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ORIGINAL PAPER

# Growth inhibition and excessive branching in *Aphanomyces cochlioides* induced by 2,4-diacetylphloroglucinol is linked to disruption of filamentous actin cytoskeleton in the hyphae

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**Abstract** We observed that 2,4-diacetylphloroglucinol (DAPG), a major antimicrobial metabolite produced by a rhizoplane bacterium *Pseudomonas fluorescens* ECO-001 inhibited mycelial growth of a damping-off phytopathogen *Aphanomyces cochlioides* AC-5 through inducing excessive branching and curling in the hyphae. This study aimed to unravel the mode of action of DAPG caused excessive branching, curling and growth inhibition of AC-5 hyphae by detecting localized changes in the cortical filamentous actin (F-actin) organization by rhodamine-conjugated phalloidin. Confocal laser scanning microscopic observations revealed that both living bacteria and DAPG severely disrupted the organization of F-actin in the *A. cochlioides* hyphae in a similar manner. Furthermore, an inhibitor of F-actin polymerization, latrunculin B also induced similar growth inhibition, excessive branching and caused disruption of F-actin in the AC-5 hyphae. Our results suggested that growth inhibition and excessive branching induced in *A. cochlioides* by DAPG is likely to be linked to the disruption of F-actin cytoskeleton in the affected hyphae. This is the first report on disruption of cytoskeleton of a eukaryotic *A. cochlioides* by a well-known biocontrol metabolite DAPG secreted from a prokaryotic bacterium ECO-001.

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## Introduction

The Peronosporomycetes (Oomycete in the old classification) are eukaryotic Stramenopiles that resemble filamentous fungi in their vegetative growth form, but are closely related to brown algae and diatoms (Dick 2001). Members of these fungus-like microorganisms such as *Phytophthora, Pythium, Aphanomyces*, and *Plasmopara*, are devastating pathogens of plants, animals, fishes and humans (Islam and Tahara 2001). Among them, *Aphanomyces cochlioides* is a serious pathogen causing damping-off disease in sugar beet, spinach and some other members of Chenopodiaceae and Amaranthaceae. Screening antagonistic rhizoplane bacteria, we observed that a strain of *Pseudomonas fluorescens* ECO-001 isolated from *Plantago asiatica* inhibited polar growth of *A. cochlioides* AC-5 on potato dextrose agar medium by inducing excessive branching and curling in the hyphae. Our further investigation confirmed that a polyketide antibiotic, 2,4-diacetylphloroglucinol (DAPG) secreted from the ECO-001 was responsible for its antimicrobial activity.

Some strains of *P. fluorescens* produce DAPG, which is a well-known antimicrobial agent that inhibits growth of the diverse group of microorganisms including fungi, bacteria, protists and nematodes (Bender and Rangaswamy 1999; Isnansetyo et al. 2003; de Souza et al. 2003; Kamei and Isnansetyo 2003). Due to the antifungal activity of DAPG, several strains of *P. fluorescens* have shown promising biocontrol activity against fungal and

peronosporomycetal phytopathogens (Bender and Rangaswamy 1999; Kamei and Isnansetyo 2003; Chin-A-Woeng et al. 1997; Bangera and Thomashow 1999). Several lines of evidence suggest that exogenous application of DAPG alters growth and development of fungal hyphae (Isnansetyo et al. 2003; de Souza et al. 2003) and plant roots (Brazelton et al. 2008).

The microfilaments of actin that are associated with the plasma membrane, are important for generating cell-surface specializations, and also provide the driving force for remodeling cell morphology and triggering cell behavior when the environment is modified (Revenu et al. 2004). It has been known that actin plays an important role during differentiation and morphogenesis of the cell (Miller et al. 1999; Hülskamp 2004). Proper actin organization is essential for normal establishment of polarity in many cells including the embryos of brown algae (Brawley and Robinson 1985), polar growth of fungal and peronosporomycetal hyphae (Heath et al. 2000) and normal morphogenesis and polar growth of trichomes (Szymanski et al. 1999) and root hairs (Miller et al. 1999) in plants.

