Light (A and B) and scanning electron micrographs (C-H) showing the halting activity and subsequent morphological changes of *Aphanomyces cochlioides* zoospores exposed to mastoparan.
A, Halted zoospores (dots) around a Chromosorb particle coated with 1×10^{-5} M solution of mastoparan (after 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 5 min around a particle coated with 1×10^{-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).

phological changes up to germination of cystospores (Fig. 8D-H). During the first 5–10 min of stimulation, zoospores were found to coil or wind up their anterior flagella on their own bodies (Fig. 8D). At this stage the posterior flagellum was attached to the body of other zoospores (in case of dense population) or remained unchanged (Fig. 8D). 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) bounded by a smooth cyst-coat (Fig. 8G). 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 (Fig. 8H). Interestingly, the sequence of morphological changes of zoospores by cochliophilin A or mastoparan was completely similar to those occurred during interaction with spinach roots (Fig. 8H).

d. 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 (1×10^{-11} to 1×10^{-6} M). The radial growth and oospore production of this fungus on a corn meal agar medium was unaffected in the presence of host-specific compounds at a range from 1×10^{-11} to 1×10^{-6} M (data not shown). The release of zoospores from the mycelia was also not at all affected up to 1×10^{-6} M concentration of host-specific compounds in the culture media (data not shown).

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

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 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[93]. Application of neomycin at 1×10^{-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).

D. Discussion

Zoospores of soilborne Oomycetes 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 (adhesive substance)[44] encyst and germinate by the recognition of host-surface components[19,64] and finally penetrate into the host directly or *via* appressoria[10,64]. 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 (Fig. 7A-C). The behavior of zoospores on around Chromosorb particles coated with cochliophilin A (Fig. 7C, E, H) was similar to that of zoospores toward spinach roots. The concentration of **5** required (1×10^{-8} M) to initiate encystment followed by germination are equivalent to the content of it estimated in fresh spinach roots[130]. 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 (Fig. 7C, 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-infection events, those are prerequisites for invasion of the zoosporic fungal pathogen into the host. Only a germinated cystospore can penetrate into root tissues directly or *via* appressorium[10,64]. Interestingly, an almost similar phenomenon was observed in bacteria. As signal for chemotaxis of rhizobia a concentration as low as 1×10^{-9} M luteolin is sufficient, and at 10^{-6} M concentration luteolin stimulates *nod* gene expression[6,33].

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** (1×10^{-11}–1×10^{-10} M) by a micro-syringe into the zoospore suspension stimulated to form clumps of encysted spores at the bottom of petri dish (Fig. 7G). 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, the 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 (Fig. 7A-E)[107].

The germ-tubes from encysted spores at a little far from the particles coated with **5** or those from autoaggregated spores showed tropism toward the aggregate center (Fig. 7B, F). Tropic responses of hyphal germlings to host-specific signals have also been observed in *Phytophthora sojae*[99] and autoaggregation of zoospores in the absence of a host appears to be characteristic of many other oomycetes[116]. 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* were found to be unaffected up to 1×10^{-6} M concentration of host-specific signals in a homogeneous solution (data not shown). This information supports that this fungus can grow well and produce 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.

Transient rises in cytoplasmic Ca^{2+} concentration have been implicated in the differentiation of zoospores by external signals[18,147]. I have examined whether cochliophilin A induced differentiation of zoospores requires Ca^{2+} fluxes across the plasma membrane. I found that Ca^{2+} channel blocker loperamide (20 mM) or Ca^{2+} chelator EGTA (10 mM) also decreased the encystment and germination power of mastoparan or host-specific attractants (data not shown), suggesting that transduction of host-specific signal or G-protein activator requires transmenbrane Ca^{2+} fluxes. The inhibitors also partly suppressed the chemotaxis of zoospores by the host-specific compounds (data not shown).

Mastoparan is commonly used as diagnostics for the participation of G-proteins in both animal and plant signal transduction pathways[100,109]. The heterotrimeric G-protein activator, mastoparan promoted both encystment and germination of zoospores at micromolar concentration (Table 2 and Fig. 8). 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 applications 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 (data not shown). These results 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 established[18,20,146,149]. To my knowledge, this is the first indication that G-protein mediated signaling mechanism is involved in oomycete zoospores. My 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[14,107,144]. 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[100,145].

Mizutani *et al*.[97] found that the interaction of two nonhost 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*. 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[129]. Rapid increase of phosphatidic acid (PA) was observed in the differentiating zoospores of *Phytophthora palmivora* by trispectate[156]. Zhang *et al*.[156] suggested that the kinetics of PA production during differentiation of zoospores might 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[70].

E. Conclusion

This study has shown that at elevated concentrations, host-specific attractant or G-protein activator triggers encystment followed by germination of *A. cochlioides* zoospores. The host signal in the range of concentration likely to be present in the rhizosphere is not toxic to the growth and reproduction of this zoosporic phytopathogen. Together the results suggest that chemotaxis and subsequent differentiation of zoospores may be initiated by an agent stimulating G-protein-coupled receptor, which activates phosphoinositide and Ca^{2+} second messenger pathways.

VI. ZOOSPORICIDAL ACTIVITY OF POLYFLAVONOID TANNIN IDENTIFIED IN *LANNEA COROMANDELICA* STEM BARK AGAINST PHYTOPATHOGENIC OOMYCETE *APHANOMYCES COCHLIOIDES*

A. Introduction

Accumulated evidence suggests that the motile zoospores of *A. cochlioides* locate their host by utilizing chemical signals released from the roots of host plant, and then undergo a series of morphological changes leading to initiate infection[58,64,68,112]. In contrast to susceptible plants, I hypothesized that nonhost plants may have some "chemical weapons" for their resistance[63]. I undertook a survey of physiologically active constituents in nearly 200 nonhost traditional medicinal plants guided by bioassay using zoospores of *A. cochlioides*. The motility and viability of the zoospores were markedly affected by some of the crude extracts. Among the activities of plant extracts, sudden inhibition of motility followed by characteristic lysis of zoospores by the stem bark extracts

of *Lannea coromandelica* (Anacardiaceae) was noticeable. This interesting observation prompted me to characterize the motility inhibiting and lytic factors of *Lannea* extracts by detailed bioassay-directed fractionation.

L. coromandelica L. is a deciduous tropical tree widely distributed in Bangladesh, India and some other tropical countries. Plants belonging to this genus are used in folk medicine for treatment of elephantiasis, impotence, ulcers, vaginal troubles, halitosis, heart disease, dysentery, gout and rheumatism[101,154]. Reports on phytochemical investigations of *Lannea* genus are scanty, although some natural products including flavonoids, hydroquinones, ferulic acid esters have been isolated from this genus[41,60,101,154].

Bioassay-guided fractionation and chemical characterization of *Lannea* extracts indicated that some polyflavonoid tannins are responsible for zoosporicidal activity[69]. This section describes the characterization of chemical properties of the zoosporicidal factor in *L. coromadelica* extracts and the bioassay results of *Lannea* extracts compared with two popular commercial polyflavonoid tannins, Quebracho and Momosa. In addition, the characteristic morphological changes of zoospores in the presence of polyflavonoid tannins are visualized by scanning electron microscopy. The potential role of condensed tannins in plant-pathogen compatibility is also discussed in relation to the biorational control of oomycete phytopathogens.

B. Materials and Methods

a. Chemicals

Two commercial polyflavonoid tannins namely Quebracho (wood tannin of *Schinopsis balansae*, Anacardiaceae) and Mimosa (bark tannin of *Acacia mearnsii*, Leguminosae) for industrial use were received as the gifts from Dr. Kazuaki Takenouchi and Mr. Masahiro Sato, Graduate School of Agriculture, Hokkaido University, Japan. The commercial tannins were extracted with 80% acetone and successively fractionated as with *Lannea* tannin described below.

b. Plant materials, extraction and fractionation

The stem bark of *L. coromandelica* was collected from Mymensingh district of Bangladesh. The dried pulverized sample (1.9 kg) was successively extracted with 80% acetone and 60% MeOH to yield 280 g and 271 g of partially concentrated extracts, respectively. The acetone extracts showed bioactivity, and were successively fractionated according to solubility with *n*-hexane, diethyl ether, EtOAc and MeOH. The motility inhibitory and lytic activity was observed in MeOH fraction (256 g), which was subjected to successive bioassay directed fractionation using SiO_2 gel, Sephadex LH-20 and RP-18 reversed phase column chromatography. But none of the chromatographic techniques were found suitable for separating active constituents, and hence, the MeOH solubles were directly used for bioassay and MALDI-TOF-MS analysis.

The UV absorbance (at 280 nm) of MeOH solubles of *Lannea* extract was *ca* 23.16/mg/ml. Vanillin-sulfuric acid reagent spray on a silica gel thin layer plate gave quick red coloration. The UV absorbances (at 280 nm) of the MeOH solubles of Quebracho and Mimosa tannins were *ca* 17.73 and 9.02/mg/ml (in MeOH), respectively. Methylation and acetylation of the MeOH fraction of acetone extracts were carried out as described before[62] and the products were purified by SiO_2 gel column chromatography. Both mixture and purified acetylation and methylation products were bioassayed and were found inactive up to 100 μg/ml concentration.

^1H and ^{13}C NMR spectra were measured in Me_2CO-d_6 at 270 MHz using TMS as the int. standard. Other instrumental analyses were conducted using a JEOL JSM AX-500 (FAB) and JEOL JSM-SX102A (FD) for mass spectrometry, and a HITACHI U-3210 spectrometer for UV spectrometry (in MeOH).

c. MALDI-TOF-MS

The tannin samples were dissolved in acetone (4 mg/ml). About 1 μl of sample (tannin extract) was placed on the MALDI target followed by equal volumes of NaCl and matrix (2,5-dihydroxy benzoic acid) solutions were added on the same MALDI target[105]. After evaporation of the solvent the MALDI target was introduced into the spectrometer. The spectra were recorded on a Voyager DE-STR/15000 instrument. The irradiation source was a pulsed nitrogen laser with a wavelength of 337 nm. The measurements were carried out using the following conditions: polarity-positive, flight path-linear, mass-high (20 kV acceleration voltage), scans averaged 256. The delayed extraction technique was used applying delay times of 300 ns.

d. Production of zoospores and bioassay

A. cochlioides (AC-5) was a gift from Prof. R. Yokosawa, which was isolated from the soil of sugar beet field. Culture of *A. cochlioides* and production of zoospores and bioassay was carried out as reported previously[63,124]. Quantitative bioassay for *Lannea* extracts and two commercial tannins were carried out as follows. Tannin extracts were first dissolved in small quantities of DMSO and then diluted with distilled water. Appropriate amounts of sample suspension were directly added to the zoospore suspension taken in one dish of Nunc Multidish (NUNC™) to make final volume as 200 μl and quickly mix well by a glass rod. The final concentration of DMSO in the zoospore suspension was maintained less than 1% in all treatments. In control treatment, only equal volume of deionized water containing equivalent amount of DMSO was added instead of sample solution. The halted zoospores rapidly settled at the bottom of petri dish and then started to burst. Time-course changes of the number of settled and burst spores were counted microscopically (X 20 magnification). Each treatment was replicated thrice and the mean value (%) of each treatment

was compared to that of the control. The percent of halted and burst zoospores was calculated by the above formula.

$$\% \text{ of halted zoospores} = \frac{H_t - H_c}{H_v} \times 100$$

$$\% \text{ of burst zoospores} = \frac{H_b}{H_v} \times 100$$

H_t = average number of halted spores per microscopic field in tannin treated dish

H_c = average number of halted spores per microscopic field in control dish

H_b = average number of burst spores per microscopic field in tannin treated dish

H_v = average number of halted spores per microscopic field in dish containing equal volume of vortexed (30 sec) zoospore suspension.

Vortexing of 30 sec caused 100% halting in 1 min. Spores were counted from at least 5 microscopic fields in each dish and averaged

e. Scanning electron microscopy

Appropriate amount of tannin extract was directly added to a zoospore suspension taken on a SEMpore membrane (pore size 0.6 μm, JEOL). After a set time (5, 30 and 60 min for *Lannea* extracts, 10 and 30 min for commercial tannins) of treatments, the specimen was fixed with 2% buffered glutaraldehyde (TAAB, Berkshire, UK) at room temperature (about 23 ˚C). After dehydration in a graded acetone series (50%, 70%, 90%, 95% and 99.5%), the spores were critical-point dried using liquid CO_2, and coated with 10–nm thick platinum-palladium using a sputter coater. The coated spores were observed under a JSM-6301F, JEOL scanning electron microscope with accelerating voltage of 5 kV[64].

C. Results and Discussion

a. Isolation of the factor responsible for motility inhibition and lysis of zoospores

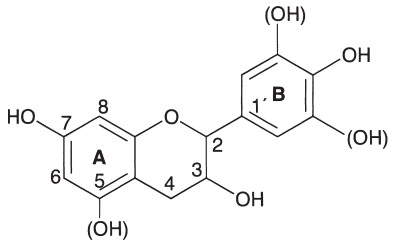
The MeOH fraction of acetone extract of *Lannea* stem bark showed bioactivity, and hence subjected to bioassay-guided fractionation by different column chromatography including SiO_2 gel, Sephadex LH-20, and RP-18 CC (data not shown). However, none of the chromatographic techniques were found suitable for separating the active factor(s) due to its high polarity and complex behavior in chromatography. The active fraction (MeOH solubles from the 80% acetone extracts) was tan colored amorphous powder soluble in 80% aqueous acetone and gave a sharp UV absorption maximum at 280 nm (in MeOH). The FD-MS and FAB-MS were found ineffective to get information of the molecular weight of bioactive constituents in the column fractions. ^{1}H NMR of the bioactive SiO_2 gel column fractions or MeOH solubles gave an identical very

broad peak at the aromatic region. The ^{13}C NMR also gave some broad peaks at δ 25–85 ppm and 96–160 ppm indicating the presence of polyflavonoid tannins in the bioactive fractions (data not shown)[110]. Based on the physicochemical properties including ^{1}H and ^{13}C NMR data of the active fractions, we assumed that the active principle in *Lannea* extracts might be a mixture of complex polyflavonoid tannins, which was highly stable in hydrolysis. To get definite evidence, we tested the activity of two popular commercial tannins (Quebracho and Mimosa) in our bioassay. Interestingly, 80% acetone extracts of both commercial tannins showed identical halting and lysis activities and supported our assumption that active principle in *L. coromandelica* extract is also polyflavonoid tannin.

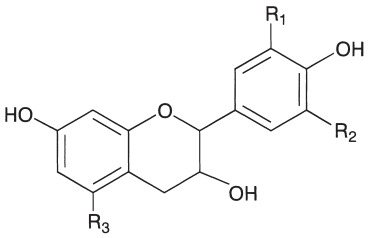
b. Characterization of *Lannea coromandelica* polyflavonoid tannin by MALDI-TOF-MS

Recently, MALDI-TOF mass spectrometry has successfully been used in determination of aspects of the structure and characteristics of the polyflavonoid tannins, which are too difficult to determine, by other technique[105]. We applied this method to determine the structural features of *Lannea* tannin along with a known commercial Quebracho tannin[105]. The Quebracho tannin gave clear spectrum showing the degree of polymerization of the building units and oligomer series with masses of the repeat units of 272.3 and 288.3 Da[105]. The predominant repeat units in this tannin are 272 Da, indicating that this tannin is predominantly consisting of profisetinidin-type unit (data not shown). The flavonoid repeating units present in the polyflavonoid tannins could be A, B, C and D having masses of 258.3, 274.3, 290.3 and 306.3 Da, respectively (Fig. 9). Combinations of these masses can be used to calculate the masses of the profisetinidin/prorobinetinidin-type of polyflavonoid tannin oligomer peaks in the spectra according to the expression, $M + Na^{+} = 23.0$ (Na) + 2.0 (endgroups, 2 × H) + k(256.3A) + l(272.3B) + m(288.3C) + n(304.3D) (k, l, m, n, are integral numbers) (Table 3) (Pasch *et al*., 2001). As can be seen in the spectra, there are more peak series, which are due to different endgroups. They have the same repeat units, for example, 586–314 and 1450–1178 Da in Fig. 10.

