J Gen Plant Pathol
DOI 10.1007/s10327-007-0026-8

FUNGAL DISEASES

Composition of culture medium influences zoosporogenesis and differentiation of *Aphanomyces cochlioides*

Md. Tofazzal Islam · Mitsuyoshi Sakasai · Yasuyuki Hashidoko · Abhinandan Deora · Yasuko Sakihama · Satoshi Tahara

Received: 26 July 2006 / Accepted: 24 January 2007

Abstract A modified medium was used to culture mycelium and produce a large quantity of zoospores of *Aphanomyces cochlioides*, a principal pathogen of damping-off disease of sugar beet and root rot disease of spinach. The semisolid medium consisted of 17 g corn meal agar (CMA) added with 4 g of yeast extract (YE) per liter of 50 mM phosphate buffer (pH 6.8–7.0). This medium supported the production of ca. 10^6 zoospores ml^{-1} in 6-day-old cultures, approximately 11-fold higher than the commonly used CMA (17 g CMA per liter of water, pH 6.0 ± 0.2). Although morphological characters of the zoospores produced from the hyphae grown on CMA and CMA + YE were almost similar, they contrasted their developmental strategy after encystment induced by mechanical agitation. Cystospores originating from the zoospores on CMA regenerated zoospores (>80%), while those from CMA + YE germinated (ca. 80%) and produced hyphae. Furthermore, 4–10% of the germinated cystospores on CMA + YE had double germ tubes. The soluble protein profiles of zoospores produced on CMA and on CMA + YE demonstrated that several proteins were either different or expressed differently. Our results suggest that the culture medium directly influences zoosporogenesis in *A. cochlioides* hyphae and the developmental strategy of the produced zoospores.

Keywords Corn meal agar · Cystospore germination · Phosphate buffer · Yeast extract · Zoosporogenesis

Md. T. Islam · M. Sakasai · Y. Hashidoko (✉) · A. Deora · Y. Sakihama · S. Tahara
Laboratory of Ecological Chemistry,
Graduate School of Agriculture,
Hokkaido University, Kita-Ku,
Sapporo 060-8589, Japan
e-mail: yasu-h@abs.agr.hokudai.ac.jp

Present Address:
Md. T. Islam
School of Agriculture and Rural Development,
Bangladesh Open University, Gazipur 1705, Bangladesh

Introduction

Aphanomyces cochlioides Drechsler is a soilborne peronosporomycete, which causes damping-off and root rot diseases in sugar beet, spinach and other members of Chenopodiaceae and Amaranthaceae. The pathogen reproduces asexually via zoosporangia on the vegetative thallus or sexually via antheridia and oogonia on diseased roots and hypocotyls (Papavizas and Ayers 1974). The nonmotile primary zoospores, released from the cylindrical zoosporangium, immediately encyst and attach in clusters on the tip of the sporangium. Laterally biflagellate uninucleate, motile secondary zoospores arising from a group of primary cystospores are thought to be the main infective agents on seedlings (Haverson and Rush 1993; Islam et al. 2001). The sexual spores, oospores, are physically tougher than zoospores and are the propagules that overwinter in decayed plant tissue (over 10 years or so), providing inoculum potential for the following crop (Payne and Asher 1989).

Once liberated from the primary cysts, the secondary zoospores of *A. cochlioides* locate host roots by perceiving cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone), a host-specific flavonoid signal released from the roots (Horio et al. 1992). When the secondary zoospores arrive at the host surface, they become immobilized by shedding flagella and are transformed into cystospores (Islam et al.

2002). The cystospores then germinate to form germ tubes and invade root tissues directly or via appressoria (Islam et al. 2001, 2002, 2003). This sequence is extremely rapid as it leads to infection within 30–40 min after the zoospores arrive at the host root surface. Disruption of any of these asexual stages in the sequence eliminates the potential for pathogenesis (Islam et al. 2005). Therefore, understanding the molecular mechanisms regulating and stimulating the asexual proliferation of *A. cochlioides* is important for the development of biorational approaches to control this serious soilborne phytopathogen.