Tips of growing hyphae of the Peronosporomycetes contain an apical cap of fine F-actin filaments adjacent to the plasma membrane, extending back along the hyphae for approximately 30 μm, where they merge with the subapical pattern of cortical actin cables and plaques (Bachewich and Heath 1998). An inhibitor of actin polymerization, latrunculin B isolated from a marine sponge *Latruncula magnifica* specifically disrupted F-actin and induced hyperbranching in *Saprolegnia ferax* (Bachewich and Heath 1998). Recently, Islam (2008a) demonstrated that induction of excessive branching, curling and stunted growth in a damping-off pathogen *A. cochlioides* by a biocontrol bacterium *Lysobacter* sp. SB-K88 is associated with disruption of cytoskeletal filamentous actin (F-actin) network in the hyphal cells.

Although inhibitory activities of DAPG on microorganisms have been documented in many articles, however, the mode of action of this well-known antimicrobial agent is poorly understood (Bender and Rangaswamy 1999). As filamentous actin (F-actin) plays a vital role in polar growth and morphogenesis of hyphal tips (Bachewich and Heath 1998; Islam 2008b), we hypothesized that inhibition of mycelial growth accompanied with excessive branching and curling of hyphae by DAPG are linked to the localized changes of cortical F-actin organization in the *A. cochlioides* hyphal cells. Therefore, the objectives of this work were to (1) evaluate inhibitory effects of DAPG on mycelial growth of a damping-off pathogen *A. cochlioides*; and (2) visualize organization of F-actin network in the cells of excessively branched and curled hyphae during interaction with DAPG by using rhodamine-conjugated phalloidin.

## Materials and methods

### Isolation and identification of DAPG producing bacterial strain

The DAPG producing bacterial strain ECO-001 was isolated from the rhizoplane of a weed *Plantago asiatica* grown in the campus of Hokkaido University in October 2003 employing a simple procedure. Briefly, thirty different individual plants were dug up with soil around the roots and shaken in the air to remove the soil. After soaking the roots in sterile water for 20 min, they were gently swirled in sterile water to remove the soil. The roots were cut into 1 cm pieces, placed on 0.5% agar plates, and then incubated at 25°C in the dark for 3 days. No mycelia were observed around the roots of *P. asiatica* but some bacteria grew instead. The grown bacteria were separated as a single colony on PDA (potato dextrose broth, 24 g/l, Difco Laboratories; agar, 20 g/l) plates. Each isolate was placed on a nutrient broth agar (NBA) plate for antibacterial test by an inoculating loop against *Bacillus subtilis* cells homogeneously inoculated on the NBA medium. After incubation at 25°C in the dark for 12 h, one isolate showed inhibitory zone around the colony. We coded it as ECO-001.

The isolate ECO-001 produced visible crystals around the bacterial colonies grown on PDA medium. The crystals were briefly washed with MeOH, then dissolved in EtOAc and tested for biological activity by thin layer bioautography using *B. subtilis* and *Cladosporium herbarum* as test microbes. Growth of both microorganisms was significantly inhibited by ECO-001 metabolite(s). Analysis on thin layer chromatography under varying ratios of $CHCl_3$ and MeOH gave a clear single spot on silica gel plates. Chemical structure of the crystals was unambiguously determined as DAPG by the spectral analysis including MS and $^1H$ NMR and direct comparison with synthetic authentic compound (Marchand et al. 2000). Considering the over production of polyketide antibiotic DAPG and other biochemical and phenotypic traits, we identified the strain ECO-001 tentatively as *P. fluorescens*.

### Test microorganism

The damping-off pathogen *Aphanomyces cochlioides* AC-5 used as a cell material for cytochemical study was kindly donated by Prof. R. Yokosawa, Health Science University of Hokkaido, Japan. This organism was originally isolated from the roots of sugar beet, routinely grown and maintained in PDA medium at 25°C (Islam 2008a).

### Hyphal cell preparation for microscopy

Interactions between *A. cochlioides* AC-5 and *P. fluorescens* were studied by collecting agar disks (6 mm in diameter) from the growing edges of AC-5 hyphae on PDA and placing each disk 3 cm apart from the colony of bacterial strain ECO-001 in four replicates. Another four replicate plates were inoculated with same size disks of AC-5 colony in plates in the absence of ECO-001. Plates were allowed to grow at 25°C in the dark for 5 days, after which time AC-5 hyphae grown on PDA were harvested for confocal laser scanning microscopy (CLSM), with a sterile cork borer (6 mm i.d.) from the colony edge growing toward the ECO-001 colonies.

