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Antagonistic rhizoplane bacteria induce diverse morphological alterations in Peronosporomycete hyphae during *in vitro* interaction

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Abstract

A total of 150 bacteria were isolated from rhizoplanes of the host and non-host plants of a phytopathogenic Peronosporomycete *Aphanomyces cochlioides*. Upon screening, 5% of the isolates were evaluated as antagonists as they inhibited radial growth of *A. cochlioides* AC-5 hyphae in a dual culture assay. In addition, those antagonistic bacteria also induced characteristic morphological alterations in the *A. cochlioides* AC-5 hyphae that grew towards bacterial colonies. Hyphal morphological alterations observed in AC-5 and other tested strains of Peronosporomycetes included excessive branching, curly growth, unusually longer and pointed tip formation and swelling; all of these were comparable to the alterations induced by known antimicrobial compounds. Among the antagonistic bacteria, *Pseudomonas* sp. strain EC-S101 induced a unique branching pattern (tree-like) in AC-5 hyphae by continuous apical bifurcation of successive hyphae, where increases in number of branches and hyphal area were linearly correlated with time up to 10 h. Our observations suggested that the pathogen might have lost its ability of normal branch production; however maintained the capability of self-branching. Soluble extracts from the culture fluids of *Pseudomonas* sp. strain EC-S101 and *Stenotrophomonas maltophilia* EC-S105 induced similar excessive branching and curly growth in *A. cochlioides* hyphae as the respective bacterium. These results revealed that bacterial metabolites appeared to be responsible for induction of morphological alterations. Interestingly, the antagonistic bacteria that induced hyphal morphological alterations, also efficiently suppressed *in vivo* damping-off disease caused by AC-5. We suggest that antagonistic rhizoplane bacteria have the capability to induce diverse morphological alterations in Peronosporomycetes hyphae during *in vitro* interactions. Hyphal morphological alterations associated with growth inhibition and the induction of characteristic morphological changes indicate antagonistic activity against the Peronosporomycete.

Introduction

The rhizoplane is considered as a suitable source of antagonistic microorganisms for the biocontrol of soilborne phytopathogens. One of the bioassays used for screening against the phytopathogens is conventional *in vitro* dual culture agar assay. Although synthetic antimicrobial compounds have been found to induce morphological alterations in the hyphae of fungal pathogens along with radial growth inhibition (Kang et al., 2001), no previous screening studies using dual culture assay have considered whether antagonistic bacteria could alter the morphology of

hyphae during growth inhibition. Therefore, we screened antagonistic bacteria on the basis of hyphal growth inhibition that is associated with induction of morphological alterations in some Peronosporomycete pathogens during interactions in a dual culture agar assay.

The tested organism selected during initial screening was a Peronosporomycete *Aphanomyces cochlioides* AC-5, causing a damping-off disease in spinach, sugar beet and some other members of Chenopodiaceae and Amaranthaceae. An additional advantage of selecting *A. cochlioides*, a soilborne pathogen, is that the hyphae of this pathogen act as a zoosporangium, from which zoospores are released. Upon germination, the encysted zoospores penetrate host roots directly or via appressoria and cause infection (Islam and Tahara, 2001). Considerable attention has been paid over the last two decades to the biological control of the pathogenic Peronosporomycetes. For example, a number of plant extracts (Islam and Tahara, 2001) and bacterial metabolites (de Souza et al., 2003) have been screened for zoospore stimulants, repellents, and halting and lysis activities, thereby targeting only the control of motile pathogenic zoospores. Despite the importance of mycelia as the source of zoospore production, scant efforts have been made to evaluate biological agents for attacking the mycelia of Peronosporomycetes, particularly antagonistic bacteria (Thrane et al., 1999). We speculate that antimicrobial compounds altering the morphology of the hyphae may also affect zoospore production and vice-versa, thereby decreasing pathogenicity.

Our aims here were to observe induction of morphological alterations in the Peronosporomycetes associated with growth inhibition in a dual culture assay by antagonistic rhizoplane bacteria and to compare their effects by using known antimicrobial chemical compounds. We isolated 150 rhizoplane bacteria both from the host and non-host plants of *A. cochlioides* and screened their ability to inhibit mycelial growth and alter the morphology of *A. cochlioides* hyphae on agar plates. Active strains were further tested for their antagonistic activity against other Peronosporomycetes. The results of *in vitro* inhibitory activities were verified by assessing the disease suppression ability in sugar beet and spinach of *Pseudomonas* sp. strain EC-S101, isolated from spinach, and *Stenotrophomonas maltophilia* EC-S105, isolated from sugar beet under controlled *in vivo* conditions.

Materials and methods

Isolation of rhizoplane bacteria

Roots of sugar beet (*Beta vulgaris*) cv. Yuden, spinach (*Spinacia oleracea*) (unknown cv.), mustard (*Brassica campestris*) (unknown cv.) and weeds grown in the fields of these crops were collected in June, 2003 from Hokkaido University experimental farm, Sapporo and in October, 2003 from a vegetable farm at Eniwa-shi, Hokkaido, Japan. Weeds collected from the sugar beet field were *Chenopodium album*, *Equisetum arvense*, *Solanum nigrum*, *Polygonum nodosum*, *Fallopia dumetorum*, *Stellaria alsine* and from the spinach field were *Portulaca oleracea*, *Capsella bursa-pastoris*, *Spergula arvensis*, *Amaranthus lividus* and *A. retroflexus*. Bacteria were isolated and purified on Nutrient Broth (Difco Laboratories, Detroit, MI, USA) solidified with agar (i.e. NA) using aliquots of root washings as described by Williams and Asher (1996), and were preserved in 20% glycerol at −80 °C. For seed-associated bacteria, spinach cv. Tonic, sugar beet cv. Abendrot, *C. album* and *A. retroflexus* seeds were kept under running water for 48 h. Imbibed seeds were surface-disinfected by 70% EtOH for 1 min followed by 2.5% NaOCl solution for 10–15 min. Sterilized seeds (two seeds of sugar beet and spinach, 10 seeds of *C. album* and *A. retroflexus*) were inoculated in a culture tube containing 10 ml of 1/5 Hoagland's S medium supplemented with 0.3% gellan gum (Hoagland's S medium (g l^{-1}): $Ca(NO_3)_2{\cdot}4H_2O$, 1.18; KNO_3, 0.505; $MgSO_4{\cdot}7H_2O$, 0.493; KH_2PO_4, 0.272). Culture tubes were then kept in the phytotron (23 °C, 16 h light and 8 h dark). After one month, 10 μl of gellan gum medium was taken out using a sterile micro-tip from the rhizosphere of individual crop seedlings and inoculated on NA plates, serially diluted by streak culture. Isolation, purification and preservation were done as mentioned above.