Little is known about the underlying molecular processes that control asexual differentiation in soilborne peronosporomycete phytopathogens (van West et al. 2003). Culturing *A. cochlioides* mycelium in a Petri dish containing the commonly used corn meal agar (CMA) medium for 5–6 days and then washing nutrients, followed by incubation in distilled water for 12–15 h could yield up to 10^5 zoospores per milliliter (Horio et al. 1992; Islam et al. 2003). Few attempts have been carried out to understand the sporulation of the phytopathogenic peronosporomycetes on various compositions of the culture medium. Hence, little information is available concerning the mechanism of zoosporogenesis and the developmental strategy of the peronosporomycete zoospores. The aim of this research was to investigate the effect of culture medium on zoosporogenesis and the developmental fate of the zoospores of *A. cochlioides*. We found that incorporation of yeast extract in CMA powder dissolved in phosphate buffer significantly increased the release of zoospores from the mycelia of *A. cochlioides*. Further bioassays confirmed that the developmental strategy and protein profiles of the zoospores produced with CMA supplemented with yeast extract and dissolved in phosphate buffer differed remarkably from those produced with CMA alone.

Materials and methods

Culture of *Aphanomyces cochlioides* and production of zoospores

A. cochlioides (strain AC-5) was cultured on a CMA plate (9 cm i.d.) at 20°C (Islam et al. 2001, 2002). The agar layer on the plate covered with mycelia (6-day old) was divided into eight parts and transferred to a sterilized rectangular plastic plate (13.5 × 9.5 cm) containing 100 ml of sterile deionized water. To wash out any nutrients from the agar medium, the water in the plate was changed three times with 100 ml of sterilized water at 20 min intervals. Finally, 40 ml of sterilized water was added, and the plates were allowed to stand for 16 h at 20°C in darkness to promote the release of zoospores.

Preliminary observations using CMA dissolved in water (pH 6.0 ± 0.2) provided the starting point for further studies on medium composition and zoosporogenesis. Several inorganic ions (e.g., Na^+, K^+, $H_2PO_4^-$, Cl^-, and Ca^{2+}) in a range of concentrations (10^{-7} to 10^{-3} M) were added individually or mixed in various proportions with CMA to study the effects on zoosporogenesis. Similarly, Bacto peptone (Difco Laboratories, Sparks, MD, USA) (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0%) or yeast extract (YE) was added to CMA in the presence or absence of phosphate buffer. Addition of Bacto peptone or inorganic salts did not remarkably increase or suppress the mycelial growth and zoosporogenesis of *A. cochlioides* under conditions tested. However, the addition of YE to CMA powder dissolved in 50 mM phosphate buffer (PB, pH 7.0) turned the final pH between 6.8 and 7.0 and significantly triggered both mycelial growth and zoosporogenesis of *A. cochlioides*. Therefore, the effects of YE on growth and zoosporogenesis were studied in detail.

Light and scanning electron microscopy

Motility of zoospores was halted by mechanical agitation (30 s by vortex mixing). Four-hundred microliters halted zoospores in suspension was added to each dish in Nunc Multidishes (Nalge Nunc International, Rochester, NY, USA) and allowed to settle to the bottom of the dish for 15 min. The settled cystospores per milliliter of water were counted as described earlier (Islam et al. 2004). The percentage of germinated, ungerminated and regenerated cystospores was also calculated using count data from micrographs taken with a light microscope after 6 h of mechanical agitation. The morphology of the germinated cystospores was observed with scanning electron microscopy (SEM) as described previously (Islam et al. 2001, 2002; Islam 2005). However, chemotactic behavior of zoospores toward the host-specific attractant cochliophilin A was tested with a light microscope using a particle bioassay method as described before (Horio et al. 1992; Islam et al. 2003).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