We synthesized sufficient amount of DAPG in the laboratory following method published by Marchand et al. (2000) for bioassay and CLSM study. The effects of synthetic DAPG and actin inhibitor, latrunculin B (Sigma) on disruption of F-actin in hyphae were tested using paper disks charged with a series of concentrations against *A. cochlioides* AC-5 on agar plates (Islam 2008a). Sterile paper disks of 8 mm diameter and 1.5 mm thickness (Advantec Toyo, Japan) were used. DAPG and latrunculin B separately dissolved in acetone were applied at a concentration of 0, 0.1, 0.5, 1.0, and 5 μg per disk, respectively. Control disks were prepared with the solvent alone. Paper disks were dried by evaporating the solvents under vacuum. The resulting disks were then placed 2 cm apart onto Petri dishes containing PDA inoculated with a 6 mm diameter mycelial plugs cut from the edge of an actively growing *A. cochlioides* AC-5 colony, placed 2 cm apart, and incubated at 25°C. The F-actin patterns in the *A. cochlioides* hyphal cells approaching toward paper disks were visualized by rhodamine-conjugated phalloidin (RP) (Molecular Probes). For comparisons, non-treated controls were harvested from PDA plates with only AC-5 for CLSM visualization.

### CLSM specimen preparation and observation

Rhodamine-conjuated phalloidin (RP) has specificity to both types of actin arrays (filaments and plaques) in the hyphal cells of peronosporomycetes (Heath et al. 2000; Islam 2008a, b). Therefore, the RP was selected for visualization of changes of the F-actin organization in the *A. cochlioides* hyphae. The F-actin organization in hyphae was visualized using a protocol outlined by Heath et al. (2000) with slight modifications (Islam 2008a,b). Briefly, a sterile cork borer (6 mm) was used to remove a plug of mycelium from the growing edge of 5-day-old plates of isolate AC-5 paired with the bacterial colony or DAPG or with latrunculin B-treated paper disks. Plugs were removed from untreated controls by a similar method. The plugs were fixed with 6% paraformaldehyde (Wako Pure Chem. Co. Inc.) in 60 mM 1,4-piperazinediethanesulfonic acid (Pipes) buffer (Sigma) (pH 7.0), with 100 μM MBS (*m*-maleimidobenzoyl *N*-hydroxysuccinimide ester, Pierce) for 30 min at room temperature. They were then rinsed three times in a buffer solution and transferred to a glass slide for sectioning. The upper portion of the agar plug was sectioned uniformly (*ca*. 0.25 mm thickness) with a sterilized stainless blade. The specimens were then stained for 30 min in 0.66 μM RP in 60 mM Pipes buffer (pH 7.0), rinsed in buffer, and mounted in 50% glycerol with 0.1% *p*-phenylenediamine. Then the specimens were observed under a confocal laser scanning microscope (CLSM) (LSM410, Carl Zeiss Co., Germany) (Islam 2008a, b). To get a fluorescence image, the averaging (overlapping) was conducted at eight scans per frame. Scan time per frame was 1.08 s. For Z-sectioning, 10–30 sections were obtained depending on the depth of the sample. To obtain a DIC image, the averaging was performed at four scans per frame. This experiment was repeated three times and all treatments were replicated at least three times. The representative images were captured and presented in the results.

## Results

### *Pseudomonas fluorescens* ECO-001 induces excessive branching and disrupts F-actin organization in affected hyphae

The ECO-001 inhibited polar growth of *A. cochlioides* hyphae in a dual culture assay on PDA medium (Fig. 1b) by inducing characteristic excessive branching in the confronting hyphae (Fig. 1d). Tubular polar growth and normal branching patterns of hyphae was observed in the control plate (Fig. 1a, c). When the excessively branched and curled *A. cochlioides* hyphae induced by ECO-001 were placed on a new PDA plate, the polar growth of the hyphae returned to normal.

To see whether growth inhibition and morphological alterations of hyphae induced by *P. fluorescens* ECO-001 are associated with changes of cytoskeleton in AC-5, we monitored organization of filamentous action (F-actin) by visualizing with RP and observed under a confocal laser scanning microscope (CLSM). CLSM revealed that hyphae stained with RP exhibited two morphological forms of plasma membrane-associated actin arrays, filaments and plaque-like. In control hyphae, F-actin was located in close proximity to the plasma membrane in plaques interconnected with fine filaments (Fig. 2a, c). However, apex and tip of the control hyphae were populated only with fine filaments.