Observation of hyphal morphological alterations

Initially, all bacterial isolates were tested for their antagonistic activity against *A. cochlioides* AC-5

on PDA (Difco™ Potato Dextrose Agar, Becton, Dickinson and Company, Sparks, MD, USA). The active isolates selected were further tested to assess their specificity against *A. cochlioides* AC-D (F1), and *A. euteiches* AE-F3, both on CMA. *Pythium aphanidermatum* PA-5, another Peronosporomycete, was tested on PDA medium. Bacteria used in dual culture assay were incubated for 24 h at 25 °C on NA. Dual culture assay was done using streak-cultured bacterial colonies and a 6 mm i.d. mycelial plug of the pathogen cut from the edge of an actively growing culture plate with a sterile cork borer, and placed 3 cm apart on the agar plate. Both pathogens and bacteria were inoculated on the agar plate at the same time, except for *P. aphanidermatum*, which was inoculated after two days of incubation of the bacteria because of its rapid growth. Inhibition zones, measured from tips of approaching hyphae to the edge of the bacterial streak, were recorded after incubation at 25 °C for 96 h and 36 h for AC-5 and PA-5, respectively. Both AC-D (F1) and AE-F3 were incubated at 22 °C for 10 days. Morphological characters of approaching hyphae were observed every day under a light microscope (400×, IX70-S1F2, Olympus Optical Co. Ltd., Tokyo, Japan) and images were recorded with a digital camera (CAMEDIA C-3040 Zoom, Olympus Optical Co. Ltd.).

Assay of known antimicrobial compounds

The effects of some commercially available fungicides, antibiotics and synthetic compounds were tested using paper discs charged with a series of concentrations against *A. cochlioides* AC-5 on agar plates. Sterile paper discs of 8 mm diameter and 1.5 mm thickness (Advantec Toyo, Japan) were used. Cytochalasin A (Sigma-Aldrich, St. Louis, MO, USA), zarilamide (synthesized in the laboratory) and calcium ionophore A23187 (Calbiochem, La Jolla, CA, USA) were dissolved in acetone, while hymexazol (Tachigaren®) (Hokkai Sankyo, Sapporo, Japan) was in acetone–water (1:1). Individual compounds were charged at 0.025, 0.25, 2.5 and 25 μg disc^{-1}, respectively. Control discs were charged with the solvent alone. Paper discs were dried by evaporating the solvents under vacuum. The resulting discs were then put onto Petri dishes containing PDA inoculated with a 6 mm diam mycelial plugs cut from the edge of an actively growing *A. cochlioides* AC-5 colony, placed 2 cm apart and incubated at 25 °C. Culture fluids of *Pseudomonas* sp. strain EC-S101 and *Stenotrophomonas maltophilia* EC-S105 were passed through a C18 column (Cosmosil 75C_{18}-PREP, Nacalai Tesque Inc., Kyoto, Japan), and metabolic substances trapped on the reversed column chromatographic adsorbent were extracted with 30%, 50%, 80% and 100% MeOH (Methanol). All of the four crude extracts obtained were concentrated by rotary evaporation, loaded on the paper discs and tested against *A. cochlioides* AC-5 as described above. Solvent alone 30%, 50%, 80% and 100% MeOH were kept as controls. The growth inhibition zone together with morphological changes of the approaching hyphae were observed and measured every day.

Identification of active rhizoplane bacteria

For the determination of 16S rDNA sequences of active isolates, chromosomal DNA extraction was done using Isoplant II (Wako Pure Chemical Industries) Kit and quantified using Genequant *pro* (Biochrom Ltd., Cambridge, U.K.). The 16S rDNA region was amplified by PCR using a HotStarTaq (Qiagen, Hilden, Germany) kit and universal primers 27F and 1525R for the 16S rRNA gene (Weisburg et al., 1991). Thermal cycling was performed with Mastercycler® Gradient (Eppendorf, Hamburg, Germany). The thermal profile used was: preheating for 15 min of *taq* polymerase activation at 95 °C, and 30 successive cycles consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 56 °C, and 1 min of extension at 72 °C. A conditioning step of 1 cycle at 72 °C for 10 min was performed and finally the reaction mixture was kept after cooling to 4 °C. PCR direct-sequencing was done using Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) for the 1.5 kb PCR product as the template with the universal primers (1080R, 1112F, 926F and 803R) under the following conditions: 1 cycle of 96 °C for 5 min, 25 cycles of 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. Labelled PCR-direct mixture were purified and analyzed by sequencer (ABI Prism® 310 Genetic analyzer, Applied Biosystems) according to the manufacturer's instructions. The 16S rDNA sequences of the strains were aligned with reference sequences obtained from the BLASTN database in DDBJ (DNA Data Base of Japan) website.

Time-course observation of hyphal branching in A. cochlioides *AC-5 during interaction with* Pseudomonas *sp. strain EC-S101*

To record the increases in area and number of tips per hyphae, we undertook a time-course observation of the *in vitro* dual culture interaction (day 3) between AC-5 hyphae growing towards the *Pseudomonas* sp. strain EC-S101 by arranging a Petri dish under light microscope (400×). Photographs of changes in hyphal growth with a set time interval (2 h) were taken with a digital camera up to 10 h. Artifacts to the images were processed by omitting the background using Microsoft® Paint (version 5.1) for estimating the area covered by mycelia on the agar surface. Area of mycelial growth was analyzed using a software, Area Measure (A. Hongu, MYKA Lab, Ver. 1.00, 1995, Japan) and numbers of tips per hyphae were counted by visual observations.