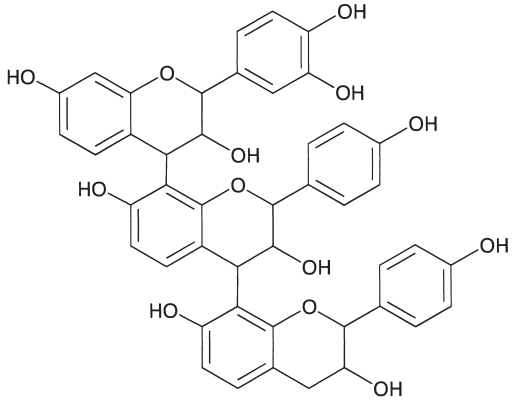
MALDI-TOF-MS analysis of *Lannea* extracts gave clear spectra exhibiting the degree of polymerization of the building units and oligomer series with masses of the repeat units of 256.3, 272.3, 288.3 and 304.3 Da (Fig. 10 and Table 3). For each oligomer, substructures with mass increments of 16 Da appear, suggesting different combinations of various substructures[105]. The MALDI-TOF-MS analysis also indicates the presence in the tannin of oligomers to the maximum of nonamer (2506.3 Da). Table 3 indicates that many valid combinations of different repeating units are possible. Interestingly, a monomer peak at 314.2 Da is composed of one C type unit plus 2H endgroups plus Na^{+}. The peak at 458.2 Da is obtained from the 568.2 Da dimer by elimination of a catecholic B-ring (568.2 −



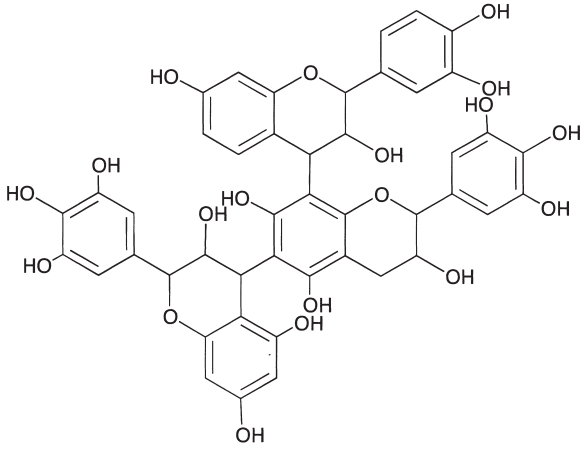
Flavan-3-ols repeating unit



A: $R_1 = R_2 = R_3 = H$
B: R_1 and $R_3 = H$, $R_2 = OH$
C: $R_1 = R_2 = OH$, $R_3 = H$
D: $R_1 = R_2 = R_3 = OH$



A possible linear type trimer (M + Na = 809.9) (central building unit shows linear-type substitution)



A possible angular-type trimer (M + Na = 905.9) (central building unit shows angular-type substitution)

Fig. 9. Structural units of *Lannea coromandelica* (stem bark) polyflavonoid tannins.

Table 3. MALDI-TOF-MS peaks for *Lannea coromandelica* stem bark extract with their possible repeat units. Note that the predominant repeat units in this tannin is 288 Da, indicating that this tannin is predominantly prorobinetinidin

$M + Na^+$	$M + Na^+$		Unit type			
Experimental	Calculated		A	B	C	D
Monomer						
314.2	313.3		-	-	1	-
Dimers						
552.2	553.6		1	1	-	-
568.2	569.6		-	2	-	-
586.2	585.6		-	1	1	-
602.3	601.6		-	-	2	-
618.2	617.6		-	-	1	1
634.0	633.6		-	-	-	2
Trimers						
810.1	809.9		2	1	-	-
890.3	889.9		-	1	1	1
		or,	-	-	3	-
904.1	905.9		-	1	-	2 Angular tannin
		or,	-	-	2	1 Angular tannin
920.0	921.9		-	-	1	2 Angular tannin
Tetramers						
1178.3	1178.2		-	-	4	-
		or,	-	1	2	1
		or,	-	2	-	2
1194.3	1194.2		-	-	3	1 Angular tannin
		or,	-	1	1	2
1211.5	1210.2		-	-	2	2 Angular tannin
		or,	-	1	-	3 Diangular tannin
Pentamers						
1450.0	1450.5					
1466.8	1466.5					
1482.2	1482.5					
1499.1	1498.5					
Hexamers						
1753.8	1754.8					
1770.5	1770.8					
1786.8	1786.8					
Heptamers						
2042.9	2043.1					
2059.0	2059.1					
2074.3	2075.1					
Octamers						
2298.2	2299.7					
2347.2	2347.7					
2365.0	2363.7					
2381.2	2379.7					
2397.0	2395.7					
Nonamer						
2506.3	2507.7					

110 = 458.2). Similarly, a major peak at 552.2 Da is also explained by the presence of a dimer composed of an A-unit plus a B-unit plus 2H endgroups plus Na^+. There are however, some cases in which unequivocal assignment of the structure can indeed be done.

This is the case of angular tannins, namely oligomers in which a repeating unit of type D is bound through both its 6 and 8 A-ring sites to B and C type units, with its C4 sites equally bound and unbound[105].

The MALDI-TOF-MS analysis shows the existence of fragments of angular tannins by the presence of definite peaks at 904.1, 1178.3, 1194.3 and 1211.5 Da in *Lannea* tannin extract (Fig. 10). Thus the *Lannea* tannin is angular-type one which is partly similar to Mimosa but rather different from Quebracho[105]. The predominant repeat units in *Lannea* tannin are 288 Da, indicating it to be predominant prorobinetinidin-type polyflavonoid tannin. Although the presence of a high proportion of phlobatannin in *L. coromandelica* stem bark has been reported earlier (Nair *et al*., 1963) but so far, report on the structural characterization of *Lannea* tannin has not been published. Therefore, it is the first report on the structural features of the polyflavonoid tannin present in *L. coromandelica*.

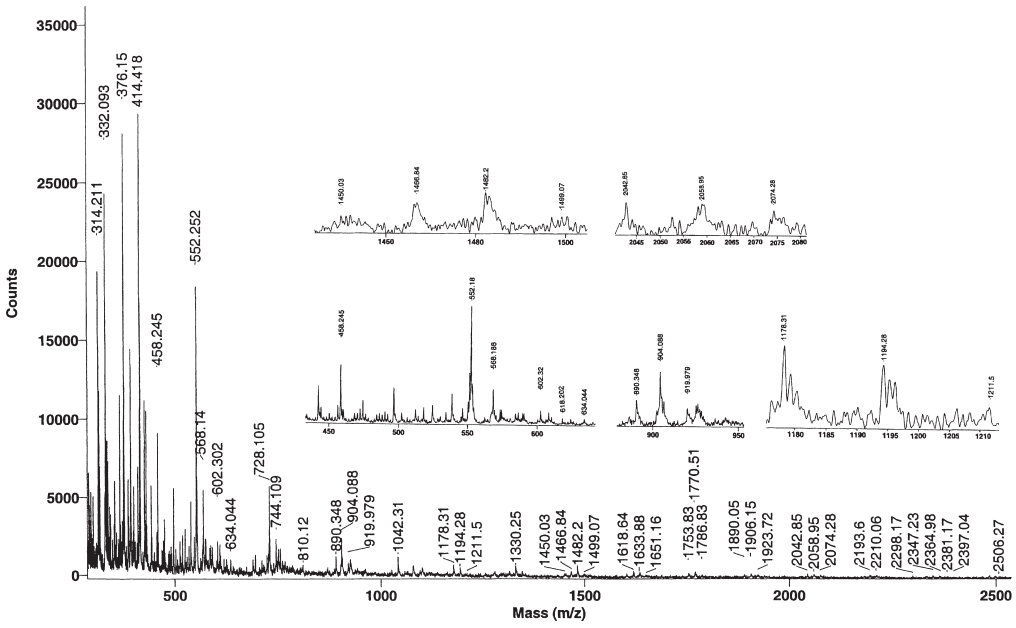


Fig. 10. MALDI-TOF mass spectrum of *Lannea coromandelica* extract. Insets showing expanded form of some important parts of the MALDI-TOF mass spectrum. For each oligomer, substructures with mass increments of 16 Da appear, indicating different combinations of various substructures (Please see Table 3).

c. Motility inhibition and lytic activities of polyflavonoid tannins against zoospores

Light microscopic observation revealed that the *Lannea* extract and commercial ploy flavonoid tannins show zoosporicidal activity against *A. cochlioides* almost in similar manner. In all cases, initially zoospores were halted and the cellular materials rapidly fragmented and formed globular structures. These globular structures achieved Brownian movement, and finally dispersed into the surrounding water medium by bursting the cell membranes within 60 min. The time-course motility inhibition and lytic activities of *Lannea* and commercial polyflavonoid tannins are presented in Table 4. It appeared that both *Lannea* extracts and commercial tannins caused motility inhibition followed by lysis of zoospores in a dose dependent manner at a range of 0.1–50 μg/ml concentration (Table 4). Among the three extracts, *Lannea* showed the higher halting and lytic activity (MIC 0.1 μg/ml) than the two commercial tannins extracts (both MIC *ca* 0.5 μg/ml). Both halting and lytic activity was increased with time and the highest activity was achieved within 60 min of treatment. When previously

Table 4. Zoosporicidal activity of *Lannea* extract and two commercial plant polyflavonoid tannins against *Aphanomyces cochlioides*

Name of tannins/extract	Dose (μg/mL)	Zoosporicidal Activity (%±SE)[a]							
		15 min		30 min		45 min		60 min	
		halted	burst	halted	burst	halted	burst	halted	burst
*Lannea*extract	0.1	10±3	0±0	19±8	2±0	22±4	5±3	25±7	7±4
	0.5	36±4	5±3	48±7	14±5	55±3	18±6	68±8	28±5
	5.0	80±8	16±4	88±7	21±8	95±5	39±4	100±0	57±8
	50.0	100±0	42±6	100±0	68±4	100±0	71±10	100±0	89±7
Quebracho	0.1	2±0	0±0	4±0	0±0	6±2	0±0	10±4	0±0
	0.5	12±6	0±0	26±5	5±2	31±4	7±2	40±7	10±2
	5.0	47±4	9±5	63±3	18±7	75±8	22±5	79±7	35±6
	50.0	96±3	26±10	100±0	46±8	100±0	51±8	100±0	60±5
Mimosa	0.1	4±0	0±0	5±0	0±0	4±0	0±0	9±2	0±0
	0.5	15±6	0±0	19±2	2±1	36±7	10±5	45±5	15±2
	5.0	61±9	12±4	70±8	19±4	78±2	30±4	81±5	39±9
	50.0	77±4	23±7	82±11	47±4	98±2	66±6	100±0	71±4
Control	-	3±1	0±0	5±2	0±0	6±1	0±0	9±3	0±0

[a]Data presented here are the average value ± SE of at least 3 replications in each dose of polyflavonoid tannin. Both Mimosa and Quebracho caused granulation or deformation of almost all rounded cysts especially at higher concentration (>5.0 ppm). Some of the pre-encysted spores were not burst completely but became enlarged and they did not germinated or regenerated up to 24 h. SE = standard error.

encysted spores (cystospores) were exposed to 5 μg/ml of *Lannea* or commercial tannin extracts, they were deformed and did not germinated or regenerated zoospores even after 12 h seemingly being killed (data not shown). Methylation or acetylation of *Lannea* extracts yielded completely non-active products (inactive at 100 μg/ml) indicating that hydroxyl groups in the polyflavonoid tannin may be essential for motility inhibition and lytic activity.

Previously, one natural product, avenacin (a saponin) from the oat roots also exhibited motility inhibition followed by lysis of some oomycetes zoospores but the active mechanism of that compound is yet to be known[21]. Recently, we found that nicotinamide from a nonhost *Amaranthus gangeticus* showed potent motility inhibiting activity against *A. cochlioides* zoospores[124]. Interestingly, nicotinamide induced halted zoospores were encysted and then regenerated zoospores instead of germination or lysis. Motility inhibiting activity of zoospores was also observed by the interactions of two chemically different factors isolated from another nonhost *Portulaca oleracea*, where all halted zoospores were first encysted, and then germinated[97].

d. Morphological changes of zoospores interact with polyflavonoid tannins

To get more insights of the zoosporicidal activity of *Lannea* and commercial tannin extracts, I studied the morphological changes of zoospores by scanning electron microscopy (SEM) (Fig. 11). Time-course SEM observation revealed that both *Lannea* extract and commercial polyflavonoid tannins caused lysis of zoospores in a similar manner (Fig. 11a-k). It appeared that tannin extracts first attacked the tripartite tubular hairs (TTHs) (characteristic hairy structures responsible for swimming) of the anterior flagellum as well as the fine structures of the posterior flagellum (responsible for swimming)[64]. The tannins reacted with TTHs, and precipitated them within 5 min (Fig. 11a). Thus, zoospores became paralyzed and rapidly halted. The surface of the affected zoospores became relatively smooth, rounded (Fig. 11e,i) and rapidly burst (Fig. 11 b,f-h,j). The inner cellular materials of the spores fragmented and formed unique globular structures and soon dispersed into surrounding medium within 60 min (Fig. 11d). In contrast, 22:1ω^7-anacardic acid isolated from the *Ginkgo biloba* caused shrinkage of zoospores followed by bursting at a single point spore without forming any characteristic fragmentation of cellular materials[7]. The morphological changes (fragmentation of cellular materials and formation of unique structures) of zoospores by polyflavonoid tannins observed in this experiment are similar to the characteristic features of apoptosis[125]. Characteristics fragmentation of nuclear DNA was also observed in tannin-induced spores by fluorescence microscopy[66]. However, no clear ladders of DNA fragments were found in agarose gel electrophoresis of DNA extracts of tannin-affected zoospores[66]. Recently, gallotannin was found to induce apoptosis in a human colon cancer line (T-84) at 10 μg/ml[36]. The pre-formed cystospores also affected by the extract where they completely

deformed and cracked down but no clear fragmentation of cellular materials was observed (photomicrgraph not shown). The fate of zoospores interacting with commercial tannins was identical to that with *Lannea* extracts indicating that the same active mechanism may be involved in both cases.

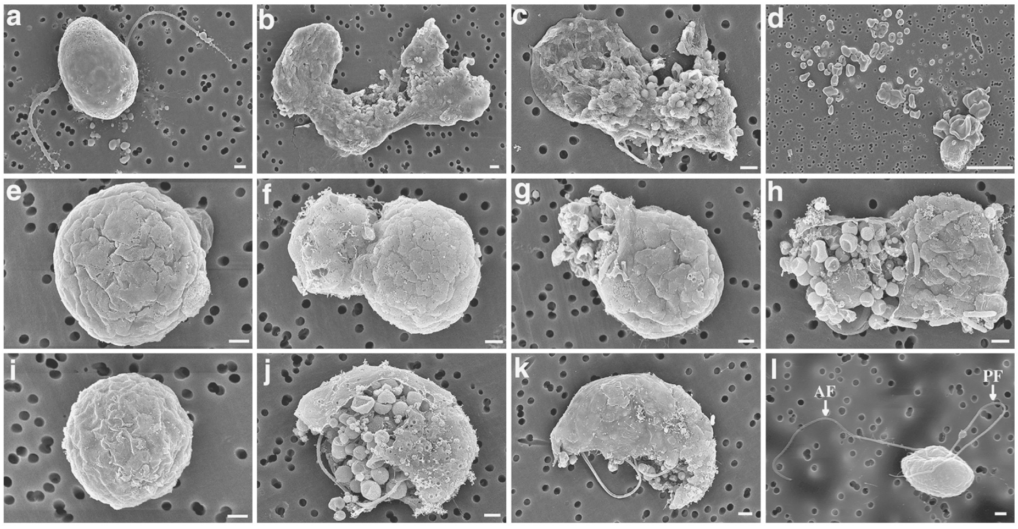


Fig. 11. Scanning electron micrographs showing zoosporicidal activity of *L. coromandelica* extracts (**a-d**), and two commercial polyflavonoid tannins (**e-h**, Quebracho; **i-k**, Mimosa) toward zoospores of the *A. cochlioides*. **a**. fine structures of the flagella of a zoospore are precipitated (5 min), **b**. a zoospore burst after fragmentation of cellular materials (30 min), **c**. a lysed zoospore (30 min), **d**. characteristic fragments of a lysed zoospore (60 min), **e-h**, characteristic zoosporicidal effects of Quebracho tannin (**e**, 10 min; **f-h**, 30 min); **i-k**, typical zoosporicidal effects of Mimosa tannins (**i**, 10 min; **j,k**, 30 min); l. a bi-flagellated (AF, anterior flagellum; PF, posterior flagellum) zoospore (control). Scale bars, **a-l** all 1 μm except **d**, 10 μm.

Tannins are secondary metabolites distributed widely in the plant kingdom, which have been closely associated with plant defense mechanisms towards phytopathogens, insects and mammalian herbivores. Recently, direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep have been demonstrated[1]. Kiuchi *et al*.[84] also found that tannins, both condensed and hydrolyzable, caused bursting of the second-stage larvae of dog roundworm (*Toxocara canis*). The characteristic zoosporocidal activity of *Lannea* and other two commercial polyflavonoid tannins shown in this section has not been reported for any other oomycete zoospores.