To examine any difference in developmental strategy between the mechanically induced cystospores obtained from CMA or YE in CMA powder dissolved in 50 mM PB medium, we extracted soluble proteins isolated from cystospores and separated them by 2D-PAGE. The zoospores produced from the mycelia grown in both media were separately filtered through double-layered cheese cloth and

then centrifuged at 600 rpm for 1 min to give a small pellet of spores. The spores were resuspended in 50 mM Tris–HCl (pH 6.8) and sonicated three times for 10 s. After centrifugation at 14,000 rpm for 10 min at 4°C, the supernatant was used for 2D-PAGE. Isoelectric focusing was carried out using a 2D gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). The soluble proteins were mixed with sample buffer containing 8 M urea, 2% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 100 mM dithiothreitol (DTT), 0.2% (v/v) Bio-Lytes at pH 3–10 and 0.001% (w/v) bromophenol blue. Protein samples (20 μg) were applied to 70 mm pH 3–10 Ready Strip Gel (Bio-Rad Laboratories). The gel strips were rehydrated for 12 h and then focused for 8 h with the preset linear method. After isoelectric focusing, strips were equilibrated for 10 min in 0.375 M Tris–HCl (pH 8.8), 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS and 0.130 M DTT and then for 10 min in the same buffer containing 135 mM iodoacetamide instead of DTT. The strips were run on 4% polyacrylamide stacking gel and 13% polyacrylamide separating gel, and then the gel was stained using a silver stain kit (Wako Pure Chemical Industries, Osaka, Japan).

Results

Radial growth and zoosporogenesis

The relative radial growth of mycelia in different concentrations of yeast extract (YE) in corn meal agar (CMA) powder was examined. In YE-combination tests, radial growth of *A. cochlioides* was unaffected by 0.2% YE, but at 0.4%, relative radial growth of the mycelia was slightly suppressed to 0.9 (control as 1.0). However, at this concentration, relative production of zoospores was approximately fourfold higher than the control. When the YE concentration was increased to 0.6 or 0.8%, relative radial growth rates were suppressed to 0.7 and 0.5, respectively. No zoospores were observed at these higher concentrations.

The pH of CMA-only medium dissolved in water was 6.0 ± 0.2. To stabilize the pH of the medium, water was replaced with phosphate buffer (PB, pH 7.0) in the test medium. Among the concentrations of 25, 50, 100, and 200 mM PB based media containing 0.4% YE in CMA, the best radial growth was observed at 50 mM PB. Also, in this medium, zoospore release was highest of all the conditions tested, almost 11-fold higher than that in CMA alone (Fig. 1). Therefore, 0.4% YE in CMA powder dissolved in 50 mM PB (YE-CMA-PB) (pH 6.8–7.0) was the best to trigger zoospore release.

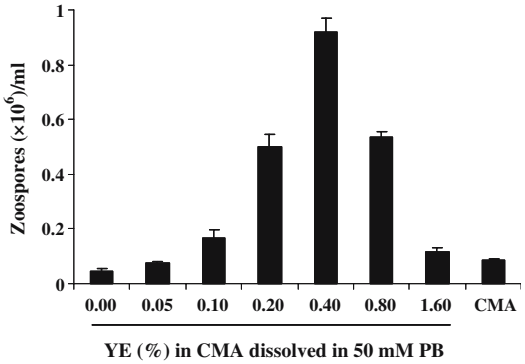

Fig. 1 Zoosporogenesis of *Aphanomyces cochlioides* from media with different concentrations of YE in CMA powder dissolved in 50 mM PB (YE-CMA-PB). *Error bar* represents the standard error of the mean of three replications. PB was added to stabilize pH in media added with varying concentration of YE. CMA, plane corn meal agar in water; YE, yeast extract; PB, phosphate buffer

Furthermore, no oogonia, antheridia or oospores were produced in the YE-CMA-PB culture medium until 7 days incubation, while 20–50 oospores per square millimeter were produced in the case of CMA alone (data not shown). This result suggests that the meiosis or sexual sporulation of *A. cochlioides* is completely suppressed in YE-CMA-PB (pH 7.0).