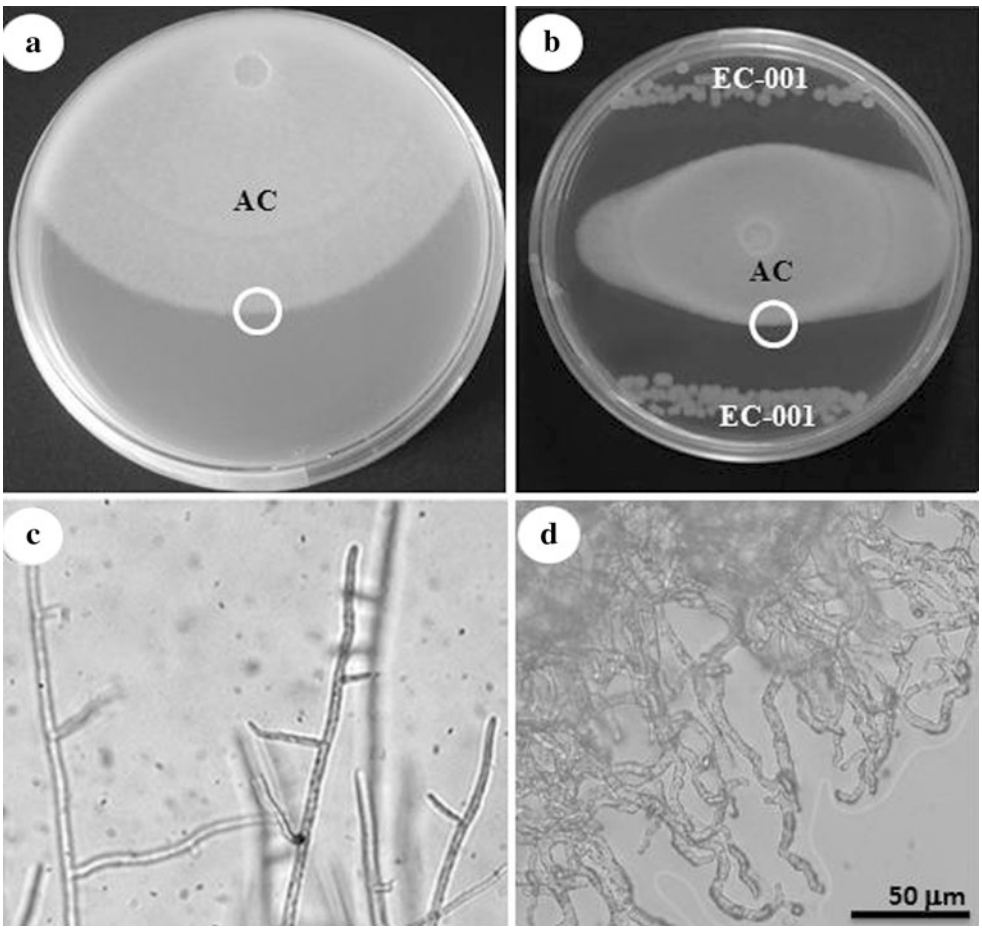


**Fig. 1** In vitro interactions between *Pseudomonas fluorescens* ECO-001 and *Aphanomyces cochlioides* in dual culture on PDA medium at day 5. **a** Normal growth of *A. cochlioides* in single culture. **b** Growth inhibition of *A. cochlioides* by *P. fluorescens* ECO-001. Characteristics round-shaped colonies of ECO-001 are seen at the periphery of the Petri dish. **c** Normal branching pattern of hyphae in the control plate (no bacteria). **d** Curly growth and excessive branching of *A. cochlioides* hyphae approaching an ECO-001 colony. The photographs **a** and **b** were taken with a digital camera (CAMDIA C-3040 zoom; Olympus Optical Co. Ltd.), micrographs in **c** and **d** were taken with the same digital camera connected to a light microscope (IX70-S1F2; Olympus). *Circled area* indicates growing hyphae. AC, *A. cochlioides* AC-5; ECO-001, *P. fluorescens*

In contrast, the plaque form of F-actin in hyphae became smaller in size at early stage of interactions (Fig. 2e, g) with ECO-001 but at the advanced stage, both plaques and filaments were almost disappeared in the hyphal cells (Fig. 2k). Interestingly, F-actin was precipitated to form characteristic structures (F-actin appeared to aggregate into heavy bars) in some of the hyphal cells before lateral branching by the induction of EC-001 (Fig. 2i). Accumulation of F-actin filaments as patches along the affected hyperbranched hyphae was also commonly observed (Fig. 2m, arrow). Although, remarkable alterations in F-actin organization were observed in all RP stained ECO-001 affected hyphal cells of AC-5, the type and degree of alterations in filament and plaque forms of actin were markedly varied among the observed cells (Fig. 2).