D. Conclusion

In conclusion, polyflavonoid tannins from *L. coromandelica* and commercial sources have motility inhibitory and unique lytic effects *in vitro* against the zoospores of a phytopathogenic oomycete *A. cochlioides*. Plant polyflavonoid

tannins could be useful for managing the infestation of crops by the notorious oomycete phytopathogens. Further studies on the zoosporicidal mode-of-action of polyflavonoid tannins and their effects on other phytopathogenic oomycetes are needed for considering their practical use as a naturally occurring oomicidal agent.

Ⅶ. INTERRUPTION OF THE HOMING EVENTS OF PHYTOPATHOGENIC *APHANOMYCES COCHLIOIDES* ZOOSPORES BY SECONDARY METABOLITES FROM NONHOST *AMARANTHUS GANGETICUS*

A. Introduction

Phytopathogenic oomycetes (Peronosporomycetes in the new classification)[27] such as *Aphanomyces, Phytophthora* and *Pythium* spp. develop a special motile spore with two dissimilar flagella called a zoospore, during their life cycle[64]. These zoospores liberated from mycelium or sporangium swim within water films, then encyst at potential sites of infection on the host and form germ tubes that may generate structures such as appressoria or penetrate the surface directly[20,64,68]. The disruption of any of these asexual stages eliminates the potential for pathogenesis. Therefore, understanding the mechanisms of molecular signaling and interactions between plants (host and nonhost) and oomycete pathogens related to their asexual life stages is important to the development of biorational approaches to the control of pathogens[63].

A. cochlioides is a serious pathogen causing root rot and damping-off diseases in economically important species belonging to Chenopodiaceae (*e.g.*, spinach, sugar beet) and Amaranthaceae (*e.g.*, feather cockscomb)[142,148]. The zoospores of *A. cochlioides* are guided to host plants by host-specific chemical signals released from the roots[58,59] after which they undergo a series of morphological changes before penetrating the host tissues to establish the disease[64,68,70]. Cochliophilin A (**5**) was identified as a host-specific attractant (active at 3×10^{-10} M by the particle method) for zoospores of *A. cochlioides* from the roots and root exudates of spinach[58]. Another novel attractant, *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) (active at 1×10^{-8} M), was identified together with **5** in the roots of *Chenopodium album*[59]. Cochliophilin A (**5**) also triggers encystment and germination of *A. cochlioides* zoospores[70]. 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 the differentiation of zoospores essential for the establishment of an infection[70,130].

In contrast to host plants, nonhost plants may contain "chemical weapons" to defend themselves from attack by soilborne oomycetes[62,69,131]. This hypothesis was tested by screening extracts from 200 non-susceptible plants, almost half of which affected directly the motility and viability of *A. cochlioides* zoospores. Among the 200 plants, *Amaranthus gangeticus* was selected for further study,

since the crude extract (whole plant) displayed both potent attractant and sudden motility inhibitory activities against zoospores. The amaranth plants are used in foods, ornaments and herbal medicines all over the world[16,85,121].

Am. gangeticus is principally incompatible with *A. cochlioides*. Hence, the unusual bioactivity of *Am. gangeticus* extracts toward the zoospores encouraged us to isolate the active principle(s) using a detailed bioassay-guided approach. Chromatographic separation of the *Am. gangeticus* constituents revealed that the cumulative effects of two chemically distinct factors regulated taxis and subsequent inhibition of the zoospore motility. Here I report chromatographic procedures for the isolation of an attractant and a motility-inhibitory factor of zoospores from *Am. gangeticus*, and discuss the significance of their presence in a nonhost plant in terms of ecological chemistry.

B. Materials and Methods

a. Plant materials

Young specimens of *Am. gangeticus* (whole plants) were collected from a greengrocery at Mymensingh, Bangladesh. The plants were washed with water, dried in the shade, and coarsely ground using an electric grinder.

b. Extraction and chromatographic procedure

Ground plant sample (108 g) was extracted with acetone and then 60% aq. methanol. The chemical fractionation and chromatographic procedures used for the bioassay-directed isolation of active molecules are presented in Scheme 1.

c. Seed germination and bioassay of excised roots

Seeds (Daigaku-Noen, Tokyo) of host and nonhost plants (Table 7) were soaked overnight in running water and then surface sterilized with 1% sodium hypochlorite for 10 min. The seeds were germinated on filter paper soaked with sterilized water in a petri dish (9-cm diameter) at room temperature. When cotyledons emerged from the seed-coat on day 6 of cultivation, the young seedlings were used in this experiment.

Zoospores of *A. cochlioides* were attracted to specific sites of the roots of germinated seedlings. The excised roots were directly immersed in 2 ml of the zoospore suspension (*ca.* 1×10^5 ml^{-1}) in a small petri dish (3 cm i.d.) for up to 24 hr to allow interactions between zoospores and roots (diffusates). The motility behavior and morphological changes of zoospores in the presence of plant roots were observed under a light microscope.

d. Production of zoospores and bioassays

A. cochlioides (AC-5) was grown for 6 days on a corn meal agar (Difco) plate (9-cm diameter) at 20°C, and zoospores were produced as described previously[63]. These zoospores remain motile for more than 20 hr in sterilized distilled water.

The "particle bioassay" was carried out as described earlier[58,63,72]. A quantitative bioassay of the biological activity of nicotinamide (**8**) was carried out using the "homogeneous solution method" as follows:

Nicotinamide was dissolved in deionized water to prepare a stock soln. of 1×10^{-3} M. A series of nicotinamide solutions (*e.g.*, 1×10^{-4}, 1×10^{-5}, 1×10^{-7}, 1×10^{-8} M *etc.*) was prepared by the addition of deionized water. An appropriate amount of nicotinamide solution was directly added to the zoospore suspension (*ca.* 10^5/ml) in one small dish (bottom area: 16 mm^2) of the Nunc Multidish to a final volume of 200 μl and quickly but gently mixed well using a glass rod. In the control treatment, an equal volume of deionized water was added instead of the nicotinamide solution. The zoospores halted by nicotinamide rapidly encysted and settled at the bottom of the dish within 5 min. Mechanical agitation by a vortex mixture for 30 sec also caused the halting and encystment of zoospores[69]. The number of settled cystospores was counted by taking photographs (X50 magnification) with a digital camera attached to the microscope in at least 5 microscopic fields and averaged. The percentage of halted zoospores was calculated according to the following formula:

$$\% \text{ Halting activity} = [\{(H_t - H_c) \div A_m\} \times A_d] \div H_v \times 100$$

H_t = average number of halted spores per microscopic field in the treatment dish; H_c = average number of settled zoospores per microscopic field in the control dish; A_m = area of the microscopic field; A_d = total area of the bottom of the dish; H_v = total number of halted spores in an equal volume of vortexed (30 sec) ($H_v = H_{v'} \div A_m \times A_d$) zoospore suspension. $H_{v'}$ = average number of settled zoospores per microscopic field in the dish of vortexed zoospores. Similarly, percentages of regenerated and germinated zoospores were calculated compared with control treatments. Each treatment was replicated five times.

e. Scanning Electron Microscopy

An appropriate concentration of test solution (in the case of nicotinamide, **2**) or Chromosorb W AW (60–80 mesh) coated with the test solution was added to 200 ml of a zoospore suspension on a SEMpore membrane. The spores were fixed with 2% buffered glutaraldehyde after varying the period (5, 10, 30, 40 and 60 min) of interaction. Further preparations for microscopy were similar to those described earlier[64,70].

Each experiment was repeated at least three times, and more than 50 cells were observed in each preparation.

C. Results and Discussion

a. Detection of zoospore attractant and motility inhibitory activities, and the active principles

On observing the behavior of *A. cochlioides* zoospores using a particle

bioassay method, it was found that the EtOAc soluble materials in the acetone extracts of whole plants had potent attractant and subsequent motility inhibitory activities toward zoospores (at 100 ppm) (Scheme 1). Small amounts of EtOAc solubles were then subjected to silica gel TLC in $CHCl_3$–MeOH (4:1 v/v) and the developed plates were divided into 10 horizontal zones from the origin to the solvent front (10 cm). Each zone was removed from the plate and eluted with EtOAc-acetone (10:1 v/v) to give TLC fractions corresponding to the 10 zones. The eluates from 5 plates were combined, concentrated, and checked for biological activity. Only the 4th (3–4 cm) and 7th zones (7–8 cm) were displayed inhibitory and attractant properties, respectively. When these two eluates were combined, the attraction followed by inhibition of motility, characteristic of the crude extract, was restored. These results indicated that *Am. gangeticus* extracts contain at least two distinct active compounds; an attractant and a motility-inhibiting factor. The characteristic behavior of *A. cochlioides* zoospores is shown in Fig. 12: zoospores aggregated and became immobile close to the Chromosorb W AW particles treated with the crude *Amaranthus* extract (A), while in the control (B), they continued swimming quite actively.

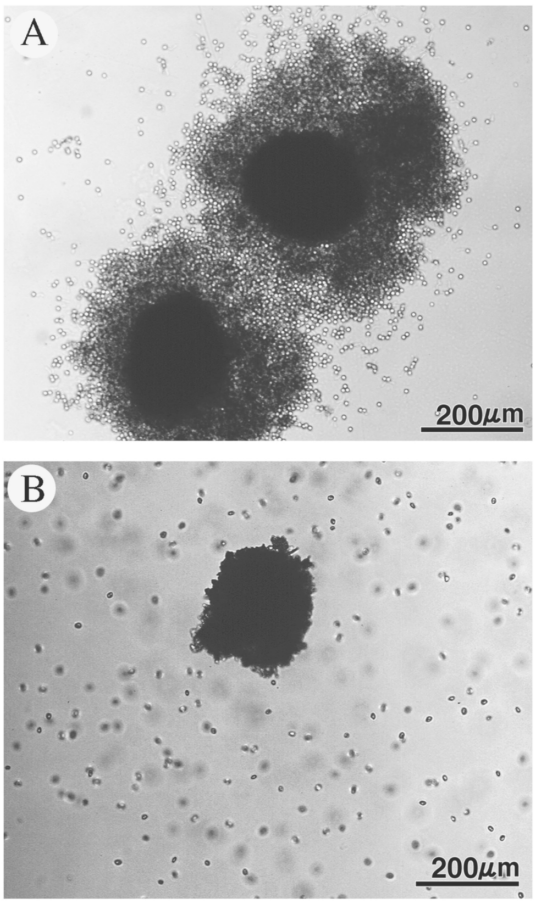
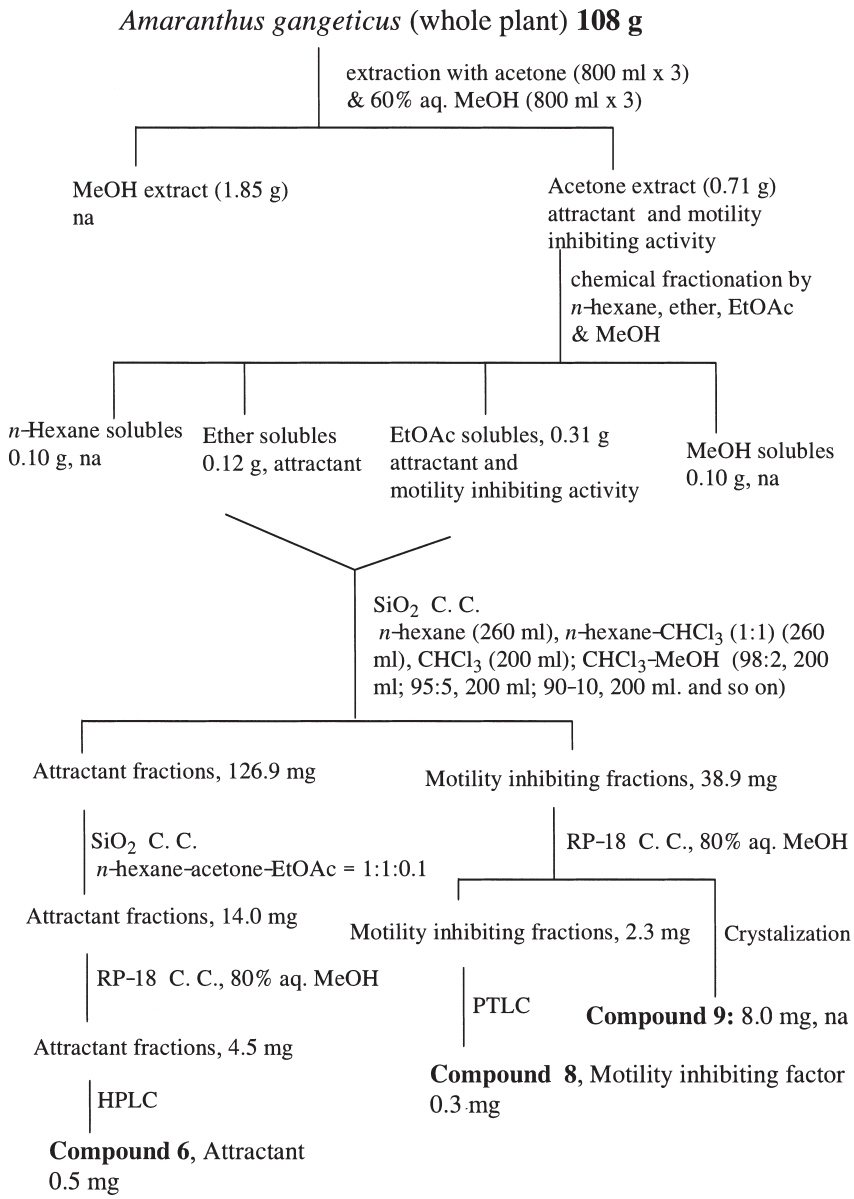


Fig. 12. Micrographs showing the attraction and subsequent halting of zoospores of *Aphanomyces cochlioides* moving toward Chromosorb W AW particles coated with crude extract of *Amaranthus gangeticus*.
A. Zoospores attracted and halted on and around the particles treated with crude extract dissolved in EtOAc (200 μg/ml); **B**. no attraction and halting of zoospores toward a particle treated with solvent alone (control).

b. Isolation of the factors responsible for attraction and motility inhibition

The coarsely powdered plant samples (108 g) were extracted with acetone and then aqueous MeOH (Scheme 1). The acetone extract (0.63 g) was fractionated using *n*-hexane, diethyl ether, EtOAc, and MeOH to give 0.10 g, 0.12 g, 0.31 g, and 0.10 g, respectively. Only the diethyl ether (attracting activity) and EtOAc



Scheme 1. Isolation procedure for compounds in *Amaranthus gangeticus* extract exhibiting attractant followed by motility-inhibiting activity against zoospores of *Aphanomyces cochlioides*.
SiO_2 C. C.: silica gel column chromatography, RP-18 C. C.: reverse phase column chromatography, HPLC: high performance liquid chromatography, PTLC: preparative thin layer chromatography, na = non-active.

(attracting and motility inhibitory activity) solubles showed bioactivity at 100 and 200 ppm, respectively. Considering the amount and properties of bioactivity, these two active fractions were combined and subjected to silica gel (45 g) CC (Scheme 1) eluted with *n*-hexane-$CHCl_3$-MeOH with increasing amounts of $CHCl_3$ and MeOH to give 8 fractions. On the basis of bioactivity (100 ppm) and TLC patterns, relevant fractions were combined. Active compounds were eluated in two groups, attractant fractions (*ca.* 55–65 ml) (eluting solvent: $CHCl_3$ to $CHCl_3$ -MeOH, 98:2 v/v; a solute, 126.9 mg) and weak attractant and strong motility inhibiting fractions (*ca.* 75–80 ml) (eluting solvent: $CHCl_3$-MeOH, 98:2 v/v; a solute, 38.9 mg).

Attractant fractions (126.9 mg) were rechromatographed in silica gel (12.6 g) eluted with *n*-hexane-EtOAc-acetone (1:1:0.1 v/v/v) to give 14.0 mg (240–400 ml) of a partially purified attractant which was then subjected to RP-18 CC with 80% aq. MeOH as an eluting solvent. Finally, the attractant was purified as a single compound (0.5 mg, **6**) by HPLC at t_R 29.25 min [Inertsil ODS 5 μm (250 × 20 mm), MeOH - H_2O (3:2) flow rate: 5 ml/min and UV detector at 320 nm] (Fig. 13).