Culture of bacteria in liquid medium

Pseudomonas sp. strain EC-S101 and *Stenotrophomonas maltophilia* EC-S105 were cultivated in a 500 ml flask containing 200 ml of AB minimal medium (g l^{-1}: K_2HPO_4, 3.0; NaH_2PO_4, 1.0; NH_4Cl, 1.0; $MgSO_4{\cdot}7H_20$, 0.3; KCl, 0.15; $CaCl_2$, 0.01; $FeSO_4{\cdot}7H_2O$, 0.0025; saccharose, 5.0) incubated at 25 °C for two weeks with shaking at 100 rpm. Culture fluids were centrifuged at 12,000 × *g* for 15 min at 5 °C and the bacterial pellets were washed as described (Bacilio-Jiménez et al., 2003).

Influence of bacterial presence on zoospore release

Zoospores were obtained from *A. cochlioides* AC-5 by washing the mycelia three times with sterile H_2O (Islam and Tahara, 2001). At the final washing, six different inocula of *Pseudomonas* sp. EC-S101 were added as water suspensions to the mycelial suspension. Bacterial populations added were counted by the serial dilution method on NA plates kept at 25 °C overnight. After 16 h of incubation at 20 °C, the number of zoospores ml^{-1} of suspension was counted microscopically (Islam et al., 2004). Each treatment was replicated three times and data were expressed as the mean value ± standard error. Data were subjected to analysis of variance using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). Pairwise significant differences were tested by Fisher's LSD-test.

In vivo *disease suppression*

Bacterial inoculation of seeds, soil used, watering of seedlings and disease suppression ability by the tested bacteria were conducted as described previously (Islam et al., 2005). Briefly, sterilized seeds of sugar beet cv. Abendrot and spinach cv. Tonic were coated with 10^8 CFU $seed^{-1}$ of *Pseudomonas* sp. strain EC-S101 and *Stenotrophomonas maltophilia* EC-S105, respectively (Nakayama et al., 1999). Partially dried seeds (four) were sown in a cell ($4.5 \times 4.5 \times 4.5$ cm^3) of a plastic pack (36 cells), each containing 30 g of fertilized soil (Forex, Japan), purchased from a nursery. Soil used in the experiment was sterilized in a hot-air oven (Sanyo Sterilizer MOV-2125, Japan) at 160 °C for 4 h. The soil was a mixture of litter compost and volcanic gravel having high capacity to retain the moisture; the porous nature helped in proper respiration. Seedlings were grown in a growth chamber under controlled conditions (23 °C, 16 h light and 8 h dark). Seedlings (12 days old) were inoculated with *A. cochlioides* AC-5 zoospores at doses of 1×10^4, 1×10^3, 1×10^2 and 0 $seedling^{-1}$, respectively. Flooded conditions (about 40 ml of water) were maintained immediately after zoospore inoculation to stimulate infection, and plants were watered every day about 20 ml per cell. To compare the efficiency of *Pseudomonas* sp. strain EC-S101 and *Stenotrophomonas maltophilia* EC-S105 to control disease, seeds were treated with a conventional fungicide, hymexazol at a dose of 7.5 g kg^{-1} of seed and an unidentified bacterium isolated from *C. album* that showed no inhibition zone and morphological alterations in dual culture assay. The number of healthy seedlings was recorded under each treatment at weekly interval after two weeks of inoculation of zoospores. Each treatment was replicated three times and data were expressed as number of healthy seedlings ± standard error. Data were subjected to analysis of variance using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). Pairwise significant differences were tested by Fisher's LSD-test.

Results

Antagonistic activity against Aphanomyces cochlioides *AC-5*

In the initial screening, 150 bacteria isolated from the rhizoplanes of 14 plant species grown in the field were tested for their ability to alter hyphal morphology along with growth inhibition of *A. cochlioides* AC-5 hyphae in a dual culture assay. Five percent of the bacterial isolates caused characteristic morphological alterations (Figure 1b) in the AC-5 hyphae growing towards bacterial colonies compared to the untreated control (Figure 1t) along with development of clear inhibition zones on the agar medium (Figure 1a). The 16S rDNA sequence of the active isolates viz. EC-S101, EC-S102, EC-S103, EC-S104, EC-S105, EC-S106, EC-S107 and EC-S108 has been deposited in DDBJ (DNA Database of Japan) under accession