## DAPG induces excessive branching and disrupts F-actin organization in *A. cochlioides* hyphae

The DAPG is one of the most intensively studied biocontrol metabolites produced by fluorescence pseudomonads, yet very little is known about its mode of action. We investigated the effect of pure DAPG on growth and morphology of the AC-5 by a paper disk method on PDA medium. The DAPG caused almost similar growth inhibition (ca. 14 mm inhibitory zone at 1.0 µg/disk) and excessive branching in the hyphae approaching toward a paper disk containing DAPG as shown by living ECO-001(data not shown). To see whether excessive branching and curling of hyphae is associated with disruption of actin cytosketon in the hyphal cells, we visualized filamentous actin (F-actin) in the cells of control and DAPG treated

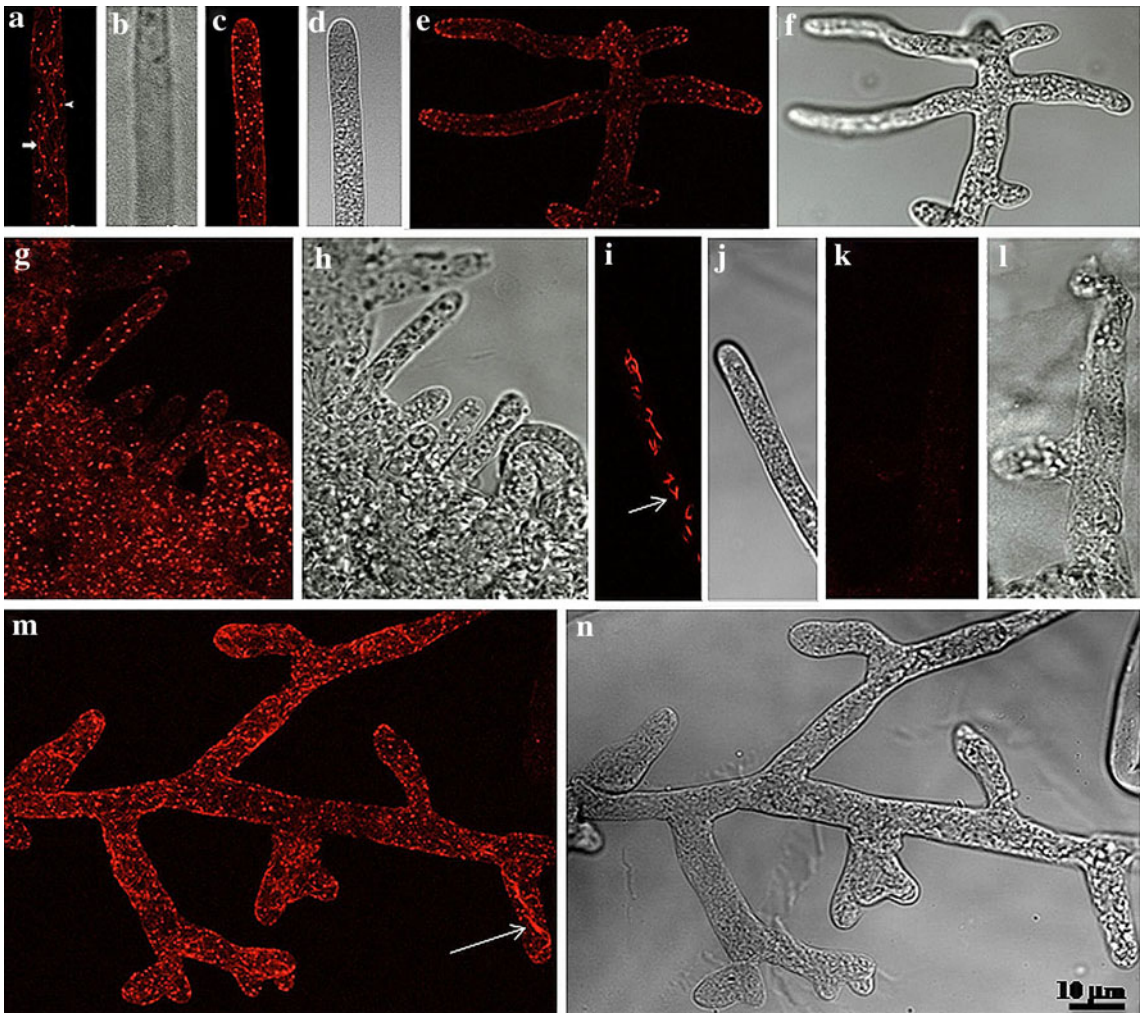


**Fig. 2** Differential interference contrast (DIC) micrographs (**b**, **d**, **f**, **h**, **j**, **l**, and **n**) and their corresponding confocal images (**a**, **c**, **e**, **g**, **i**, **k**, and **m**) showing F-actin organization in control (**a**–**d**) and excessively branched and curled hyphae of *Aphanomyces cochlioides* by *Pseudomonas fluorescens* ECO-001). **a** and **c** Normal organization of F-actin network in *A. cochlioides* hyphae (control). *Arrow and arrowhead* in **a** indicate typical filament and plaque forms of F-actin in the hyphae. **e** Disruption of F-actin in a *P. fluorescens* ECO-001-induced hyperbranched hypha. **g** Disruption of F-actin in ECO-001 affected (highly stunted growth) hyphae. **i** F-actin precipitated or aggregated into characteristic heavy