We added several ions such as Ca^{2+}, Na^{+}, K^{+}, and $H_2PO_4^{-}$ in the common CMA medium at a range of concentrations (10^{-7}–10^{-3} M), but none had any positive effect on asexual sporulation of *A. cochlioides* hyphae. Different levels (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1.0%) of mycological peptone into CMA also did not increase zoospore production from the hyphae.

Chemotactic and other properties of zoospores

Zoospores produced from the mycelia grown on CMA or YE-CMA-PB medium were equally attracted to Chromosorb W AW particles coated with as low as 10^{-9} M of the host-specific attractant cochliophilin A or to a root tip of a sugar beet seedling (Islam et al. 2001). Higher levels of cochliophilin A ($\geq 10^{-8}$ M), which attracted zoospores, equally triggered their encystment and subsequent germination of the cystospores as observed in CMA alone. However, results with cystospores produced from zoospores after mechanical agitation for 25–30 s and standing for 6 h in the Nunc multidish were dependent on the culture medium used for the mycelia (Table 1). The cystospores from CMA medium mainly regenerated (ca. 80%) zoospores within 3 h. Only 10–12% of the cysts germinated and produced a single germ tube. In contrast, cystospores from the YE-CMA-PB medium had mostly germinated (ca. 79%)

Table 1 Number of zoospores produced from hyphae grown on CMA or YE in CMA in 50 mM PB (YE-CMA-PB) and their developmental transition observed 3 h later than encystment by mechanical agitation with vortexing for 30 s

Culture media	No. of zoospores produced per milliliter	Fate of zoospores 3 h after vortexing (%)		
		Germinated	Regenerated	Ungerminated
CMA	$8.6 \pm 0.17 \times 10^4$	10 ± 2	82 ± 11	6 ± 3
YE-CMA-PB	$9.2 \pm 0.53 \times 10^5$	79 ± 5	13 ± 3	8 ± 4

CMA corn meal agar, *YE* yeast extract, *PB* phosphate buffer

Values are averages ± standard error of five microscopic observations from five replicates.

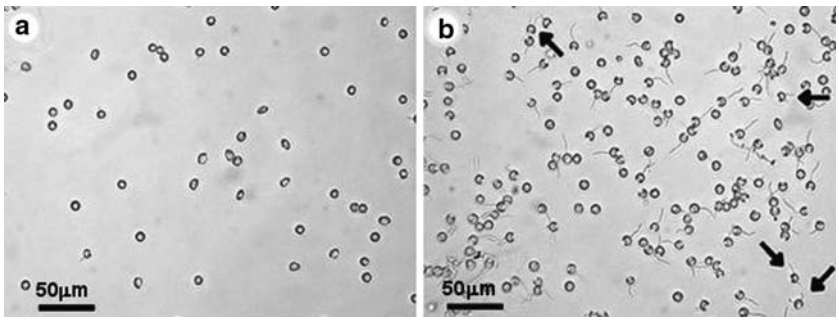

Fig. 2 Developmental transition of mechanically induced cystospores from zoospores produced in YE-CMA-PB after 30 min of encystment. For proper counting, zoospore suspension from YE-CMA-PB was diluted five times before mechanical agitation for 30 s (**b**), but no dilution was done in control (**a**). After encystment, zoospores from YE-CMA-PB germinated to form germ tubes within 30–40 min (**b**); however, zoospores did not germinate in CMA alone (**a**). *Arrows* in **b** indicate double germ tube production