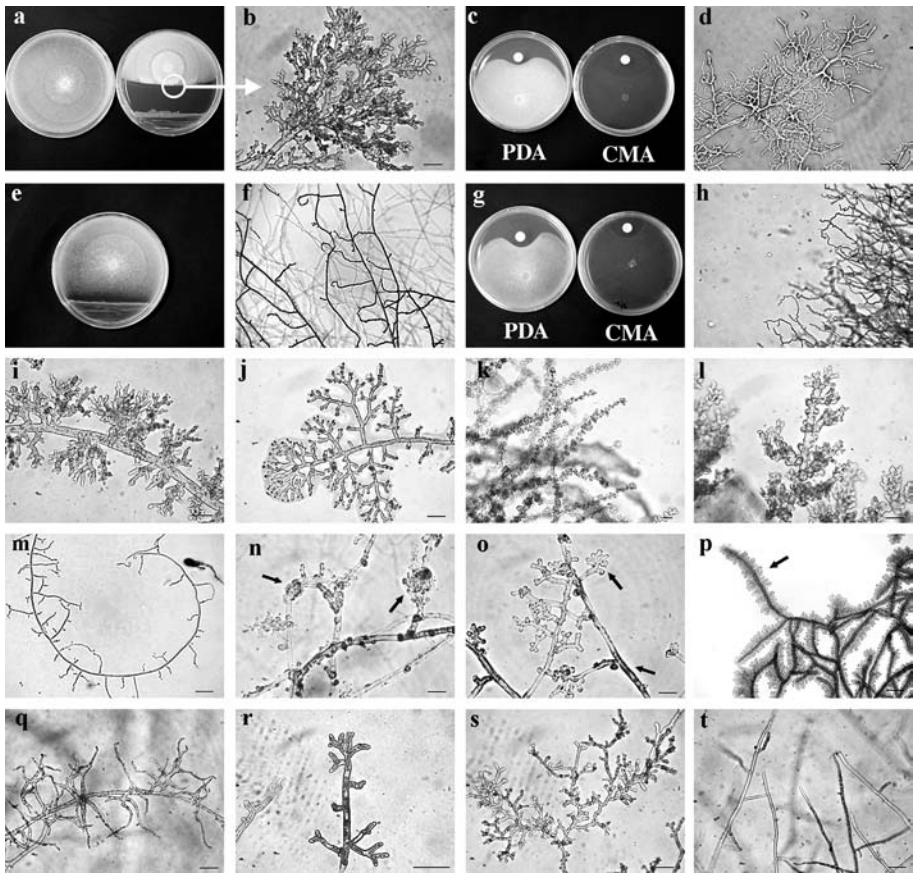

Figure 1. Photomicrographs showing hyphal morphological alterations in *Aphanomyces cochlioides* hyphae caused by rhizoplane bacteria/bacterial metabolites (a) to (t). Interactions were studied on PDA, unless medium not specified. (a) *Pseudomonas* sp. strain EC-S101: inhibition zone; (b) *Pseudomonas* sp. strain EC-S101: excessive branching; (c) Methanol (100%) solubles of *Pseudomonas* sp. strain EC-S101: inhibition zone; (d) Methanol (100%) solubles of *Pseudomonas* sp. strain EC-S101: excessive branching; (e) *Stenotrophomonas maltophilia* EC-S105: inhibition zone; (f) *Stenotrophomonas maltophilia* EC-S105: curling; (g) Methanol (100%) solubles of *Stenotrophomonas maltophilia* EC-S105: inhibition zone; (h) Methanol (100%) solubles of *Stenotrophomonas maltophilia* EC-S105: curling; (i) *Pseudomonas* sp. strain EC-S101: early stage; (j) *Pseudomonas* sp. strain EC-S101: later stage on CMA; (k) *Pseudomonas* sp. strain EC-S102: early stage; (l) *Pseudomonas* sp. strain EC-S102: later stage; (m) *Stenotrophomonas maltophilia* EC-S106: curling; (n) *Bacillus subtilis* EC-S108: gall-like appearance and decrease in normal branching; (o) *Bacillus subtilis* EC-S108: necrosis and swelling of apex; (p) *Bacillus subtilis* EC-S108: dense colonization of bacteria to the hyphae; (q) *Delftia* sp. EC-S107: longer and pointed tips with irregular growth; (r) *Klebsiella oxytoca* EC-S104: apical branching and extensive vacuolation; (s) *Klebsiella oxytoca* EC-S104: apical branching; (t) control. Scale bar – 50 μm.

numbers AB190286, AB200255, AB200256, AB200254, AB200253, AB200258, AB200259 and AB200257, respectively. Interestingly, the morphological alterations noted were initiated before the visible zones of growth inhibition developed in a particular interaction. For example, *Pseudomonas* sp. strains, EC-S101 and EC-S102 (from spinach and cabbage, respectively) induced excessive branching in AC-5 hyphae (Figures 1i and k) from day 1 of the interaction. The relative radial growth of AC-5 hyphae was lower as compared to untreated AC-5 hyphae; however, a visible radial growth inhibition zone between AC-5 hyphae and EC-S101 was developed at day 3 (data not shown).

After testing all of four crude MeOH extracts obtained from culture fluids, 100% MeOH solubles of both *Pseudomonas* sp. strain EC-S101 and *Stenotrophomonas maltophilia* EC-S105 (Figures 1c and g, respectively) induced similar excessively branched and curled hyphae (Figures 1d and h, respectively) to that induced in dual culture by the respective bacterium (Figure 1b and f) when tested towards *A. cochlioides* AC-5. In the presence of EC-S101, the approaching hyphae displayed a remarkable increase in branch frequency per unit length by decreasing the internodal distance (Figure 1b) as compared to that of the control (Figure 1t). On maturity of the mycelium, assemblages of tree-like branches were observed (Figure 1j). A similar pattern of branching was also observed for strain EC-S102 (Figure 1l).

Interactions with *Stenotrophomonas maltophilia* EC-S105 and EC-S106 (from sugar beet and *A. retroflexus*, respectively), resulted most of the surface hyphae losing their radial growth and curling in an anti-clockwise direction (Figures 1f and m). *Bacillus subtilis* EC-S108 (from *Spergula arvensis*) induced overlapping of the hyphae, giving a gall-like appearance (Figure 1n, arrows). The induction of apical branching with swelling of hyphal tips and necrosis (darkened hyphae) were commonly seen (Figure 1o, arrows). Hyphal diameter frequently varied in the colony. Later, the swarming bacteria colonized the hyphoplane of approaching hyphae (Figure 1p), and finally caused lysis of hyphae (data not shown).

Delftia sp. strain EC-S107 (from spinach roots) caused an increase in branching of hyphae with unusual longer and pointed-tip formation (Figure 1q). In addition, increase in diameter at the base of emergence of secondary branches was observed along with irregular growth directions. At advance stages of interaction with bacteria, extensive vacuolation was evoked (data not shown). Increase in apical branching, mainly in the primary hyphae, and extensive vacuolation were observed in AC-5 on interaction with *Klebsiella oxytoca* EC-S103 (Figure 1r) and EC-S104 (Figure 1s), (from *A. retroflexus* and *C. album*, respectively).