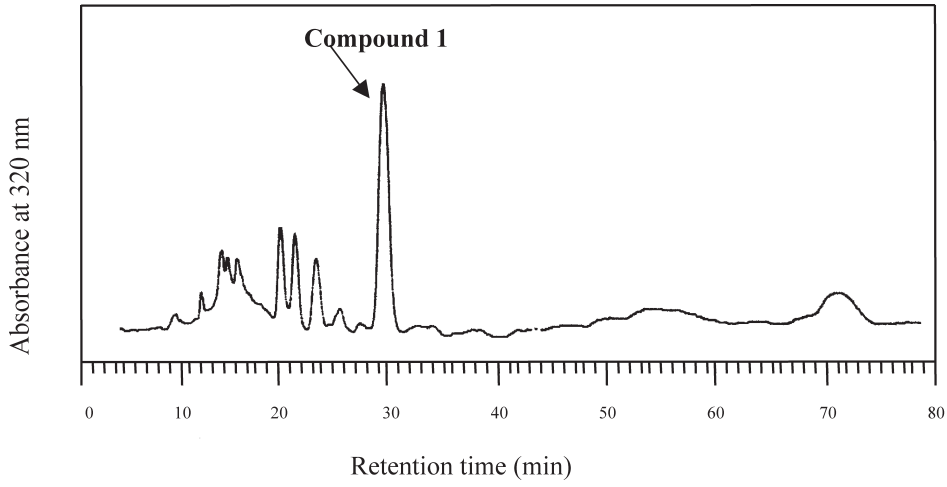
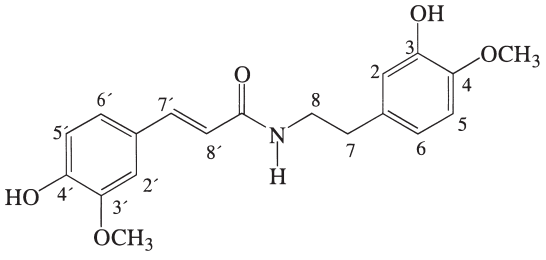


Fig. 13. HPLC profile of the RP-18 column fraction of *Amaranthus gangeticus* extract containing compound **1** (retention time: 29.25 min). Inertsil ODS 5μm (250 × 20 mm); solvent: MeOH-H_2O = 3:2; flow rate: 5 ml/min.

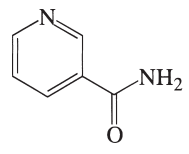
Zoospore motility inhibiting fractions (38.9 mg) were repeatedly chromatographed over RP-18 CC eluted with 80% aq. MeOH to give a partially purified halting factor (2.3 mg, **8**) together with a non-active triterpenoid saponin (8.0 mg, **9**). The partially purified compound **8** was finally purified (0.3 mg) by PTLC using diol thin layer plates (solvent: $CHCl_3$-MeOH, 12:1 v/v). The halting factor gave a round quenching spot under UV light at 254 nm on silica gel thin layer plates ($60F_{254}$ plate, 0.25 mm thickness, *Rf* 0.31 in $CHCl_3$-MeOH, 4:1 v/v).

c. Characterization of the zoospore attractant and the motility inhibiting factor

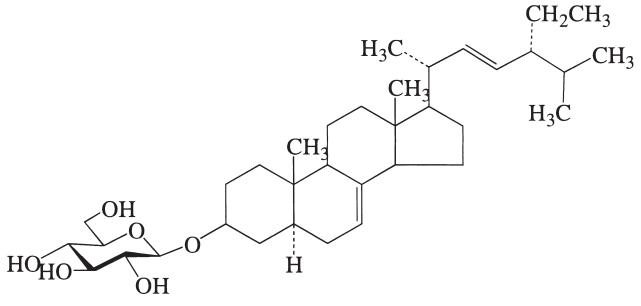
Compound **6**: Gibbs reagent very quick gave a purple blue coloration when the thin-layer plates were exposed to NH_3 vapor. The compound **6** is a yellow oil and soluble in diethyl ether, EtOAc, $CHCl_3$, acetone, and MeOH. The FD-MS of this compound gave a base peak at m/z 343 ($[M^+]$) and the molecular formula of compound **6** was determined to be $C_{19}H_{21}NO_5$. HR-FD-MS, m/z: 343.1394 ($[M]^+$ calcd. for $C_{19}H_{21}NO_5$: 343.1450). In the ^{1}H-NMR spectrum of **6**, signals of two methylene groups, two methoxy groups, six aromatic protons, and one *trans*-olefinic group were observed. The ^{1}H-NMR (Me_2CO-d_6) signals (7.44, 1H, *d*, J = 15.7 Hz, H-7′; 7.16, 1H, *d*, J = 1.8 Hz, H-2′; 7.04, 1H, *dd*, J = 8.1 and 1.8 Hz, H-6′; 6.85, 1H, *d*, J = 8.1 Hz, H-5; 6.83, 1H, *d*, J = 8.1 Hz, H-5′; 6.74, 1H, *d*, J = 1.8 Hz, H-2; 6.66, 1H, *dd*, J = 8.1 and 1.8 Hz, H-6; 6.49, 1H, *d*, J = 15.7 Hz, H-8′; 3.88, 3H, *s*, feruloyl-OCH_3; 3.81, 3H, *s*, amine-OCH_3; 3.50, 2H, *m*, J = 7.3 Hz, H-8; 2.73, 2H, *dd*, J = 7.3 Hz, H-7) along with other physicochemical data, and the bioactivity of compound **6** were indistinguishable from those of *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) previously isolated as a host-derived attractant only from the roots of *Chenopodium album*[59]. This is the second report of this compound from a natural source having a rare 4-*O*-methyldopamine moiety in its structure.



6. *N*-*trans*-feruloyl-4-*O*-methyldopamine



8. nicotinamide



9. chondrillasterol 3-*O*-β-D-glucopyranoside

Fig. 14. Structures of *Amaranthus gangeticus* isolates (**6**, 8 and **9**).

However, *N-trans*-feruloyl-3-*O*-methyldopamine was previously isolated from Chenopodiaceae[17,128] and Lauraceae

The physicochemical properties (FD-MS, HR-EI-MS, UV, and ^{1}H NMR) of the isolated compound **8** were identical to those of authentic nicotinamide. The relationships between the structure and motility-inhibiting activity of nicotinamide-related compounds were reported elsewhere[124]. Compound **9** was purified by crystallization from a $CHCl_3$–MeOH solution as trapezoidal plates (8.0 mg), which had mp 272.5–274.0°C (Lit. 272–275°C)[124]. FD-MS gave a base peak at m/z 574 corresponding to a molecular formula of $C_{35}H_{58}O_6$. The ^{1}H and ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY and HMQC data of this isolate reasonably matched those reported for 3-*O*-glucopyranoside of chondrillasterol, which was isolated from *Am. Cruentus*[76]. This compound was also isolated for the first time from *Am. gangeticus*.

d. Biological activity of the isolated compounds

The biological activity of **6**, **8** and a host-specific plant signal compound cochliophilin A (**5**) were tested using the particle bioassay method. Compound **6** showed attractant activity without inhibiting the motility of zoospores at 1×10^{-8} M, while compound **8** caused motility-inhibition and encystment of zoospores at 5×10^{-6} M (particle bioassay method) (Table 5). In the case of compound **8**, the halted zoospores changed into cystospores within 10–20 min and surprisingly, those cystospores regenerated into tertiary zoospores instead of germinating within 3 hr (Table 5). However, concomitant application of two compounds (**6** at 1×10^{-6} M and **8** at 1×10^{-5} M), resulted in attractant followed by motility inhibiting activity of zoospores (Table 5). Almost 60% of the halted zoospores encysted within 20 min and germinated within 40 min. This result is comparable to that obtained with cochliophilin A at 1×10^{-7} M. The bioassay results for compound **8** using the homogeneous solution method are presented in Table 6. Compound **8** caused strong motility inhibition followed by the regeneration of zoospores at a concentration as low as 5×10^{-8} M.

The cystospores obtained with nicotinamide did not adhere to the surface of the petri dish, and were easily washed away from the bottom of the dish with a gentle water flow. But cystospores produced with cochliophilin A (**5**) or a concomitant application of **6** and **8** adhered tightly to the surface of the petri dish via adhesive substances released from the spores during their maturation, and were not washed away. This indicated that the nicotinamide-induced cystospores might lack adhesive material. It is known that zoospores release adhesive glycoproteins during encystment on the host surface or are induced to encyst by a host-specific plant signal[64].

Nicotinamide (**8**) displayed shell-opening activity against common bivalve mollusks[102] and repellent activity toward the blue mussel (*Mytilus edulis*)[150]. The mechanisms behind these biological effects of compound **8** are still unknown.

Table 5. Effects of *N-trans*-feruloyl-4-*O*-methyldopamine **(6)**, nicotinamide **(8)** and cochliophilin A **(5)** on attraction, encystment, germination and regeneration of zoospores of *Aphanomyces cochlioides*

Concentration (1 × M)			Behavior and developmental strategy of zoospores*			
6	**8**	**5**	Attraction	Encystment	Germination	Regeneration
10^{-7}	10^{-7}	-	+++	no	no	no
10^{-7}	10^{-6}	-	+++	++	+	no
10^{-6}	10^{-5}	-	+++	+++	++	no
10^{-5}	10^{-5}	-	+++	+++	+++	no
10^{-7}	10^{-5}	-	++	+++	+	+
10^{-8}	10^{-5}	-	+	++	no	++
-	5×10^{-6}	-	no	+	no	+
10^{-8}	-	-	+	no	no	no
-	10^{-5}	10^{-7}	++	+++	+	no
10^{-7}	-	10^{-7}	+++	+++	++	no
-	-	10^{-7}	+++	+++	++	no

* Particle bioassay method; solutions of 2 or 3 compounds were mixed to obtain appropriate concentrations of each compound in the mixture and then the mixture was used to treat Chromosorb particles.
"+" indicates degree (+ = low, ++ = moderate, and +++ = high) of attractant activity. In the case of encystment, germination and regeneration: + = ~20%, ++ = 20-60%, and +++ = 60-100%.
In this bioassay system, cochliophilin A shows attractant activity at 3×10^{-10} M.

Table 6. Effects of nicotinamide **(8)** on motility inhibition and developmental strategy of *Aphanomyces cochlioides* zoospores

Concentration	Motility inhibition (%)		Developmental transitions of cystospores after 6 hrs (%)*		
(1×M)	After 5 min	After 10 min	Germinated	Regenerated	Ungerminated/lysed
10^{-5}	100±0	100±0	0±0	52±8	46±5
10^{-6}	100±0	100±0	4±3	78±7	19±6
10^{-7}	95±2	100±0	7±2	87±5	9±3
5×10^{-8}	91±9	98±3	9±3	85±9	8±5
10^{-8}	7±4	20±7	-	-	-
Mechanical agitation (30 sec)	100±0	100±0	9±4	84±11	8±4
Control	4±3	10±4	-	-	-

*Homogeneous solution method. Data are mean value±standard error of 3 replications. Percent of germinated, regenerated and ungerminated/lysed cystopsores were calculated after 6 hrs of incubation at 20 °C. Both mechanical agitation (vortexing) and nicotinamide induced halted zoospores changed into round shaped cystospores within 7-15 min of treatments.
'-' means not calculated.

Indeed, nicotinamide is a product of the cleavage of NAD^+ by ADP-ribosyltransferase, and serves as an effective inhibitor of enzyme activity[114]. It was also observed that nicotinamide directly inhibited vascular smooth muscle

cell contraction, possibly by blocking external Ca^{2+} entry or the release of Ca^{2+} from intracellular stores[12]. Indeed, Ca^{2+} ions are also critical to the motility and differentiation of zoospores[149] therefore, the motility-inhibiting activity of nicotinamide may be related to the Ca^{2+} influx/efflux in zoospores. Structure-activity relationships of the naturally occurring attractant *N-trans*-feruloyl-4-*O*-methyldopamine (**6**)[82] and motility-inhibitory properties of nicotinamide-related compounds[124] have been reported previously.

e. Morphological changes of zoospores exposed to *Amaranthus gangeticus* constituents

Light microscopic observation revealed that zoospores halted by nicotinamide alone or nicotinamide (**8**) and either *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) or cochliophilin A (**3**) initially underwent similar morphological changes *i.e.* they soon shed their flagella and became round-shaped cystospores. However, only the nicotinamide-induced cysts regenerated zoospores, while in other cases, the cysts subsequently germinated to form germ tubes. To see whether there is any morphological difference among the cysts obtained using the three different chemical inductions, I examined morphology by scanning electron microscopy (Fig. 15). Interestingly, the cysts induced by nicotinamide alone had smooth coats even after 30 min of chemical induction (Fig. 15B), whereas, cochliophilin A (**5**) or the combination of nicotinamide (**8**) and *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) produced cysts with wrinkled coats in 15–20 min (Fig. 15C). However, samples fixed in a shorter time (10 min or so) after induction by cochliophilin A (**5**) or the combination of nicotinamide (**8**) and compound **6** gave cystospores (immature!) with smooth cyst-coats (Fig. 15B).

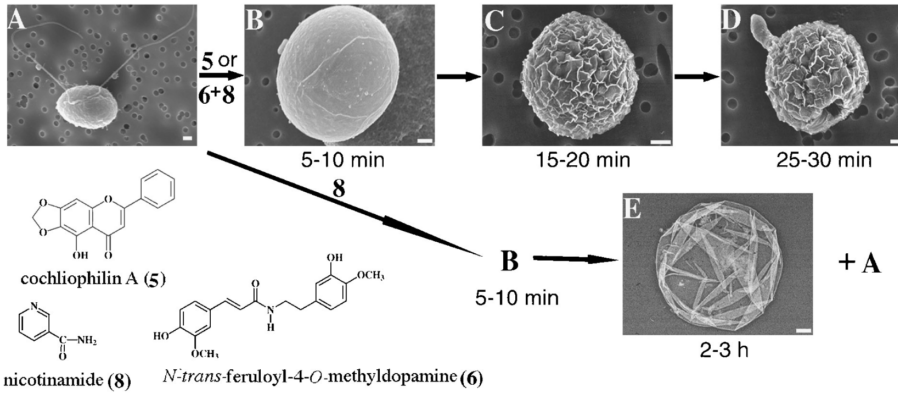


Fig. 15. Scanning (**A**-**D**) and transmission (**E**) electron micrographs showing a zoospore of *Aphanomyces cochlioides* and it's developmental transition triggered by plant secondary metabolites.
A. zoospore; **B**. cystospore (immature) with a smooth surface; **C**. cystospore (mature) with a rough surface; **D**. germinated cystospore; **E**. empty cyst-coat (ghost). Scale bars, **A**-**E**, 1 μm.

This indicates that nicotinamide-induced cysts may regenerate into zoospores before proceeding to the mature stage (wrinkled surface) (Fig. 15A, B and E). Furthermore, mechanical vortex-induced cysts which usually regenerate into zoospores also had smooth coats and lacked adhesive materials. When newborn zoospores emerged from these cysts, they left their coats (ghost) (Fig. 15E). In contrast, when a cystospore germinates to form a hypha, it also leaves its outer coat at one end[68]. These results indicate that the outer cyst-coat, which is formed at the initial stage of encystment, is not reused during regeneration of the zoospore or germination of the cystospores of *A. cochlioides*.

f. Responses of zoospores to the root tips of host and nonhost plants

I investigated the behavior of zoospores toward seedlings (5-days old) of a number of host and nonhost plants, namely, *Spinacia oleracea*, *Chenopodium album*, *Beta vulgaris*, *Am. gangeticus*, *Am. caudatus*, *Am. tricolor*, *Am. magnostanus*, *Celosia cristata* L. var. *childsii* Hort (orange), *C. cristata* L var. *childsii* Hort (yellow), *C. cristata* L. var. *childsii* Hort (red), *Raphanus sativus*, *Arabidopsis thaliana* and *Zea mays*. At first, whole seedlings were immersed into the zoospor-

Table 7. Responses of *Aphanomyces cochlioides* zoospores toward excised root tips of host and nonhost plant seedlings

Name of plant	Responses of zoospores toward the excised root tips*				
	Attraction	Halting	Encystment	Germination	Regeneration
Host plants					
Spinacia oleracia L.	+++	+++	+++	+++	no
Chenopodium album L.	+++	+++	+++	+++	no
Beta vulgaris L.	+++	+++	+++	+++	no
Celosia cristata L. var. *childisii* Hort (yellow)	+++	+++	+++	+++	no
Celosia cristata L. var. *childisii* Hort (orange)	+++	+++	+++	+++	no
Celosia cristata L. var. *childisii* Hort (red)	+++	+++	+++	+++	no
Amaranthus retroflexus L.	+++	+++	+++	+++	no
Nonhost plants					
A. gangeticus L.	+	+++	+++	+	+++
A. tricolor L.	++	+++	+++	no	+++
A. caudatus L.	+	++	++	no	++
A. magnostanus L.	+	++	++	+	++
Zea mays L.	no	+	+	+	no
Raphanus sativus L	repellent	+	+	+	no
Arabidopsis thaliana L	no	+	+	+	+

* Root tips (5-8 mm) were seperately dipped into 2 ml of zoospore suspension taken on a small petri dishes, and the changes of the development of zoospores around them were observed microscopically until 3 hours. Each treatment was replicated for three times for confirming the quality of zoospores responses toward the root tips. "+" indicates degress (+ = low, ++ = medium, and +++ = high) of attraction. In the cases of halting, encytment, germination, and regeneration: + = ~20%, ++ = 20-60%, +++ = 60-100%.

e suspension. Finally, only the excised root tips were tested because zoospores were preferentially attracted to root caps or behind the root caps. The behavior of zoospores and the development of root-induced cystospores were observed microscopically until 24 hr. Among the plant roots tested, the zoospores showed almost similar sensitivity toward all host root tips (Table 7). The zoospores also showed high to moderate sensitivities toward the roots of *Amaranthus* spp., and almost no sensitivity toward *Z. mays*, *R. sativus*, and *Arabidopsis thaliana* (Table 7).