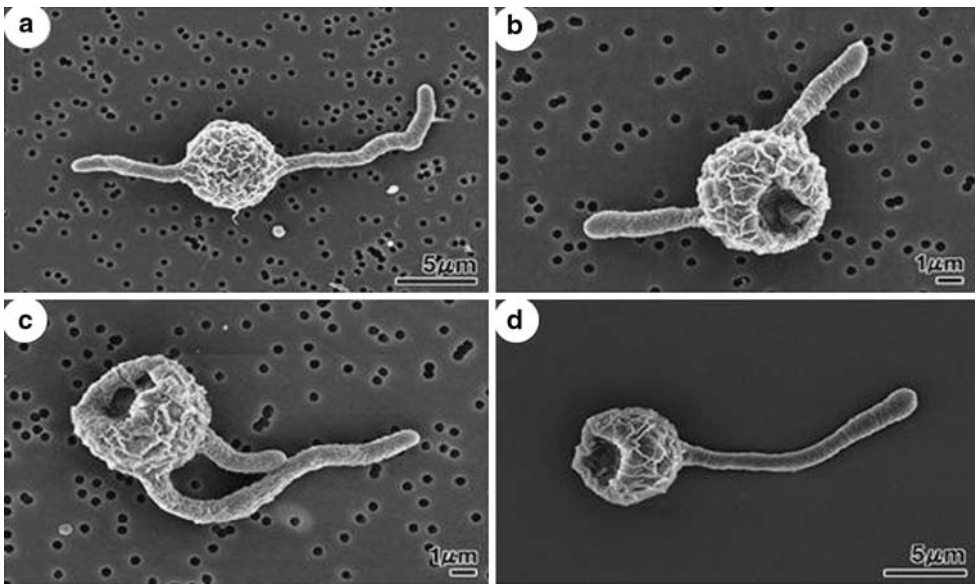

Fig. 3 Scanning electron micrographs of germinated cystospores produced from YE-CMA-PB. Production of double germ tubes from mechanically induced zoospores released from YE-CMA-PB in 50 mM PB (**a**–**c**) and a single germ tube from CMA alone (**d**)

within 60 min (Fig. 2). Interestingly, 4–10% of the cystospores of YE-CMA-PB medium produced double germ tubes. Scanning electron micrographs showed that both germ tubes can arise from the cystospore with various angles between the two tubes (Fig. 3). These results clearly showed that the physiological properties of zoospores from CMA and YE-CMA-PB medium are different.

Two-dimensional PAGE analysis of proteins

To compare the protein composition of the *A. cochlioides* zoospores released from the mycelia grown in CMA and YE-CMA-PB medium, the proteins were separated on 2D gels and silver stained (Fig. 4). The molecular masses of the majority of the polypeptides were evenly distributed

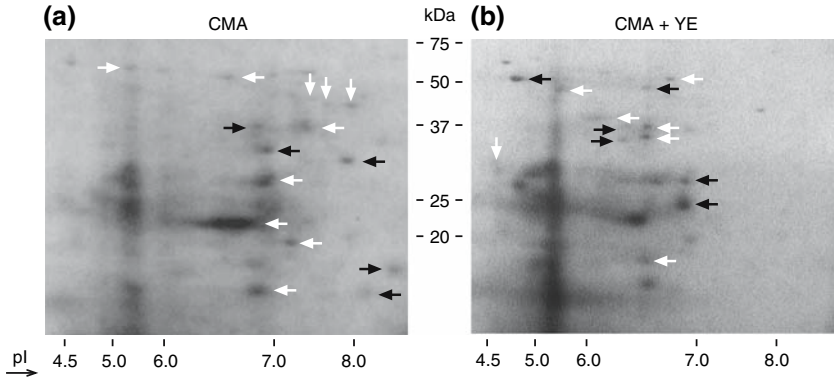

Fig. 4 Two-dimensional PAGE of *Aphanomyces cochlioides* zoospores. Proteins extracted from the mycelia cultured on CMA and YE-CMA-PB. Protein spots that are unique or more intense in one gel than in the other are indicated with *arrows*. *Black arrows* indicate the spots that are detected in CMA or YE-CMA-PB gel only. *White arrows* indicate the spots that are more intense in one gel than the other. The position of molecular weight standards is indicated on the middle (kDa) and approximate isoelectric point is indicated beneath

through the size range 10–55 kDa, and most had isoelectric points of 4.5–5.5. Comparison of the two gels showed that several proteins spot were either unique to or more intense in gels from zoospores in one medium than in the other. Five spots (black arrows in Fig. 4a) were present in the protein extract of zoospores obtained from the mycelia grown on CMA medium only; six spots (black arrows in Fig. 4b) were in that from YE-CMA-PB medium only; ten spots (white arrows in Fig. 4a) were more intense in that from CMA than YE-CMA-PB medium; and seven spots (white arrows in Fig. 4b) were more intense in that from YE-CMA-PB than CMA medium (Fig. 4).