The morphological alterations in the hyphae of *A. cochlioides* occurring during interaction with bacterial isolates were comparable to those caused by antimicrobial compounds against Peronosporomycetes. An increase in branching was observed in the approaching hyphae of AC-5 towards the paper disc loaded with cytochalasin A (Figure 2a) and Ca-ionophore A23187 (Figure 2b) at 25 and 2.5 μg disc^{-1}, respectively. In contrast to the induction of excessive branching, a decrease in normal branching (Figure 2d) appeared in hyphae close to hymexazol (at 2.5 μg disc^{-1}), and zarilamide (at 2.5 μg disc^{-1}). In addition, other inhibitory effects such as swelling and extensive vacuolation in the AC-5 hyphae caused by EC-S108 were also observed on treatment with zarilamide (Figure 2c).

Table 1. Number of bacteria from different plant sources found to be antagonists against *Aphanomyces cochlioides* AC-5 in dual culture assay

Plant source	Total number of bacteria isolated	Number of isolates showing antagonism (%)
Beta vulgaris	22	1 (4)
Spinacia oleracea	20	2 (10)
Brassica campestris	9	1 (11)
Chenopodium album	15	1 (7)
Equisetum arvense	9	0 (0)
Solanum nigrum	5	0 (0)
Polygonum nodosum	6	0 (0)
Fallopia dumetorum	8	0 (0)
Stellaria alsine	7	0 (0)
Portulaca oleracea	7	0 (0)
Capsella bursa-pastoris	5	0 (0)
Spergula arvensis	12	1 (8)
Amaranthus lividus	10	0 (0)
Amaranthus retroflexus	15	2 (13)

Values in the parenthesis indicate percentage of isolates from each plant species showing antagonism against AC-5.

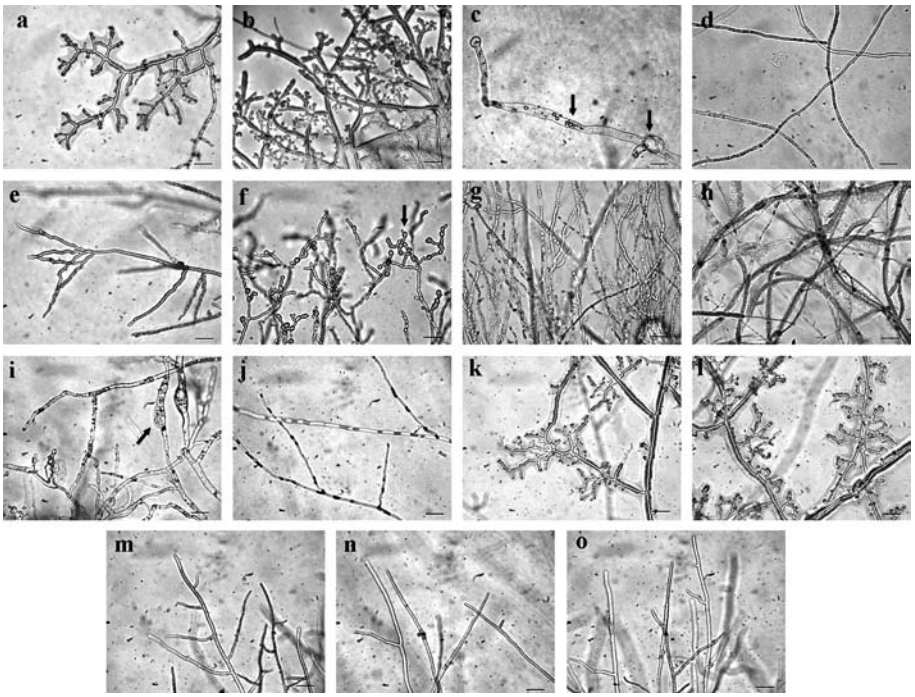

Figure 2. Photomicrographs showing hyphal morphological alterations in Peronosporomycetes caused by rhizoplane bacteria/synthetic compounds. (a) to (d) *A. cochlioides* AC-5: (a) Cytochalasin A: excessive branching; (b) Calcium ionophore A23187: excessive branching; (c) Zarilamide: swollen hyphae and suppression of branching; (d) Hymexazol: decrease in normal branching. (e) to (i) *P. aphanidermatum* PA-5; (e) *Pseudomonas* sp. strain EC-S101: apical branching; (f) *Pseudomonas* sp. strain EC-S102: swelling; (g) *Pseudomonas* sp. strain EC-S102: extensive vacuolation; (h) *Bacillus subtilis* EC-S108: dense colonization of bacteria to the hyphae; (i) *Bacillus subtilis* EC-S108: decrease in normal branching, swollen hyphae and necrosis. (j) and (k) AE-F1; (j) *Bacillus subtilis* EC-S108: extensive vacuolation; (k) *Pseudomonas* sp. strain EC-S101: excessive branching. (l) and (m) AC-D (F3); (l) *Pseudomonas* sp. strain EC-S101: excessive branching; (m) control; (n) AE-F1: control; (o) PA-5: control. Scale bar – 50 μm.

Antagonistic specificity of the active isolates to A. cochlioides *AC-5*

Among the eight isolates displaying remarkable inhibitory activities against AC-5, three isolates (EC-S101, EC-S102 and EC-S108) induced inhibition zones along with morphological alterations in *Pythium aphanidermatum* PA-5. *Pseudomonas* sp. strain EC-S101 caused an increase in branching after 24 h (Figure 2e), but the branching pattern was less dense than that induced in AC-5. *Pseudomonas* sp. strain EC-S102 induced swelling of hyphae along the length and at tips after 24 h (Figure 2f) and extensive vacuolation after 48 h (Figure 2g). Both excessive branching and extensive vacuolation were also induced by *Pseudomonas* sp. strain EC-S101 after 48 and 72 h, respectively. Along with bacterial colonization of the hyphae (Figure 2h), similar morphological alterations (Figure 2i) appeared in PA-5 due to *Bacillus subtilis* EC-S108 as those that occurred in AC-5. Testing against *A. euteiches* AE-F1, EC-S108 caused extensive vacuolation of hyphae (Figure 2j). By comparison, *Pseudomonas* sp. strain EC-S101 induced excessive branching in AE-F1 hyphae similar to that observed in AC-5 (Figure 2k). A similar effect of *Pseudomonas* sp. strain EC-S101 could be seen on *A. cochlioides* AC-D (F3) (Figure 2l).