Zoospores of *A. cochlioides* are usually attracted to the host roots (spinach and sugar beet) preferably behind the root cap[68]. Interestingly, in *C. cristata* L. var. *childsii* Hort (orange), zoospores consistently aggregated just on the root cap (Fig. 16D). The aggregated spores formed a mass of cystospores around the root tips (like a ball) within 30 min. All adhered cystospores germinated within 30–40 min (Fig. 16D). Other regions of root also attracted zoospores, but no mass of cystospores was observed. The specific aggregation of zoospores at root caps of *C. cristata* L. var. *childsii* Hort. suggests some powerful attractant is exuded from the root caps. TLC examination revealed that both root exudates and root extracts of seedlings showed identical attractant and differentiation activities toward zoospores. Detail bioassay-directed fractionations are necessary to identify the attractant(s) from this host plant.

Zoospores responded differently toward the root tips of *Amaranthus* spp. In most cases, their movement was halted before approaching the root tips. Very few cystospores attached to the root tips of *Am. tricolor*, *Am. magnostanus*, and *Am. caudatus*. Among the *Amaranthus* spp., *Am. gangeticus* attracted the fewest number of zoospores not to the root cap but to the root hair region (Fig. 16B). Interestingly, about 80% of the cysts regenerated into zoospores in the dishes of *Am. tricolor* and *Am. gangeticus*, and were found to be motile after 24 hr of treatment (Fig. 16C). However, only a very small number of motile zoospores (1–5%) were observed in the dishes of other species after 24 hr of plant-pathogen interaction. The cystospores adhered to the roots of plants were found to form filamentous mycelial structures around the roots within 24 hr regardless of plant species. However, light microscopic photographs did not show whether the germ tubes of these germinated spores really penetrated the root tissues or not.

When a root tip is immersed into a zoospore suspension, it is feasible that the plant root tissue exudes chemical substances including secondary metabolites through specific sites and a gradient of chemicals develops around the roots. Zoospores seemed to respond according to the nature and site of the chemical gradients. Preliminary bioassays of the eluants from different bands of silica gel TLC plates charged with crude root extracts revealed that the roots of all members of Amaranthaceae contain at least two active compounds (*N-trans*-feruloyl-4-*O*-methyldopamine, **6** and nicotinamide, 8) in varying quantities depending on the plant species. However, we detected only compound 8 in the root

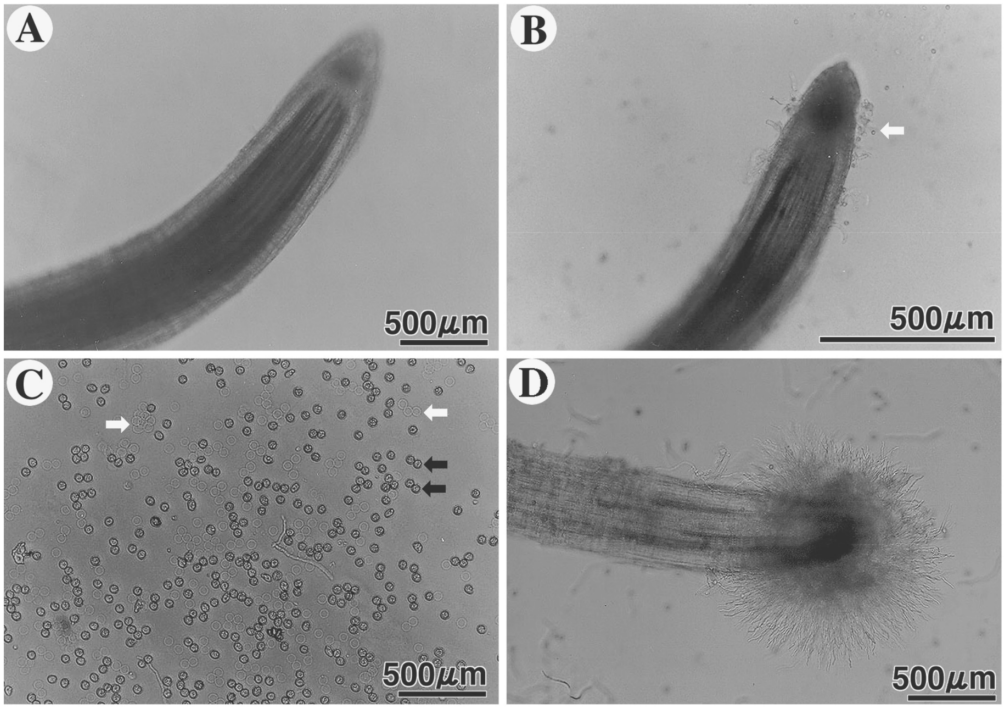


Fig. 16. Micrographs showing responses of *Aphanomyces cochlioides* zoospores toward excised root tips of *Amaranthus gangeticus* (nonhost) and *Celosia cristata* (host) dipped into a zoospore suspension (10^5/ml) in a small petri dish (3-cm diameter).
A. No aggregation of zoospores at the root tip of *Am. gangeticus* (after 30 min); **B**. a few germinated cystospores are seen (white arrow) on and around the root tip of *Am. gangeticus* (after 60 min); **C**. halted and encysted zoospores on the bottom of petri dishes containing *Am. gangeticus* root tips regenerated leaving empty cyst-coats (white arrow). Some immature cystospores (black arrows) are seen along with empty cyst-coats (after 60 min); **D**. aggregated zoospores encysted and all cystospores germinated on the root cap of *C. cristata* (after 3 hr).

exudates of *Am. gangeticus* seedlings in small-scale TLC examinations suggesting that *Am. gangeticus* exudes a sufficient amount nicotinamide to interrupt the homing of *A. cochlioides* zoospores. A precise conclusion can be made only after a quantitative analysis of the root exudates of *Am. gangeticus*.

g. Putative role of nicotinamide in the resistance of *Amaranthus gangeticus* against *Aphanomyces cochlioides*

In a detailed study on host-pathogen compatibility, Ui and Nakamura found that the pathogenic oomycete *A. cochlioides* was highly compatible with Chenopodiaceae and slightly less compatible with Amaranthaceae[142]. It had low compatibility with the members of Portulacaceae and Caryophyllaceae, and was incompatible with members of Cruciferae, Commelinaceae, Polygonaceae,

Compositae, Cucurbitaceae, Solanaceae, Leguminosae, Linaceae, and Gramineae. In another study, Watanabe found that *A. cochlioides* is a serious pathogen of *Celosia cristata* (Amaranthaceae), and was also compatible with *Am. Lividus*[148]. The crude root extracts[130] and root tips from the plants belonging to Chenopodiaceae and Amaranthaceae also showed strong attractant activity toward *A. cochlioides* zoospores (Table 7) which reflected the positive correlation between plant-pathogen compatibility and the zoospore attractant activity.

A bioassay-guided isolation of the active constituents in plants of Chenopodiaceae (*S. oleracea, B. vulgaris, C. album*) showed that all the hosts of *A. cochlioides* contained either cochliophilin A (**5**) *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) or both[58,59]. Moreover, in a preliminary study, the extract of *C. cristata* was revealed to contain compound **6** and probably also compound **5** (data not shown). In the current study, I found that *A. gangeticus* which is less compatible with *A. cochlioides* contained compound **6** along with a motility-inhibiting factor, nicotinamide (**8**). The roots of *Am. gangeticus* appeared to exude large amounts of zoospore motility inhibitor, nicotinamide (**8**) indicating that **8** directly interrupt the homing of zoospores for the establishment of infection. It has been found that root extracts of *Portulaca oleracea*, which had very low compatibility with this pathogen, strikingly inhibited the motility of zoospores[97]. My findings suggest that the exudation of nicotinamide (**8**) from *Am. gangeticus* is one of the reasons for the incompatibility that restricts the attack of soilborne *A. cochlioides*. All cystospores on and around the host roots germinated while the halted zoospores regenerated in the case of *Am. gangeticus* and other nonhost Amaranthaceae. The roots of *Amaranthus* spp. may exude a larger amount of nicotinamide (**8**) with a trace amount of compound **6** because the high concentration of nicotinamide (**8**) (1×10^{-5} M) halted the motility of zoospores even in the presence of a trace amount of compound **6** (1×10^{-8} M), which resulted in the regeneration of cystospores instead of germination (Table 5). Therefore, it is possible that Amaranthaceae plants exude these two compounds from their roots in varying proportions and thus regulates the degree of host-pathogen compatibility in the natural ecosystem. To test the above correlation more precisely, further study is necessary to quantify the amount of attractants and motility-inhibiting factor (nicotinamide as a defense agent) in the roots and root exudates of the members (both susceptible and resistant) of Amaranthaceae. Cultivated *Amaranthus* spp. that can exude large amounts of zoospore motility inhibitor could be used as a decoy crop to naturally block the homing of soilborne phytopathogenic *Aphanomyces*[124].

D. Conclusion

Our bioassay-guided investigation of *Am. gangeticus* constituents revealed that the pre-infection stages including aggregation, attachment, and encystment of zoospores of a soilborne phytopathogenic oomycete *A. cochlioides* and subse-

quent regeneration or germination processes of cystospores are directly regulated by plant secondary metabolites. The signal transduction mechanism for all these events has yet to be clarified. An understanding of the requirements for and mechanism involved in oomycete zoospore differentiation (germination and regeneration) promises to provide a basis for the development of novel disease control measures that will target and inhibit the events of life-cycle development.

Ⅷ. REPELLENT ACTIVITY OF ESTROGENIC COMPOUNDS TOWARD ZOOSPORES OF THE PHYTOPATHOGENIC OOMYCETE *APHANOMYCES COCHLIOIDES*

A. Introduction

In a parallel screening, bisphenol A (BPA) (**10**), a reputed xenoestrogen[134], exhibited potent repellent activity against the zoospores of *A. cochlioides*. BPA (**10**) is a constituent of polycarbonate plastics, epoxy and polystyrene resins that are used intensively in the food-packing industry and in dentistry. In several experiments, effects of BPA (**10**) on mammalian systems resemble those of 17β-estradiol[88,127]. The actions of **10** were found to be mediated through the estrogen receptor, a ligand-dependent transcription factor that regulates estrogen-responsive genes[55,134].

Occurrence of mammalian sex hormones in plants has been often reported[37,49,51]. Heftmann[49] and Harborne[43] discussed their sporadic availability and the chemical role in plants. The presence of estrogens and estrogen receptor-like proteins in plant has recently been confirmed[95]. The fact that human sex hormones, both male and female, occur in trace amounts in a number of plants is now well founded, but there is as yet no explanation for these occurrences[43]. It is difficult to screen plants for their occurrence due to their low level in plants. This prompted me to study the effects of mammalian sex hormones, and their derivatives on the motile zoospores of phytopathogenic *A. cochlioides* .

B. Materials and Methods

a. General

The silica gel 60 ASTM mesh 230–400 was used for column chromatography while purity of the samples was checked on Merck Kieselgel 60 F_{254}, 0.2 mm thick TLC plates. The spots were viewed under 254 and 365 nm UV light and spraying with 5% H_2SO_4 in EtOH. The mass spectra were recorded on a JEOL JMS-SX102A (FD) mass spectrometer and a JEOL JNM-EX 270 for recording the ^1H NMR. TMS was used as the internal standard in NMR spectrometry.

b. Materials

All chemicals commercially available were of the highest purity and unless otherwise stated were used without further purification. Bisphenol A (**10**) dieth-

ylstilbestrol (DES) (**11**) estrone (**12**) 17α-estradiol (**13**) 17β-estradiol (**14**) estriol (**15**) dienestrol (**16**) testosterone (**17**) pregnenolone (**18**) progesterone (**19**) and 3-*O*-benzoyl-17β-estradiol (**20**) were purchased from reputed pharmaceutical companies. Formononetin (**21**) (was isolated from *D. odorifera*[67]) and miroestrol was (a gift) from Dr. J. L. Ingham, University of Reading, UK. One metabolite of testosterone (TES-1, **22**) and two metabolites of pregnenolone (PRE-4, **23**; PRE-5, **24**) were obtained from the biotransformation of **17** and **18**, respectively by *Botrytis cinera*[31]. Cochliophilin A (**5**) and *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) used in this experiment were synthesized by Horio *et al*.[58,59].

c. Derivatization of DES and 17β-estradiol

Commercially pure DES (**11**) and 17β-estradiol (**14**) (each 100 mg) were acetylated at room temperature for 12 h with equal volume of pyridine and acetic anhydride. After the acetylation process, the reaction mixture was purified by preparative thin-layer chromatography (PTLC) in *n*-hexane - EtOAc = 3:1 v/v to give di-acetates of **11** and **14**. Mono- and di-methylation of DES and mono-methylation of 17β-estradiol (**14**) were done using suitable ratio of dimethyl sulfate in a mixture of K_2CO_3 and acetone, and the purification was done using similar technique applied to the acetates.

The aromatic hydroxyl group of 17β-estradiol (**14**) was selectively acetylated by reacting with an equal mol of KOH in acetone followed by the addition of 1. 2 mol of acetic anhydride at room temperature for 6 h. 3-*O*-Acetyl-17β-estradiol (**25**) was purified by PTLC using the above solvent system. 17-*O*-Acetyl-17β-estradiol (**26**) was prepared[8] by the hydrolysis of 3,17-di-*O*-acetyl-17β-estradiol using 10 equivalents of n-butylamine (*n*-$BuNH_2$) in benzene at room temperature for 24 h and purified by silica gel CC using *n*-hexane-EtOAc-MeOH = 30:10:3 v/v. The identity of all derivatives was confirmed by mass spectroscopy and 1D ^{1}H NMR.

d. Culture of *Aphanomyces cochlioides* and bioassay

The fungus *A. cochlioides*(AC-5) was cultured on corn meal agar media and the method of zoospore production and bioassay of pure compounds were carried out as described previously (Islam *et al*., 2004). Cochliophilin A (**5**) a host specific attractant of *A. cochlioides* zoospores[58] was used as the standard compound.