bars in an affected hypha before lateral branching. **k** Complete elimination of F-actin (both filament and plaque forms) in a severely affected hypha (advanced stage of interaction) by ECO-001. **m** Characteristic disruption and accumulation of F-actin in a hyperbranched and curled hypha. *Arrow* indicates accumulation of filaments into patches along the hypha. Magnifications of all micrographs are same as indicated in *bar* (10 µm) in (**n**)

hyphae by RP and observed by confocal laser scanning microscopy (CLSM). CLSM revealed that the DAPG also disrupted both filament and plaque forms of F-actin organization in the AC-5 hyphae in a dose-dependent manner (Fig. 3). At a lower dose (0.1 µg/disk) actin filaments were slightly disrupted (Fig. 3a). At 0.5 µg/disk, both filaments and plaques were disrupted and decreased in the cells (Fig. 3e). Filaments were almost eliminated and plaques became smaller in size at 1.0 µg/disk of DAPG (Fig. 3c, g). However, at a higher dose (5 µg/disk), the over all intensity of actin staining was drastically reduced, plaques were almost eliminated (3 k) and the filaments were accumulated and aligned transversely as patches (Fig. 3i, k, arrows) in the hyphal cells. The patterns of F-actin disruption in *A. cochlioides* caused by DAPG were similar to that of living ECO-001 (Fig. 2).

## Latrunculin B induces F-actin disruption and corresponding changes in hyphal morphology

Both microfilaments and plaques of F-actin were disrupted in the hyphae approaching toward a paper disk containing latrunculin B in a dose-dependent manner as shown in Fig. 3 [3m (0.1 µg/disk), 3o (0.5 µg/disk), and 3q and 3s