Time-course observation of hyphal branching in A. cochlioides *AC-5 during interaction with* Pseudomonas *sp. strain EC-S101*

The increase in branching and hyphal area in AC-5 hyphae on interaction (day 3) with *Pseudomonas* sp. strain EC-S101 was in a linear correlation with time up to 10 h (Figure 3). Interestingly, induction of excessive branching appeared to be in a unique pattern as the apices of hyphae were bifurcated,

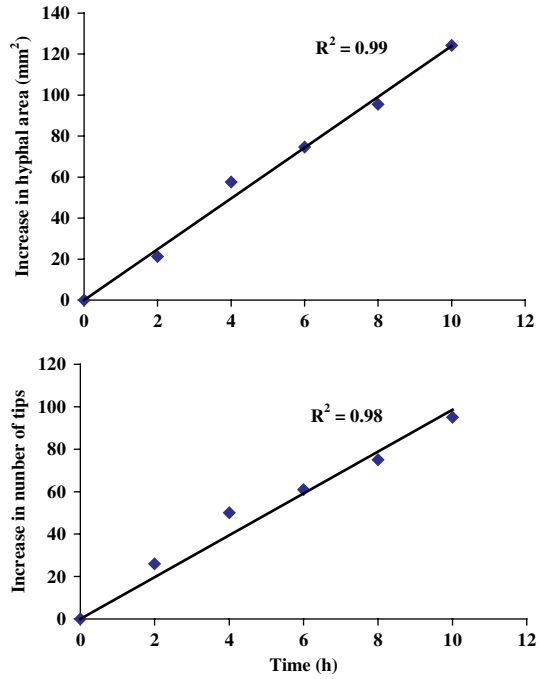

Figure 3. Increase in hyphal tips and area in the *A. cochlioides* AC-5 hyphal interaction (day 3) with *Pseudomonas* sp. strain EC-S101 (At 0 h, number of tips were 66 and hyphal area was 58.62 mm^2).

unlike the control hyphae where apices did not divide (Figure 1t). Hyphal branching continued by apical division of new hyphae and finally formed a tree-like structure (Figure 4).

Influence of bacterial presence on zoospore release

The number of zoospores released from *A. cochlioides* AC-5 mycelia was significantly reduced ($P < 0.001$) in proportion to the presence of different population sizes of *Pseudomonas* sp. strain EC-S101 (Figure 5) 16 h after the addition of bacterial cells. Remarkably, all of the zoospores were encysted in the presence of higher populations of bacteria (6.4×10^8, 2.1×10^8 and 7.2×10^7 CFU ml^{-1}). Even in the presence of lower populations of bacteria, zoospores were not as active as those in the control.

In vivo *disease suppression*

Results of *in vivo* studies indicated that none of the bacteria had negative effects on seed germination. Additionally, damping-off caused by *A. cochlioides* AC-5 infestation was significantly reduced ($P < 0.001$) after treatment with *Pseudomonas* sp. EC-S101 and *Stenotrophomonas maltophilia* EC-S105 as the number of healthy seedlings in the bacterial seed treatment was higher as compared with the control under different doses of inoculated zoospores (Table 4). Seedlings free from black and shrink to dark, slender thread hypocotyls or roots were considered as the healthy seedlings. However, in comparison with the bacterial treatment, the commercial fungicide hymexazol exerted a stronger disease suppression in both spinach and sugar beet, especially at the higher doses of zoospores (Table 4).

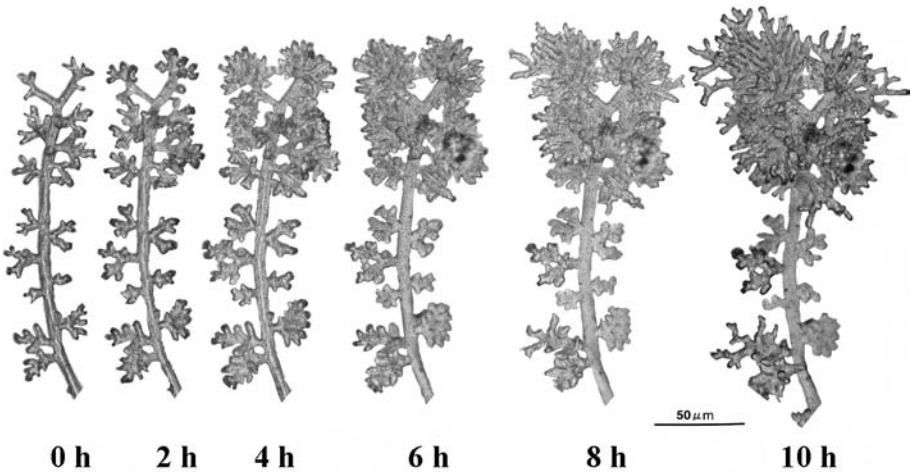

Figure 4. Time-course changes in the differentiated hyphae of *A. cochlioides* AC-5 during interaction (day 3) with *Pseudomonas* sp. EC-S101.

Table 2. Classes of morphological alterations induced in *A. cochlioides* AC-5 hyphae during interaction in dual culture with antagonisitc bacteria isolated from different plant sources

Bacterial isolate/chemical compound	Plant source	Class of morphological alterations induced				
		Branching	No branching	Swelling	Curling	Pointing tips
Pseudomonas sp. strain EC-S101	*Spinacia oleracea*	+	−	−	−	−
Pseudomonas sp. strain EC-S102	*Brassica campestris*[a]	+	−	−	−	−
Klebsiella oxytoca EC-S103	*Amaranthus retroflexus*	+	−	−	−	−
Klebsiella oxytoca EC-S104	*Chenopodium album*	+	−	−	−	−
Stenotrophomonas maltophilia EC-S105	*Beta vulgaris*	−	−	−	−	−
Stenotrophomonas maltophilia EC-S106	*Amaranthus retroflexus*	−	−	−	+	−
Delftia sp. EC-S107	*Spinacia oleracea*	+	−	−	−	+
Bacillus subtilis EC-S108	*Spergula arvensis*[a]	−	−	+	−	−
Zarilamide		−	+	+	−	−
Hymexazol		−	+	−	−	−
Cytochalasin A		+	−	−	−	−
Calcium ionophore A23187		+	−	−	−	−

[a]Non-host plants of *A. cochlioides*.