C. Results

a. Effects of estrogenic compounds and their derivatives

Some reputed commercial mammalian sex hormonal substances were purchased, for example, bisphenol A (**10**) diethylstilbestrol (DES) (**11**) estrone (**12**) 17α-estradiol (**13**) 17β-estradiol (**14**) estriol (**15**) dienestrol (**16**) testosterone (**17**) pregnenolone (**18**) and progesterone (**19**) (Fig. 17). Also two natural estrogenic

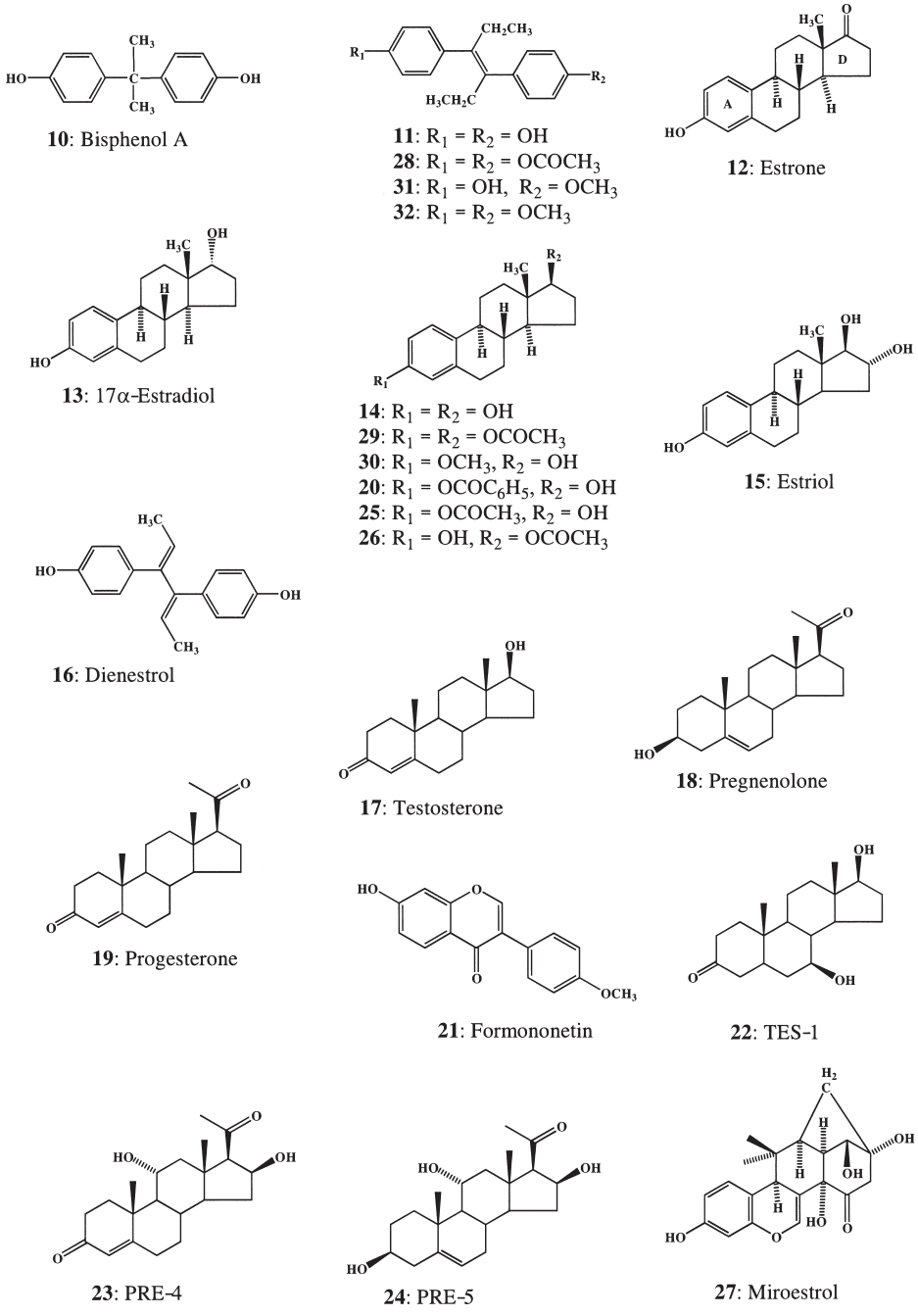


Fig. 17. Structures of two host-specific attractants, and some mammalian sex hormonal substances and their derivatives tested for the motility behavior of zoospores of the phytopathogenic oomycete *Aphanomyces cochlioides*.

mimics, formononetin (**12**) and miroestrol (**27**)[75] were included into the zoospore bioassay. After a preliminary bioassay using particle method, I prepared two representative estrogenic compounds, DES (**11**) and 17β-estradiol (**14**) to their methylated and acetylated derivatives (compounds **28**–**32**) to estabilish the relationship between structure and repellent activity. In the bioassay, we also added one metabolite of testosterone (TES-1, **22**) and two metabolites of pregnenolone (PRE-4, **23**; PRE-5, **24**)[31] and a synthetic 3-*O*-benzoyl-17β-estradiol (**20**). Two acetyl derivatives of 17β-estradiol (**14**) compounds **25** and **26** were also prepared and tested.

The bioassay results of mammalian sex hormonal substances and their derivatives toward the zoospores of *A. cochlioides* zoospores are presented in Table 8. Most of the hormonal substances (compounds **10**–**18**) exhibited repellent activities against the motility of the zoospores except progesterone (**19**) and the natural mimic miroestrol (**27**). The highest activity was recorded in DES (**11**) 17β-estradiol (**14**) and estriol (**15**) (active at 0.5 μg/ml), followed by 17α-estradiol (**13**) estrone (**12**) dienestrol (**16**) (active at 1.0 μg/ml), testosterone (**17**) (active at 50 μg/ml) and pregnenolone (**18**) (active at 100 μg/ml). The natural mimic, miroestrol (**27**) did not show any activity up to 1000 μg/ml whereas another estrogenic natural product, formononetin (**21**) showed attractant/stimulant activity towards *A. cochlioides* zoospores at 50 μg/ml. It revealed that most of the active estrogenic compounds except pregnenolone (**18**) showed higher repellent activity than that of an androgen, testosterone (**17**) The xenoestrogen, bisphenol A (**10**) showed clear repellent activity at 5 μg/ml under the same bioassay condition.

The acetylated and methylated products (**28**–**30**, **25** and **26**) of DES (**11**) and 17β-estradiol (**14**) showed relatively lower but the same kind of bioactivity as the mother compounds (Table 8) except the methylated products of DES (**31** and **32**). Interesting to note that methylated DES (**31** and **32**) showed a completely different bioactivity towards the zoospores. DES dimethyl ether (**32**) displayed potent attracting activity followed by encystment of zoospores within 4–5 min at 10 μg/ml concentration whereas DES (**11**) is a potent repellent. The encysted zoospores were found to germinate at the bottom of petri dish after 40–60 min of treatment. However, lower concentration (1.0 μg/ml) of **32** just attracted and stimulated zoospores without ceasing the motility of attracted zoospores. It was observed that the attracted zoospores seemed to search something by their flagella on the surface of treated particles. Before releasing their flagella at 10 μg/ml, the zoospores also wound up their flagella around their body or moved in a circular fashion. On the other hand, DES monomethyl ether (**31**) showed attracting and stimulating activity at 10 μg/ml without impairing the motility of zoospores. Mizutani *et al.*[97] observed that zoospores of *A. cochlioides* were halted by the action of two different compounds, while individually applied one compound (1-linoleoyl-2-lysophosphatidic acid monomethyl ester, **33**) had repellent and the other (*N-trans*-feruloyltyramine, **34**) exhibited stimulant activity. The microbial

Table 8. Bioactivity of some mammalian sex hormonal substances and their derivatives toward *Aphanomyces cochlioides* zoospores.

Name of estrogenic compounds and their derivatives	Observed activity of estrogenic compounds and their derivatives (μg/ml)*							
	1000	100	50	10	5	1	0.5	0.1
Diethylstilbestrol (DES) (**11**)	nt	---	---	--	--	-	-	±
17α−Estradiol (**13**)	nt	---	---	--	--	-	±	na
17β-Estradiol (**14**)	nt	---	---	-	--	-	-	±
Estrone (**12**)	nt	---	---	--	--	-	±	na
Estriol (**15**)	nt	---	---	--	--	-	-	±
Dienestrol (**16**)	nt	---	---	--	-	-	±	na
Bisphenol A (**10**)	---	---	--	--	-	-	na	nt
Testosterone (**17**)	---	--	-	na	nt	nt	nt	nt
Pregnenolone (**18**)	--	-	±	na	nt	nt	nt	nt
Estradiol benzoate (**20**)	na	na	nt	nt	nt	nt	nt	nt
Progesterone (**19**)	na	na	nt	nt	nt	nt	nt	nt
PRE-4 (**23**)**	na	na	nt	nt	nt	nt	nt	nt
PRE-5 (**24**)	na	na	nt	nt	nt	nt	nt	nt
TES-1(**22**)***	na	na	nt	nt	nt	nt	nt	nt
Miroestrol (**27**)	na	na	nt	nt	nt	nt	nt	nt
Formononetin (**21**)	+++/sss	++/ss	+/s	na	na	nt	nt	nt
DES diacetate (**28**)	---	---	-	±	na	na	nt	nt
Estradiol diacetate (**29**)	---	--	-	-	±	na	na	nt
3-*O*-Methyl estradiol (**30**)	---	-	-	±	na	na	nt	nt
DES Dimethyl ether (**32**)	+++ & e	+++ & e	+++ & e	++ & e	+ & s	+ &s	±	na
DES Monomethyl ether (**31**)	+++/sss	+++/sss	++/ss	+/s	s	±	na	na
Estradiol 3-acetate (**25**)	---	--	---	--	-	±	na	na
Estradiol 17-acetate (**26**)	---	--	---	--	-	-	±	na

Note: * Particle method; na = non-active; nt = not tested; - = repellent, + = attractant; e = encysting; s = stimulant; ± = activity is not clear well.

** PRE-4 (**70**) and PRE-5 (**71**) are two metabolites of **61** (Farooq and Tahara, 2000).

*** TES-1 (**69**) is a metabolite of **60** (Farooq and Tahara, 2000) .

metabolites of testosterone (TES-1, **22**) and pregnenolone (PRE-4, **23**; PRE-5, **24**) and 3-*O*-benzoyl-17β-estradiol (**20**) did not show any activity toward the zoospores of *A. cochlioides* at 1000 μg/ml.

b. Effects of estrogens in co-existence with the host-specific attractant, cochliophilin A on zoospores

The effects of potent repellent factor DES (**11**) in co-existence with host-specific attractant cochliophilin A (**5**) toward *A. cochlioides* zoospores are presented in Table 9. Compound **5** alone at lower concentrations (0.0005–0.001

Table 9. Bioactivity of DES in co-existence with a host-specific attractant cochliophilin A toward *A. cochlioides* zoospores.

Tested compounds (μg/ml)		Observed bioactivity of DES in co-existence with cochliophilin A				
Diethylstilbestrol	Cochliophilin A*	Attractant	Repellent	Stimulant	Encystment	Germination
1.0	0.0005	±	-	-	-	-
1.0	0.001	++	-	+	-	-
1.0	0.01	+++	-	+	++	+
1.0	0.1	+++	-	-	+++	+++
10.0	0.0005	-	+	+	-	-
10.0	0.001	++	-	+	-	-
10.0	0.01	+++	-	-	++	+
10.0	0.1	+++	-	-	+++	+++
50.0	0.001	-	+	+	-	-
50.0	0.01	+	-	+	-	-
50.0	0.1	+++	-	-	++	++
100.0	0.001	-	++	+	-	-
100.0	0.01	-	+	+	-	-
100.0	0.1	++ **pb**	-	+	+	+
100.0	1.0	+++ **pb**	-	-	+++	+++

*Cochliophilin A (**5**) showed clear attractant activity at 0.0005 μg/ml in the tested condition; β-estradiol (**14**) also showed similar trend of bioactivity in co-existence of cochliophilin A (data not shown); **pb** = zoospores pushed back from the particle upto 5-6 min and then attracted in usual manner. The frequency of '+' sign indicates the strength of bioactivity, and '-' sign indicates no activity under specific treatment.

Bioassay: Two treated particles (separately by two compounds) were dropped simultaneously on the petri dish containing 2 ml of zoospore suspension (particles quickly come very close to each other) and the activity of the particles toward the behavior of zoospores were observed by microscope for about 15 min for each treatment. The results presented here were confirmed by at least three times repetition under equivalent conditions.

μg/ml) shows attractant activity, whilst, at higher concentrations (0.01–1.0 μg/ml) it caused encystment followed by germination of the attracted zoospores. On the other hand, DES (**11**) exhibits only repellent activity in a dose dependent manner in a range of 1.0–100 μg/ml. Application of 1.0 μg/ml of **11** with 0.0005 μg/ml of **5** completely inactivated each other (Table 9). However, concomitant application of 0.001 μg/ml of cochliophilin A (**5**) with 1.0 μg/ml of DES (**11**) caused the zoospores weaker attractant and prominent stimulant responses. Clearly, the repellent activity of DES (**11**) was fully suppressed in this combination but the attractant activity of cochliophilin A (**5**) also weakened and a little modified due to the presence of **11**. Gradual increment of the concentration of **5** in combination with a constant concentration of **11** (1.0 μg/ml) displayed attractant, encystment and germination activity, and complete suppression of the activity due to **11**. On the other hand, concomitant application of higher doses of **11** and lower doses of **5** (10.0 μg/ml DES + 0.0005 μg/ml **5** or 50.0 μg/ml DES + 0.001 μg/ml **5** or 100.0 μg/ml DES + 0.01 μg/ml **5**) exhibited clear repellent and stimulant activity towards the zoospores (Table 9). The quality of zoospore responses was altered due to the mixing ratios of opposite active principles (attractant and repellent). Interestingly the attracted zoospores were pushed back instantly from the particles coated with higher amounts of both compounds (100.0 μg/ml **54** and 0.1 μg/ml **5**) and this phenomenon continued up to 5–6 min. Finally, the attractant and encystment activity of cochliophilin A (**5**) emerged but some of the pushed back zoospores were encysted within 4–6 min. All encysted zoospores germinated after 40–60 min. The receptor for estrogenic repellents seemed not to be affected directly by attractants (**5** and **6**) because the repellent activity of estrogens was observed in the zoospores suspended in the homogenous solution of **5** or **6**.

D. Discussion

These results show that the steroidal compounds with an aromatized A ring possess higher bioactivity toward *A. cochlioides* zoospores except 3-*O*-benzoyl-17β-estradiol (**20**). The acetylation and methylation of 17β-estradiol (**28**, **30**, **25**, and **26**) and acetylation of DES (**28**) seemed to slightly decrease the repellent activity. Interestingly, however, mono- and di-O-methylation products of DES (**31** and **32**) exhibited a completely opposite biological activity (Table 8). In case of the benzoate group at C-3–OH in 17β-estradiol (**14**) a zero activity was observed at 1000 μg/ml whereas acetylation at the same position showed a little lower repellent activity than the mother compound (**14**). Acetylation of the hydroxyl group at C-17 (**26**) did not affect much the repellent activity indicating that the free hydroxyl group at C-3 is more significant than that on C-17. Testosterone (**17**) and pregnenolone (**18**) showed lower activity than the estrogenic compounds having aromatization in the A ring of their steroidal skeleton (**12**–**16**). With respect to the structure-activity relationship, it appears that aromatization of A ring together with a free hydroxyl group at C-3 position is necessary for higher

repellent activity of estrogenic compounds. Other substituents (compounds **30**, **31** and **20**) at the same position (C-3) decreased the bioactivity, which is related to the size of the substituents. There may be a correlation between estrogenic activity and repellent activity of steroids. In animals, the sex hormones are formed from progesterone by successive oxidation steps, both at C-17 and at C-19. The oxidative removal of the side chain leads to the C-19 series with androgenic activity, and the oxidative removal of the angular methyl group at C-10 and aromatization of A ring leads to the C-18 series with estrogenic activity (Heftmann, 1970). The powerful synthetic estrogenic compound, DES (**11**) displayed potency an equivalent to 17β-estradiol (**14**) and estriol (**15**) (all active at 0.5 μg/ml). On the other hand, a xenoestrogen, bisphenol A (**10**) exhibited a repellent activity at 5 μg/ml. It has been reported that in the mammalian system, compound **10** acts through the estrogen receptor[134].

Derivatives (**31** and **32**) resulting from methylation of DES (**11**) showed completely different active principles clearly different from that of the mother compound. Both mono- and di-methylation products exhibited attractant and stimulant activity toward the zoospores of *A. cochlioides*. Moreover, the activity of the di-methylated product (**32**) resembled the attractive activity of cochliophilin A (**5**) or *N-trans*-feruloyal-4-*O*-methyldopamine (**6**) isolated from the host plants of *A. cochlioides*. Compound **5** and **6**, at around a 1×10^{-7} and 1×10^{-6} M concentration, respectively, strongly attract zoospores to the treated particles and subsequently cause encystation and germination of the attracted spores within 40–60 min. Compound **32** may act through a specific receptor because it was found to be active when compound **5** or **6**, or other estrogenic compounds were administered homogeneously into the motile zoospore suspension.

The major mammalian sex hormones (both androgens and estrogens), like estrone (**12**) 17β-estradiol (**14**) estriol (**15**) testosterone (**17**) have been isolated from several higher plants[37,43,49,95]. Basically, steroid hormones are a group of substances derived from cholesterol, which exert a very wide range of effects on biological processes such as growth, metabolism and sexual differentiation[83]. However, our current knowledge of their effect on non-mammalian biological systems like microorganisms is limited. Both growth-inhibiting and growth promoting effects of steroidal hormones have been observed by Fitzgerald and Yotis[34] but more interestingly, testosterone (**17**) and estradiol (**14**) have been found to have sex hormone activity on yeast[136]. An insect-repellent steroid was isolated from the Peruvian weed *Nicandra physalodes*[5]. Antheridiol has been identified as the chemotactic hormone of the water mold, *Achlya bisexualis* (Saprolegniaceae)[4]. Heftmann *et al.*[50] observed that a structurally related sterol of antheridiol, 5α-stigmast-22-en-3β-ol, which is produced by *Dictyostelium discoideum*, triggers the remarkable differentiation, which this slime mold undergoes. 17β-Estradiol (**14**) was found to stimulate hyphal growth of endomycorrhizal fungus, *Glumus intraradices*[111].