Discussion

The present study showed that the composition of culture medium remarkably influenced not only the zoosporogenesis but also the physiological characters of *A. cochlioides*. The supplementation of YE (0.4%) in the CMA powder dissolved in 50 mM PB (YE-CMA-PB) (pH 6.8–7.0) produced approximately 11-fold more zoospores than those produced in commonly used CMA medium (pH 6.0 ± 0.2) (Figs. 1, 2). Furthermore, zoospores from the YE-CMA-PB differed significantly in germination and in soluble protein profiles than those from CMA medium. The yeast extract is a complex mixture of vitamins, nutrients, mineral elements and other stimulants for microbial growth. Therefore, our results suggest that yeast extract might contain a specific principle(s) that stimulated asexual sporulation in *A. cochlioides* hyphae.

To investigate the role of mineral elements in YE, we completely burned an equivalent amount of YE, and the resulting ash was added to the CMA medium. No effect of the ash was found on zoospore release from the hyphae, clearly indicating that the active principle is an organic compound. Because the medium was autoclaved at 120°C for 30 min before inoculation of *A. cochlioides*, the active factor may be a heat stable compound(s). The role of a heat stable endogenous factor in sexual and asexual spore differentiation in *A. euteiches* was also suggested by earlier investigators (Mitchell and Yang 1966).

The composition of culture medium influenced not only zoosporogenesis, but also remarkably changed the developmental strategy of the mechanically induced cystospores. Spores originating from hyphae grown on YE-CMA-PB medium directly germinated (ca. 80%) to form germ tubes instead of transforming into zoospores. Further, a small portion (4–10%) of the spores formed double germ tubes. It may indicate that zoospores released from the hyphae grown in YE-CMA-PB medium contain factor(s) that enhanced germination of the cystospores. Previous studies revealed that *A. cochlioides* zoospores are attracted to roots of host plants by a host-specific plant signal released from the roots and then change morphologically on the root surface triggered by the same plant signal necessary for infection (Horio et al. 1992; Islam et al. 2003). In contrast, mechanically induced cystospores (zoospores obtained from mycelia grown in CMA medium) or cystospores produced by the induction of nonhost metabolites such as nicotinamide regenerate zoospores instead of germination (Islam et al. 2004). The cyst germination proteins of the potato pathogen *Phytophthora infestans* share homology with human protein mucins (Görnhardt et al. 2000).

In the present study, hyphae grown in YE-CMA-PB medium branched excessively but not in CMA alone (data not shown). In addition, oospore production was completely suppressed in the presence of YE. These results indicated that YE contains a factor that suppresses sexual reproduction and enhances asexual reproduction of *A. cochlioides* under the conditions tested. It is, therefore, necessary to further study the effect of YE on other

Aphanomyces species that produce few zoospores and on other species of peronosporomycetes to understand whether the effect of YE on zoosporogenesis is specific or general.

In this study, we used a modified medium to produce a high density of *A. cochlioides* zoospores in vitro. At present, we do not know whether the effect of yeast extract on zoosporogenesis is common to all peronosporomycetes. However, the medium described here offers a rapid and reproducible method for producing numerous secondary zoospores of *A. cochlioides*. The medium is a simple and semidefined one that supports increased hyphal branching and growth, triggers only asexual sporulation, and completely suppresses sexual reproduction (oosporogenesis). It thus provides an opportunity for investigating molecular processes regulating asexual reproduction and the morphological regulation of the *Aphanomyces* species.