The number of healthy seedlings after treatment with unidentified non-antagonistic bacteria from *C. album*, was lower ($P < 0.001$) compared to other treatments including the control.

Discussion

In the present study, we observed that antagonistic rhizoplane bacteria exerted characteristic morphological alterations in soilborne Peronosporomycetes along with inhibition zone in a dual culture assay. The morphological alterations induced by antagonistic bacteria were comparable to those induced by known antimicrobial chemical compounds. It appears that the sensitivity of *A. cochlioides* to such rhizoplane bacteria is relatively higher than that of the other Peronosporomycetes tested (Table 3). Since most of the antagonistic bacterial isolates were from the host

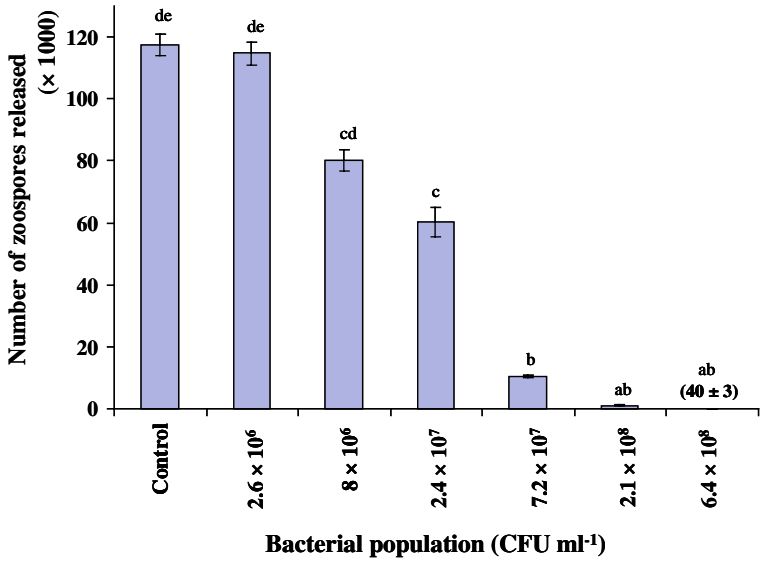

Figure 5. Number of zoospores released from *A. cochlioides* hyphae in presence of different populations of *Pseudomonas* sp. EC-S101. Bar indicates standard error of values. Data points bearing different letters are significantly different at $P < 0.001$ by Fisher's pairwise comparison. Value in parenthesis (40 ± 3) corresponds to number of zoospores released in presence of EC-S101 at 6.4×10^8 CFU ml^{-1}.

Table 3. *In vitro* antagonism of rhizoplane bacteria against phytopathogenic Peronosporomycetes

Isolate	*Aphanomyces cochlioides*			*A. euteiches*	*Pythium aphanidermatum*
	AC-5		AC-D (F1)	AE-F3	PA-5
	PDA	CMA	CMA	CMA	PDA
Pseudomonas sp. strain EC-S101	++++[a]	+++	+	++	+
Pseudomonas sp. strain EC-S102[b]	++++	+++	+	++	+
Klebsiella oxytoca EC-S103	+	+	+	++	–
Klebsiella oxytoca EC-S104	+	+	+	+	+
Stenotrophomonas maltophilia EC-S105	+	+	+	++	–
Stenotrophomonas maltophilia EC-S106[c]	+	+	–	–	–
Delftia sp. EC-S107	+	+	–	–	–
Bacillus subtilis EC-S108[b]	+++	++	++	++	+

Note: All bacteria isolates are from non-host plants of *A. euteiches* and *P. aphanidermatum*.
Among the compounds tested against AC-5; zarilamide, hymexazol, cytochalasin A and calcium ionophore A23187 caused inhibition zone at 25 μg disc^{-1} as +++, ++++, +, ++ and at 2.5 μg disc^{-1} as ++, +, –, + respectively. Compounds that did not cause growth inhibition up to 25 μg per disc were: nicotinamide, polyflavonoid tannin, commercial tannin, paclitaxel, dimethyl DES, β-estradiol, benlate, MBC, cytochalasin B and colchicine.
[a]For growth inhibition zone, each treatment was averaged over two replicates and evaluated as: ++++, >20 mm; +++, >10–20 mm; ++, 5–10 mm; +, 0–5 mm; –, no inhibition.
[b]Bacteria isolated from non-host plants of *A. cochlioides*.
[c]Seed-associated bacteria.

plants of *A. cochlioides* that were also nonhosts of *A. euteiches* and *P. aphanidermatum* (Table 3), it can be assumed that these host plants might have a higher ability to harbour such antagonistic bacteria that can protect them from their pathogens. Interestingly, bacteria that induced morphological alterations also showed potent disease suppression activity against *A. cochlioides*.

Induction of excessive branching in AC-5 on encountering *Pseudomonas* sp. strain EC-S101 was apparently similar to those caused by antimicrobial compounds viscosinamide and phenazine-1-carboximide in other Peronosporomycetes and other fungi (Thrane et al., 1999; Bolwerk et al., 2003). Both of these compounds are secondary metabolites produced by *P. fluorescens* strains DR54 and WCS365, respectively (Chin-A-Woeng et al., 1998; Nielsen et al., 1999). We also found that Methanol (MeOH) solubles obtained from culture fluid of *Pseudomonas* sp. strain EC-S101 induces excessive branching in the hyphae of AC-5; this, indicates the involvement of secondary metabolites produced by EC-S101 in the induction phenomenon of excessive branching.