In the present study, the estrogenic and repellent activities of known estrogenic compounds revealed to be correlated. The particle bioassay method is very simple and convenient for testing the motility behavior of fungal zoospores. Thus, the present bioassay system seems to be useful for pre-screening the detection of estrogenic activity of naturally occurring compounds. Therefore, it may be important to carry out further work to evaluate the usefulness of this repulsion test for the bioassay-guided isolation of similar structures of phytoestrogenic compounds. Furthermore, high repellent activity of mammalian sex hormones towards fungal zoospores may be biologically important because such high negative chemotaxis was not yet reported for any zoosporic fungi. It may be important for biorational control of zoosporic fungi and/or in studying the molecular basis of chemoresponses of zoosporic fungi.

Phytoestrogen can sometimes be involved in plant growth and development even in the sexual expression in plants[37,49] but, to our knowledge, the negative chemotaxis toward fungal zoospores recorded herein has not been formerly described. Chemotaxis of fungal zoospores has been reported in many papers[20,58,98,151,152] but, reports on negative chemotaxis are very few[67,97]. Previously, it was found that monoacylated phosphatidic acid derivatives containing at least one hydroxyl group at the phosphoryl unit showed repellent activity against *A. cochlioides* zoospores[97]. (±)-Medicarpin (**33**) an isoflavonoid isolated from *Dalbergia odorifera*, also exhibited repellent activity against the zoospores of *A. cochlioides*[67]. Furthermore, 8-prenylated naringenin showed repellent activity toward zoospores at 1 μg/ml[66]. Interestingly, this compound also had estrogenic activity[135]. The mechanism of high repellent activity by mammalian sex hormonal substances on fungal zoospores is difficult to explain by our current knowledge.

E. Conclusion

Negative chemotaxis exhibited by mammalian sex hormonal substances, most of which are found in plants, raises a question whether the occurrence of this phenomenon is benefitable for oomycete zoospores from a view point of ecology, particularly in host-parasite interaction, and a speculation that minor constituents of phytoestrogens may contribute to defend nonhost plants against pathogens.

IX. GENERAL DISCUSSION

A. Introduction

Oomycetes are devastating pathogens of plants, animals and humans (Kamoun, 2001). The disease cycle is initiated by the release of biflagellate zoospores that develop within sporangia. It is believed that the phytopathogenic oomycete zoospores locate their hosts by utilizing specific chemical signals

released from the roots and then change morphologically to penetrate into root tissues. The homing sequence of the oomycete phytopathogen is very rapid and the success of infection depends on the successful completion of all pre-infection events in a sequence. The host-specificity of the highly active agents to the pathogenic zoospores, and the exudation of the specific chemical signal(s) from the roots of host plants are two important factors for the establishment of the host-pathogen relationships. In contrast to susceptible plants, non-susceptible plants may contain some "chemical weapons" to defend themselves from the attack of heterotrophic microorganisms. In the preceding sections, I have described my experimental results on the ecochemical interactions between plants (both host and nonhost) and zoospores of the *A. cochlioides*. The objective of this chapter is to review and integrate experimental findings of earlier sections into some simplified models to illustrate the critical processes involved in oomycete-plant interactions.

B. Host-pathogen interactions

a. Morphology of zoospore

The morphological features of zoospores and their flagella have been suggested to vary widely among the zoosporic fungi, even within the same genus[26,27]. My electron microscopic illustration of zoospores revealed that *A. cochlioides* zoospores are reniform-ovate shaped and a little smaller sized than the zoospores of *Phytophthora*. The zoospore has two heterokont flagella, both inserted in a ventral groove. The anterior flagellum possessed two rows of tripartite tubular hairs (TTHs) distributed throughout its length, whereas the posterior flagellum was ornamented with two rows of highly dense fine tubular hairs, except its tapered terminal part. Interestingly, the tip of the posterior flagellum has a bunch of similar fine hairs like those on the flagellum shaft[64]. Recently it is known that the TTHs on anterior flagella of oomycetes are responsible for forward motion during swimming of a zoospore[13]. Since it was hypothesized that flagella may be involved in host recognition, and also precise landing on host surface[19,26] the characteristic fine hairs observed in *A. cochlioides* may be important in precise orientation to the host. In contrast, similar hairy structures called fimbriae, surrounding the bacterial cells, play vital roles in adhesion of bacteria to the host surface[86].

b. Homing responses of zoospore

The biflagellated *A. cochlioides* zoospores rapidly aggregated to the spinach root specifically behind the root cap, adhered, encysted by shedding flagella, germinated at a fixed point to form germ tubes, and finally invaded the root tissues *via* the appressoria within 50–60 min. Precise docking has been reported in several other organisms including *Phytphthora* and *Pythium*, suggesting that it is a common or even universal feature of motile eukaryotic cells[19,20]. The

chytrid *Rozella allomycis* always encysted on the host fungus *Allomyces* with defined lateral regions of the zoospore next to the host[52,53]. Similarly, zoospores of the *Plasmodiophora brassicae* attach to root hairs so that the side of the zoospore opposite the point of flagellar insertion is next to the host. The precise docking at the ventral groove of zoospores to the host surface seemed to be partly regulated by flagellar activity as proposed by earlier investigators[19]. The time-course microscopic observations resulted some evidence on this view and discussed below.

c. Role of flagella, and developmental transitions in zoospores

Time-course microscopic study visualized that when a zoospore approached very close to the host root, it seemed to halt, and then coiled its anterior flagellum on its body. The halted zoospore appeared to contact the host surface with its posterior flagellum, which gradually drew the encysting zoospore onto the root surface. The spore then docked precisely on the root surface at its ventral face with the help of the posterior flagellum and anchored itself by releasing some adhesive materials. The adherent spore became a spherical after shedding its flagella and rapidly turned into expanded cysts forming a smooth cyst coat around it, and finally changed into a smaller cystospore coverd with a wrinkled surface. Thus, time-course microscopic observation clearly indicates that the posterior flagella of *A. cochlioides* are involved in landing and courting of the zoospores to the potential infection sites of spinach roots. This phenomenon may be common in other oomycetes phytopathogens. Considering the precise orientation of encystment by *Pythium* and *Phytophthora*, Deacon[19] speculated that the flagella may be involved in the recognition event. The biochemical mechanism of this flagellar activity is unknown, and it merits further investigation.

The time-course microscopic observation also revealed that a zoospore changed into a cystospore *via* a distinct transitional stage, *i.e.*, enlarged, smooth-surfaced cysts. My scanning electron microscopic investigation is the first to illustrate this intermediate stage as a pre-infection event of a phytopathogenic Oomycete. At present, we have no information about the composition of the smooth coats of immature cystospores. But I found that both vortex- and root diffusate-induced cysts shed their smooth cyst coats during regeneration into zoospores and germination to produce new hyphae, respectively. These results suggest that the outer, smooth cyst coats of cystospores, which formed at an early stage of encystment, may serve only as protective shields around the cysts; they are not re-used in the new generation of zoospores or growing hyphae.

The molecular mechanisms mediating pathogenicity of oomycetes are largely unknown. Molecular biological techniques have been successfully applied to one of the oomycetes *Phytophthora* spp. only recently[39]. Analysis of protein extracts of *in vitro* grown fungal structures at several developmental stages (hyphae, cysts, germinating cysts, and appressoria) by two-dimensional sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed large, stage-specific changes in protein synthesis[39,87]. Recently, Gornhardt *et al.*[39] discovered a novel gene family known as *car* (cyst-germination-specific acidic repeat) that is activated during the cyst-germination phase of *Ph. infestans*. Interestingly, Car proteins, which share high sequence homology with the human mucins, are transiently expressed during germination of cysts and formation of appressoria and are localized at the surface of germlings[39]. It is suggested that Car proteins may serve as a mucus cover protecting the germling from desication, physical damage, and adhesion to the plant surface. Therefore, the developmental transitions observed in *A. cochlioides* zoospores during interaction with host must be controlled by the expression of some stage-specific genes. A detailed molecular biological approach may clarify the responsible genes involved in each step of morphological development.

I also observed that before encystment by chemical elicitors, zoospores on artificial membranes or glass petri dishes aggregated by using their posterior flagella. These results suggest that *A. cochlioides* zoospores may use their posterior flagella not only for successful docking on the host surface but also for aggregation of encysting spores in the absence of the host. In addition, the cystospores those germinated close the excised root tip or cochliophilin A (**5**) -coated particle showed germ tube tropism toward the root. Tropic responses of hyphal germlings to host have been observed in *Phytophthora sojae*, and autoaggregation of zoospores in the absence of a host appears to be characteristic of many other oomycetes[99]. However, the mechanistic role of the posterior flagellum of oomycete zoospore in docking on host surface or aggregation of encysting spores has not been reported before. The bunch of fine hairs on the shaft and tip of the posterior flagellum may be involved in tightly adhering the flagellum to the root surface as done by bacterial fimbriae[86]. This may give some advantage to the pathogenic spores to initial hook up to their target host and to prevent water flow in the soil from washing them off their host.

d. Host-specific plant signal triggers differentiation of zoospores

The precise homing sequence of *A. cochlioides* zoospores toward host reflects guidance by a host-specific plant signals to orient to the potential infection sites of host, and then triggers differentiation of zoospores to cystospores, formation of infection structure and or germ tube tropism for completing infection in haste. Horio *et al.*[58] isolated cochliophilin A from the roots of spinach as a potent attractant of *A. cochlioides* zoospores. High content of cochliophilin A (**5**) was also found in fresh roots as well as root exudates of spinach. But the chemical signal, which triggers encystment and subsequent germination of cystospores on host root, has not been identified.

I investigated the role of cochliophilin A on the differentiation of zoospores by a partially modified particle method. Interestingly, I found that the gradient

of cochliophilin A triggered encystment of attracted zoospores followed by germination of cystospores. The dose ($<1 \times 10^{-8}$ M) of cochliophilin A needed for differentiation of zoospores was about 10–100 times higher than that required for chemotaxis (3×10^{-10} M). *In vitro* studies revealed that this compound did not affect the growth and reproduction of this oomycete up to 1×10^{-6} M concentration in the culture medium. The concentration of cochliophilin A (**5**) required (1×10^{-8} M) to trigger encystment followed by germination are equivalent to the content of it estimated in fresh spinach roots[130]. Bioassay revealed that the behavior and morphological changes of zoospores on Chromosorb particle coated with cochliophillin A (**5**) was identical to that of zoospores toward spinach roots. SEM revealed that time-course morphological changes of zoospores by cochliophilin A on Chromosorb particles were identical with the changes of zoospores interacting with spinach roots. These obsevations suggest that cochliophilin A (**5**) is indeed a host-specific plant signal, which plays essential roles in both locating host roots and initiating encystment and germination. Encystment and germination are two important pre-infection events, which are pre-requisites for invasion of the oomycete pathogen into the host. Only the germinated cystospores can penetrate into root tissues directly or *via* appressoria[10,64]. The triggering of developmental transitions in *A. cochlioides* zoospores by a host-specific plant signal shown here has not been reported in other oomycetes. Interestingly, a similar phenomenon has been observed in rhizobia, where luteolin at 1×10^{-9} M attracts the bacteria and 10^{-6} M induces *nod* expression[6].

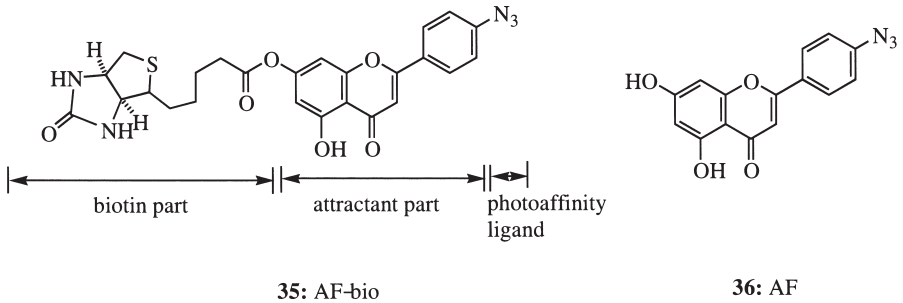
Direct application of cochliophilin A homogeneously to the zoospore suspension did not affect normal motility of zoospores even at a high concentration (1×10^{-6} M). However, very slow release of highly diluted solution of host signal by a micro-syringe to the zoospore suspension showed strong stimulation followed by encystment and germination as observed in particle bioassay. These results suggest that a gradient of host signal is essential for taxis and differentiation of zoospores. Thus, particle bioassay method appears to be a suitable method for studying chemotaxis and subsequent differentiation of zoospores where a gradient of chemical signal is essential for the response of cells. Other host-derived attractants of oomycetes zoospores may have similar effects on zoospore differentiation.

e. Putative signal transduction pathways

G-proteins are believed to be key components of signal transduction pathways in chemotaxis of many other motile cells[144]. Mastoparan is commonly used as a diagnostics for the participation of G-proteins in both animal and plant signal transduction pathways[100,109]. Interestingly, the hetrotrimeric G-protein activator, mastoparan showed encystment activity followed by germination activities at micromolar concentration. The synthetic peptide analog Mas 17,

predicted not form and amphipathic helix at 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 appeared to further enhance encystment of zoospores and rapid germination of cystospores. In addition, chemicals interfering with phospholipase C activity (neomycin) and Ca^{2+} influx/release (EGTA and loperamide) suppressed cochliophilin A and mastoparan induced encystment and germination. These results suggest that the zoospore differentiation by host-specific cochliophilin A (**5**) may be mediated by G-protein-coupled receptors to activate both phosphoinositide and Ca^{2+} second messenger pathways. Changes of Ca^{2+} fluxes during differentiation of zoospores have been observed by early investigators[18,149]. To the best of my knowledge, this is the first indication that G-protein mediated signaling mechanism is involved in oomycete zoospores. Further research on the quantification of lipid metabolism and PLC activity may open the black box of the signal transduction pathways. Since the components of the pathway represent attractive targets for developing alternative disease control methods, agricultural practice may benefit from such kind of research results in the long term.

In a biochemical study, Sakasai[120] found a putative cochliophilin A (**5**) receptor protein in the membrane of *A. cochlioides* zoospores. He designed a cochliophilin A analog, AF-bio (**35**) according to the results of structure-activity relationships analyzed by Kikuchi *et al.*[82] and Takayama[138]. The analog (**35**) consists of the required part structure as an attractant, a biotin part to be trapped by a horseradish peroxidase-avidin conjugate, and an azido group, which is for photoaffinity labelling of the zoospore proteins(s). AF-bio (**35**) showed attractant activity toward *A. cochlioides* zoospores and competition against cochliophilin A (**5**) itself in the zoospore chamotaxis. A fresh zoospore suspension containing **35** was treated by UV-light, then the membrane proteins were fractionated and subjected to SDS PAGE. The proteins in the gel were transferred to a polyvinilidine difluoride (PVDF) membrane and treated with a horseradish peroxidase-avidin conjugate. Peroxidase active region on the PVDF membrane was detected by ECL™ (enhanced chemi luminescence) method.



35: AF-bio **36:** AF

Fig. 18. Structures of cochliophilin A analog, AF-bio (**35**) and AF (**36**).

Finally, he found AF-bio (**35**) binding protein at *ca* 70 kDa, presumably a reputed receptor protein for cochliophilin A (**5**) because the band disappeared completely when the zoospores treated with **35** for photoaffinity labelling in the presence of excess amounts of AF (**36**) lacking a biotin part structure (Fig. 18). Further progress of characterization of this AF-bio binding protein is eagerly waited.

C. Nonhost plant-oomycete interactions

a. Hypothesis of nonhost defence and screening of nonhost extracts

In recent years, there has been renewed interest in examining interactions between nonhost plants and oomycetes[77]. The molecular basis of nonhost resistance remains one of the major unknowns in the study of plant-microbe interactions. Performed barriers and compounds such as saponins are ubiquitous in plants and play important roles in nonhost resistance in filamentous fungi[103,104]. Studies concerning nonhost resistance against oomycetes by plant secondary metabolites are very few. Screening extracts of 200 nonhost plants revealed that nearly half of the extracts had direct effects on motility and viability of *A. cochlioides* zoospores. None of the plant extracts showed attractant and subsequent differentiation activities as shown by cochliophilin A (**5**) in earlier sections. In addition, nonhost extracts exhibited some deleterious activities for example, repellent, stimulant, halting, lysis *etc.* against zoospores. Screening results thus indicated that many nonhost plants might use secondary metabolites to directly defend themselves from the attack of oomycetes phytopathogen[66]. Isolation of different nonhost defence factors (chemical weapons) against oomycetes may give some new interesting targets for controlling oomycete phytopathogens.