Analysis of protein profiles by 2-dimensional electrophoresis revealed that the difference in the developmental transition of zoospores produced in CMA or YE-CMA-PB medium was associated with de novo synthesis of specific proteins in the hyphal cells. The variation in *A. cochlioides* zoospore components in association with the composition of the mycelia culture media described here will form the basis for future studies that complement biochemical and cell biological approaches with molecular genetic techniques. Microsequencing of proteins separated by 2D-PAGE and cloning and sequencing of genes will help elucidate the nature and functions of proteins in zoospores from the mycelia in different media and will increase our understanding of their roles in *A. cochlioides* spore biology and pathogenicity. Clarification of the molecular mechanisms of asexual differentiation in the peronosporomycetes would ultimately allow us to develop a biorational control measure against these notorious soilborne plant parasites.

Acknowledgments We are thankful to Professor R. Yokosawa, Health Science University of Hokkaido, for his kind gift of *Aphanomyces cochlioides* (AC-5). We are also grateful for technical support from Mr. Toshiaki Ito, Laboratory of Electronic Microscopy, Faculty of Agriculture, Hokkaido University, for the SEM study. This study was supported by a Grants-in-Aid for Scientific Research from JSPS (the Japan Society for the Promotion of Science) (no. 14206013 to S.T., no. 15.3116 to M.T.I. and Y.H.) and a Postdoctoral Fellowship to M.T.I. are also very much appreciated.

References

Görnhardt B, Rouhara I, Schmelzer E (2000) Cyst germination proteins of the potato pathogen *Phytophthora infestans* share homology with human mucins. Mol Plant Microbe Interact 13: 32–42

Haverson RM, Rush CM (1993) An environmentally controlled experiment to monitor the effect of Aphanomyces root rot and rhizomania on sugar beet. Phytopathology 83:1220–1223

Horio T, Kawabata Y, Takayama T, Tahara S (1992) A potent attractant of zoospores of *Aphanomyces cochlioides* isolated from its host, *Spinacia oleracea*. Experientia 48:410–414

Islam MT (2005) Ecochemical interactions between plants and phytopathogenic *Aphanomyces cochlioides* zoospores. J Grad Sch Agr Hokkaido Univ 71:123–218

Islam MT, Ito T, Tahara S (2001) Morphological studies on zoospore of *Aphanomyces cochlioides* and changes during interaction with host materials. J Gen Plant Pathol 67:255–261

Islam MT, Ito T, Tahara S (2002) Microscopic studies on attachment and differentiation of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides*. J Gen Plant Pathol 68:111–117

Islam MT, Ito T, Tahara S (2003) Host-specific plant signal and G-protein activator, mastoparan trigger differentiation of zoospores of the phytopathogenic oomycete *Aphanomyces cochlioides*. Plant Soil 255:131–142

Islam MT, Hashidoko Y, Ito T, Tahara S (2004) Interruption of the homing sequence of phytopathogenic *Aphanomyces cochlioides* zoospores by secondary metabolites from Nonhost *Amaranthus gangeticus*. J Pestic Sci 29:6–14

Islam MT, Hashidoko Y, Deora A, Ito T, Tahara S (2005) Suppression of damping-off disease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked to plant colonization and antibiosis against soilborne Peronosporomycetes. Appl Environ Microbiol 71:3786–3796

Mitchell JE, Yang CY (1966) Factors affecting growth and development of *Aphanomyces euteiches*. Phytopathology 56:917–922

Papavizas GC, Ayers WA (1974) *Aphanomyces* species and their root diseases in pea and sugarbeet. United States Department of Agriculture, Agricultural Research Service, Techl Bull No. 1485

Payne PA, Asher MJC (1989) Disease of sugar beet seedling by *Pythium* and *Aphanomyces*. Br Sugar Beet Rev 57:44–47

van West P, Appiah AA, Gow NAR (2003) Advances in research on oomycete root pathogen. Physiol Mol Plant Pathol 62:99–113