Similarly, the curly growth of AC-5 hyphae in dual culture with *Stenotrophomonas maltophilia* EC-S105 or EC-S106 and by MeOH solubles from culture fluid of strain EC-S105 tested by paper disc, was similar to that observed in *A. cochlioides* AC-5 due to a biocontrol rhizoplane bacterium belonging to *Lysobacter* (Islam et al., 2005), a related genus of *Stenotrophomonas* (Sullivan et al., 2003) that produces xanthobaccin A (Nakayama et al., 1999). Curly growth of the *Fusarium oxysporum* f. sp. *radicis-lycopersici* hyphae was also found during interaction with *P. fluorescens* WCS365 (Bolwerk et al., 2003). By comparison, necrosis, bulbous structure formation, extensive vacuolation and varying diameter of AC-5 hyphae by *Bacillus* sp. EC-S108 are similar to effects of zarilamide. It was observed that bacteria travelled about 1–2 cm towards growing hyphae within a week of incubation and densely colonized the hyphoplane (Figure 1q), resulting in necrosis of the hyphae (data not shown). The migration of the bacteria to the hyphae and local colonization suggest that chemotactic substances could be involved. However, it is not yet known whether the lethal action behaviour of *Bacillus subtilis* EC-S108 is due to toxin production or competition with the pathogen.

We found that the increase in branching frequency and area in *A. cochlioides* AC-5 hyphae during encounter with *Pseudomonas* sp. strain EC-S101 was linearly correlated with time up to 10 h. The branching pattern induced in AC-5 hyphae

seemed to be unique with the continuous bifurcation of the apex of each successive hyphae leading to a tree-like structure. The visible decrease in internodal distance in the affected hyphae compared to controls indicates that the pathogen might have lost its ability to regulate the normal branching process, but maintained capability of self-branching. Time-course studies using the confocal laser scanning microscope and transmission electron microscope revealed that *Pseudomonas* sp. strain EC-S101 exerts physiological stresses such as cell wall thickness, extensive vacuolation and change in shape of nuclei, mitochondria and lipid bodies in *A. cochlioides* AC-5 hyphae; finally resulting in the death of the pathogen (unpublished data).

Reduction in zoospore release and the induction of encystment in *A. cochlioides* AC-5 in the presence of *Pseudomonas* sp. strain EC-S101 were similar to that in *Pythium ultimum* zoospores released from the hyphae treated with viscosinamide or viscosinamide-producing *P. fluorescens* DR54 (Thrane et al., 2000). However, AC-5 hyphae in dual culture assay with *Pseudomonas* sp. strain EC-S101 showed no oospore formation similar to the response shown in viscosinamide-treated *P. ultimum* hyphae. Therefore suppression of damping-off *in vivo* by *Pseudomonas* sp. strain EC-S101 and the *Stenotrophomonas maltophilia* EC-S105 treatment are also likely to be due to the presence of active agents produced by them. In addition to antibiosis, the efficient colonization ability of *Pseudomonas* sp. strain EC-S101 over the host root surface (data not shown) might also be involved in nutrient and niche competition with the pathogen (Lugtenberg et al., 2002).

In conclusion, our dual culture observations showed that antagonistic rhizoplane bacteria induced diverse characteristic morphological alterations in the hyphae of phytopathogenic Peronosporomycetes. Hyphal morphological alterations were associated with growth inhibition and the induction of characteristic morphological changes and indicated antagonistic activity against the Peronosporomycete. However, it is necessary to carry out further studies on the isolation of the metabolites responsible for these changes and to understand their mode of action and their usefulness as biocontrol agents.

Table 4. In vivo damping-off disease suppression ability of rhizoplane bacteria in sugar beet and spinach seedlings

Treatment	Zoospores inoculated per seedling*	Number of healthy seedlings	
		Sugar beet	Spinach
Pseudomonas sp. EC-S101[x]	1×10^4	3 ± 0.6 bc	3 ± 0.0 bc
	1×10^3	9 ± 0.6 cd	6 ± 0.6 bc
	1×10^2	12 ± 0 d	10 ± 0.3 cd
	0	12 ± 0 d	12 ± 0.0 de
Stenotrophomonas sp. EC-S105[x]	1×10^4	5 ± 0.3 bc	3 ± 0.6 bc
	1×10^3	8 ± 0.9 c	5 ± 0.9 bc
	1×10^2	12 ± 0.0 d	10 ± 0.3 cd
	0	12 ± 0.0 d	12 ± 0.0 de
Unidentified strain[x]	1×10^4	1 ± 0.3 ab	0 ± 0.0 ab
	1×10^3	1 ± 0.3 ab	2 ± 0.3 ab
	1×10^2	2 ± 0.7 ab	4 ± 0.7 bc
	0	12 ± 0.0 d	12 ± 0.0 de
Hymexazol[y]	1×10^4	5 ± 0.3 bc	11 ± 0.3 cd
	1×10^3	10 ± 0.3 cd	12 ± 0.0 de
	1×10^2	12 ± 0.0 d	12 ± 0.0 de
	0	12 ± 0.0 d	12 ± 0.0 de
Control[z]	1×10^4	0 ± 0.0 ab	0 ± 0.0 ab
	1×10^3	2 ± 0.3 ab	3 ± 0.6 bc
	1×10^2	5 ± 0.7 bc	5 ± 0.3 bc
	0	12 ± 0.0 d	12 ± 0.0 de

Values of number of healthy seedlings ± standard error were based on three replicates, each including four plants. Values followed by same letters are not significantly different at $P < 0.001$ by Fisher's pairwise comparison.
[x]Bacteria were pelleted to the seeds at 10^8 CFU seed^{-1}.
[y]Hymexazol was applied at 7.5 g kg^{-1} of seed.
[z]Seeds without bacteria or fungicide applied.
*Zoospores were inoculated on 12 day old seedlings.

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