Molecular biological investigations suggest, however, that multiple layers of gene-for-gene interactions from initial defense barrier to *Phytophthora*[77]. Several oomycetes, such as *Perenospora parasitica, Albugo candida* and *Ph. pori* are known to infect the model plant Arabidopsis[57,77,115]. However, most oomycetes can not infect *Arabidopsis*[77] and induce active defense responses involving a typical hypersensitive responses (HR) and upregulation of defense genes in this plant species. With the completion of *Arabidopsis* genome project, nonhost (*Phytophthora*) resistance genes have now been cloned and sequenced, and the challenge is now is to identify them from the 26,000 genes or so present in *Arabidopsis* genome. Considering the impressive set of functional genomics tools that are available, the *Arabidopsis* system offers good prospects for dissecting the complex interactions that take place between a nonhost plant and an oomycete pathogen. At present, we have no information whether *Arabidopsis* contains some secondary metabolites for defending themselves from the attack of oomycetes.

b. Regulation of developmental transition by *A. gangeticus* metabolites

Bioassay guided chromatographic separation of *A. gangeticus* constituents

revealed that the taxis and subsequent motility inhibition of zoospores were regulated by the cumulative effects of two chemically different factors. The attractant was identified as *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) and the motility-inhibiting factor as nicotinamide (**8**). Bioassay revealed that compound 6 showed attractant activity up to 1×10^{-8} M concentration in a dose dependent manner without halting motility of zoospores even at a very high concentration (1×10^{-5} M). The direct application of compound **6** had also no effect on the motility of zoospores. On the other hand, nicotinamide (**8**) showed immediate halting activity followed by encystment in both particle (MIC 1×10^{-5} M) and homogenous solution methods (MIC 2×10^{-8} M). Interestingly, cysts produced by 8 regenerated zoospores (85–90%) instead of germination within 2–3 h in homogeneous solution method or only 20–30 min (*ca* 90%) in particle method. However, concomitant application of compounds **6** (1×10^{-6} M) and **8** (1×10^{-5} M) showed encystment of zoospores followed by germination (100%) of cystospores within 30–35 min (particle method). SEM observation revealed that nicotinamide induced cysts regenerate zoospores leaving their smooth cyst coat whereas cysts produced by concomitant application of compounds **6** and **8** germinated as shown by **5**. When an excised root of a 6 days old seedling of *A. gangeticus* was immersed into zoospore suspension in a small petri dish, all zoospores around the root tip were halted. The halted spores encysted and then regenerated after 3 h indicating the possibility of exudation of nicotinamide (**8**) predominantly from the root *A. gangeticus*. On the other hand, zoospores were specifically attracted to the root tip of *Celosia cristata* (a host), aggregated and encysted to form a mass of cystospores within 30 min. Almost 100% cysts were germinated within 40 min instead of regeneration. However, different levels of motility inhibition followed regeneration were commonly observed in case of all nonhost species in Amaratnaceae. Preliminary studies (TLC examination of root extracts and bioassay) revealed that all nonhost roots (Amaranthaceae) contain high proportions of nicotinamide (**8**). In contrast, *C. cristata* (host) roots contain high proportion of *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) with low amount of nicotinamide (**8**). It appeared from the results that the ratio of *N-trans*-feruloyl-4-*O*-methyldopamine and nicotinamide in the root exudates of Amaranthaceae might determine the compatibility of pathogen to host. Therefore, studies on the contents of *N-trans*-feruloyl-4-*O*-methyldopamine and nicotinamide in host and nonhost members of Amaranthaceae, and their exudation from the roots may clarify the precise roles of these two secondary metabolites in plant-pathogen compatibility.

c. Zoosporicidal polyflavonoid in *Lannea coromandelica*

The separation of active principles from *L. coromandelica* extracts by chromatographic techniques was unsuccessful due to their complex behavior for usual chromatography. In course of chromatographic studies, five inactive

dihydroflavonols were identified and their structures were elucidated by spectroscopic methods. Two of them were new natural products. Identification of zoosporicidal principle in *Lannea* extracts was done by MALDI-TOF-MS as polyflavonoid tannins which was confirmed by parallel experiments using commercial polyflavonoid tannins in zoospore bioassay. Like *Lannea* extracts, commercial tannins showed identical halting and characteristic lytic activities against *A. cochlioides* zoospores. Polyflavonoidal tannins are found in many plants. Their roles against herbivores and microorganisms are well-known, but the zoosporicidal effects of polyflavonoidal tannins are not reported yet. So, the present findings raise a possibility of using those natural products in biorational control of oomycetes. However, at present I have no information, whether their effect is common against all oomycetes or very specific against *A. cochlioides*.

To get more insights of the zoosporicidal activity of *Lannea* and commercial tannin extracts, I studied the morphological changes of zoospores by scanning electron microscopy (SEM). The morphological changes (fragmentation of cellular materials and formation of unique structures) of zoospores by polyflavonoid tannins observed in this experiment are similar to the characteristic features of apoptosis[125]. Scanning electron microscopic observation visualized that both *Lannea* and commercial tannins caused lysis of cell membrane followed by fragmentation of cellular materials.

Lannea stem bark contains high proportion of polyflavonoid tannins (*ca.* 13%) and, thus raises a possibility of using those naturally occurring compounds as a zoosporicidal agent. To the best of my knowledge, this is the first report of zoosporicidal activity of natural polyflavonoid tannins against an oomycete phytopathogen. Further studies on the zoosporicidal mode-of-action of polyflavonoid tannins and their effects on other phytopathogenic oomycetes are needed for considering their practical use as a naturally occurring oomicidal agent.

d. Repellent activity of mammalian estrogens

Bisphenol A (BPA) (**10**) a reputed xenoestrogen[134] exhibited potent repellent activity against the zoospores of *A. cochlioides*. Following this finding, I tested a number of androgen and estrogenic compounds (*e.g.* testosterone, progesterone, estradiols, diethylstilbestrol, estrone, estriol, pregnenolone, dienestrol *etc.*) on the motility behavior of *A. cochlioides* zoospores. Interestingly, most of the estrogenic compounds exhibited potent repellent activity (1 μg/ml or less by the "particle method") toward the motile zoospores of *A. cochlioides*. Structure-activity relationships study revealed that aromatization of the A ring with a free hydroxyl group at C-3 position of a steroidal structure is necessary for higher repellent activity. Interestingly, methylation of diethylstilbestrol (DES) yielded completely different activity i.e. both mono- and di-*O*-methyl ethers of DES (compounds **31** and **32**) showed attractant activity. Moreover, the attracted zoospores were

found encysted and then germinated in the presence of di-*O*-methyl ether of DES (**32**). So far, the repellent activity of estrogenic compounds toward trivial fungal zoospores has not been reported.

Endocrine disrupters (bisphenol A, diethylstilbestrol *etc.*) are supposed to be a pollutant in our environment and pose a serious concern in humans health. The minute amount of these compounds in our environment is difficult to detect mainly due to lack of a simple and sensitive bioassay method. In the present study, the estrogenic and repellent activities of known estrogenic compounds revealed to be correlated. The particle bioassay method is very simple and convenient for testing the motility behavior of fungal zoospores. Thus, the particle test appears to be a useful method for detecting repellents as estrogens from the natural sources or for pre-screening the detection of estrogenic activity in the environmental samples. Therefore, it may be important to carry out further work to evaluate the usefulness of this repellent test for the bioassay-guided isolation of environmental pollutants or phytoestrogenic compounds. Furthermore, high repellent activity of mammalian sex hormones towards fungal zoospores may be biologically very interesting because such high negative chemotaxis was not yet reported for any zoosporic fungi. It may be important for biorational control of zoosporic fungi and/or in studying the molecular basis of chemoresponses of zoosporic fungi.

Most of the mammalian estrogens have been also reported in plants. However, none of them has been found in the known hosts of *A. cochlioides*. The mechanism of high repellent activity by mammalian sex hormonal substances on fungal zoospores is difficult to explain by my current knowledge. This negative chemotaxis raises questions on the occurrence of this phenomenon particularly during early stage of plant-parasite interactions, and the speculation that minor constituents of phytoestrogens may contribute to defense of non-host plants against pathogens.

D. Conclusion

The characteristic features of zoospores and their sensory responses toward host signaling substances observed in several experiments clarified some questions on the chemical ecology of an oomycete phytopathogen *A. cochlioides*. The host-specificity in *A. cochlioides* must be determined by the exudation of cochliophlin A (**5**) or other signaling compound(s) from the roots of plants. In contrast, experiments on nonhost plants revealed that the nonhost plants might directly defend oomycetes attack by using "chemical weapons" or indirectly by other means. In general no nonhost plants contain any signaling compounds effective for chemotaxis and subsequent morphological changes of zoospores essential for infection.

X. SUMMARY AND CONCLUSIONS

Members of oomycete genera *Phytophthora, Pythium* and *Aphanomyces* are the most devastating pathogens of plants, animals, fishes and humans. Their zoospores are believed to locate the host plant by chemotaxis and change morphologically before penetrating root tissue. The phytopathogenic oomycete, *Aphanomyces cochlioides* is a serious pathogen of sugar beet, spinach and some other members of Chenopodiaceae and Amaranthaceae. Understanding the mechanism of ecochemical interactions between zoospores and host and nonhost plants are fundamental requirements for developing an alternative biorational control measure for low input sustainable agriculture. Therefore, the objectives of this study were to clarify the mechanism of interactions between zoospores of the *A. cochlioides* and host- and nonhost-plants *via* their secondary metabolites. To achieve the research objectives, several independent but related experiments were carried out and important findings are summarized as follows:

A. Behavioral and morphological features of *Aphanomyces cochlioides* Zoospores

The morphological diversity of zoospores and their dynamic changes when interacting with the host materials (roots and cotyledons of spinach) were investigated by light and electron microscopy. The reniform-ovate zoospore has two heterokont flagella both inserted in a ventral groove and ornamented with characteristic hairs. The anterior flagellum possesses two rows of tripartite tubular hairs (TTHs) distributed throughout its length, whereas the posterior flagellum was ornamented with two rows of fine hairs, except its tapered terminal part. The tip of posterior flagellum has a bunch of similar fine hairs like those on the flagellum shaft. The zoospores quickly aggregated on the specific sites of host root, adhere to the root surface, encysted by shedding the flagella, germinated at a fixed point to form germ tubes and finally invaded the root tissue via the appressoria within 50–60 min.

B. Interaction of *Aphanomyces* zoospores with host plant tissues

The mode of contact, aggregation, attachment and differentiation of zoospores when interacting with the host root was investigated by SEM and light microscopy. When a zoospore approached very close to the host root, it seemed to halt, and then coiled its anterior flagellum on its body. The halted zoospore appeared to contact the host surface with its posterior flagellum, which gradually drew the encysting zoospore onto the root surface. The spore then docked precisely on the root surface at its ventral face with the help of posterior flagellum and anchored itself by releasing some adhesive materials. The adherent spore became a spherical after shedding its flagella and rapidly turned into an expanded cyst forming a smooth cyst coat around it, and finally changed into a

smaller cystospore covered with a wrinkled surface. These results Indicate that *A. cochlioides* zoospores may use their posterior flagella for successful docking on the host surface. Some host-specific triggers may be involved in rapid developmental transitions of a zoospore to a germinated cystospore on host surface.

C. Host-specific attractant, cochliophilin A triggers differentiation of zoospores

Investigation on the host signal regulating developmental transitions in zoospores on spinach roots revealed that the gradient of a host-specific attractant, cochliophilin A (**5**) triggers encystment and germination of zoospores at a concentration likely to occur within the rhizosphere. Particle bioassay method was modified and improved to evaluate the effect of compound **5** on the differentiation of zoospores. Bioassay demonstrated that compound **5** attract zoospores at a minimum concentration of 3×10^{-10} M and causes their encystment at 1×10^{-8} to 1×10^{-6} M. Cystospores adhering to the surface of particles germinated within 30–35 min and those close to the particles exhibited germ tube tropism toward the particles. SEM observation revealed that the differentiation of zoospores triggered by cochliophilin A (**5**) is identical to that observed on spinach roots: first they shed flagella and become round smooth-surfaced cysts; then those develop a rough surface and germinate. These observations suggest that cochliophilin A (**5**) is indeed a host-specific plant signal, which plays essential roles in both locating host roots and initiating encystment and germination.

D. G-protein mediated signal transduction pathway in Oomycete zoospores

Mastoparan is a cationic amphipathic tetradecapeptide isolated from wasp venom and act as a generic activator of animal heterotrimeric GTP-binding regulatory proteins (G-proteins). Mastoparan agonised differentiation of zoospores, which was suppressed by PLC inhibitors and Ca^{2+}–flux regulators. 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. Taking together, these results indicate that zoospore may perceive host signal by a G-protein-coupled receptor and then translate into responses (taxis and differentiation) via phosphoinositide-Ca^{2+} signaling cascades.

E. Nonhost plants possess "chemical weapons" to defend Oomycetes

A hypothesis of "non-susceptible plants contain chemical defences against oomycetes" was investigated by screening extracts of 200 non-susceptible plants. Almost half of the extracts were affected motility and viability of zoospores[66]. Two plants *viz., Lannea coromandelica* (halting and lytic factors) and *Amaranthus gangeticus* (attractant and halting factors) were selected for further investigations to identify their active principles. The active priciple of *L. coromandelica*

was characterized by MALDI-TOF-MS as a mixture of angular polyflavonoid tannins. Both *Lannea* and industrial polyflavonoid tannins showed motility inhibition followed by characteristic lysis of zoospores.

In addition, the mammalian estrogens which are found in plants, for example, 17β-estradiols (**14**) displayed potent repellent activity toward the trivial zoospores. Structure-activity relationships study revealed that aromatization of the A ring with a free hydroxyl group at C-3 position of a steroidal structure is necessary for higher repellent activity. Interestingly, methylation of a synthetic estrogenic compound, diethylstilbestrol (DES) (**11**) (yielded completely different activity *i.e.*, both mono- and di-*O*-methyl ethers of DES (**31** and **32**) showed attractant activity. The estrogenic and repellent activities of known estrogenic compounds revealed to be correlated.

F. Triggering developmental transitions in zoospores by host and nonhost metabolites

The compounds responsible for attractant and halting activities in *A. gangeticus* extracts were revealed by chromatography to be *N-trans*-feruloyl-4-*O*-methyldopamine (**6**) and nicotinamide (**8**) respectively. Compound **6** had no inhibitory effect on zoospores whereas compound **8** immediately halted motility and caused encystment. Interestingly, the cysts induced by compound **8** regenerated zoospores instead of germinating. Compounds **6** and **8** together produced cysts that germinated within 30–35 min just like those exposed to cochliophilin A (**5**). When an *A. gangeticus* root was immerged in a zoospore suspension, zoospore development around the root tip was halted; the spores then encysted and regenerated after 3h, suggesting that *A. gangeticus* root exude nicotinamide.

G. Conclusion

Chemotaxis and subsequent differentiation of zoospores by a host-specific plant signal (cochliophilin A, **5**) appears to be essential factor for *A. cochlioides* pathogenesis. The nonhost plants contain some diverse "chemical weapons" to protect themselves directly from the attack of oomycetes. Discovery of some potential antagonists/inhibitor from the natural sources, and elucidate the signal transduction mechanism of the developmental transitions of zoospores by host and nonhost metabolites would offer attractive targets for alternative methods of disease control. The easy production of zoospores and their synchronous developmental changes to cystospores and subsequent transition to hyphae by host-specific plant signal in laboratory condition would allow using *A. cochlioides* as an excellent model system to study all aspects of cell differentiation at single-cell level as well as to elucidate molecular mechanisms involved in plant-pathogen interactions.

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Note Added in Proof

A related paper dealt with the interaction between membraneous proteins in *Aphanomyces cochlioides* zoospores and the compound **35** (AF-bio) designed as a probe for the receptor recognizing a host-specific attractant cochliophilin A (**5**) has been published recently [Sakihama, Y., Shimai, T., Sakasai, M., Ito, T., Fukushi, Y., Hashidoko, Y. and Tahara, S., 2004. *Arch. Biochem. Biophys.*, **432** : 145–151].