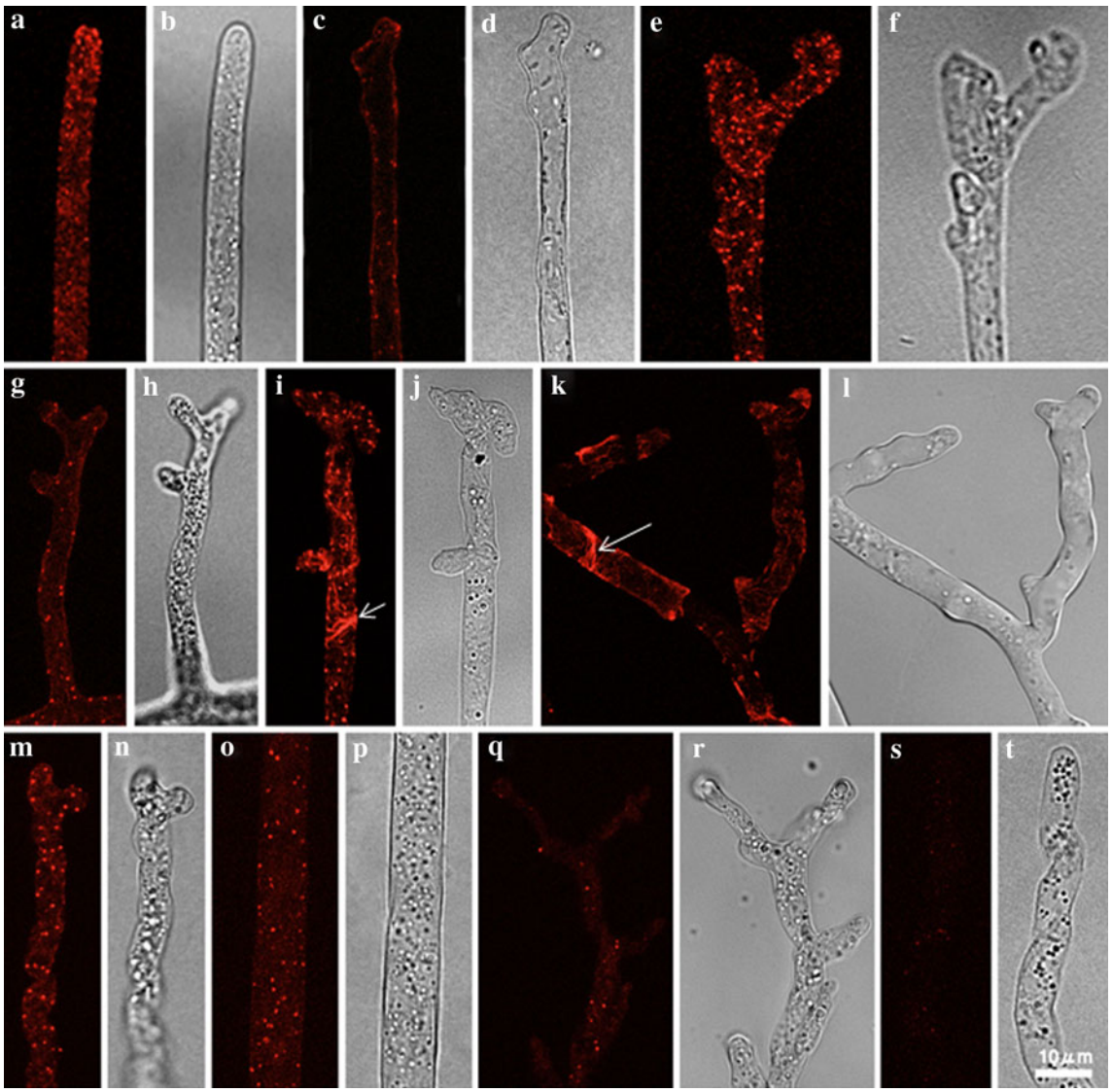


**Fig. 3** Differential interference contrast (DIC) micrographs (**b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**, **r**, and **t**) and their corresponding confocal images (**a**, **c**, **e**, **g**, **i**, **k**, **m**, **o**, **q**, and **s**) showing F-actin organization in DAPG (**a–k**) and latrunculin B (**i–n**) affected excessively branched and curled hyphae of *Aphanomyces cochlioides*. **a** Slightly disrupted F-actin organization in an unbranched and less affected hypha (0.1 μg DAPG/disk). **c**, **e** and **g** Severe disruption and elimination of both plaques and filaments of F-actin in excessively branched and curled hyphae (**c** and **g**, 1.0 μg DAPG/disk), and excessive plaques in a malformed hypha (**e**, 0.5 μg DAPG/disk). **i** and **k** Severe loss of polar growth and disruption of F-actin is seen in hyphae at 5.0 μg DAPG/disk. *Arrows* indicate accumulation of filaments transversely in the hyphae as patches. **m** Disruption of F-actin in a curled and apically branched hypha by latrunculin B at 0.1 μg/disk. **o** Smaller sized plaques and disruption of F-actin filaments by latrunculin B at 0.5 μg/disk. **q** and **s** Severe disruption of F-actin by latrunculin B in an excessively branched (**q**) and curled hypha (**s**) at 5 μg/disk. Magnifications of all micrographs are same as indicated in *white bar* (10 μm) in (**t**)

(5 μg/disk)], and thereby inhibited polar growth by inducing excessive branching in the hyphae (Fig. 3r). However, when a latrunculin B induced excessively branched *A. cochlioides* hyphae were placed on a new PDA plate, the polar growth of hyphae returned to normal (data not shown). Hyphae approaching the paper disk charged with a higher dose (5 μg/disk) of latrunculin B had severely disrupted F-actin (Fig. 3s).

## Discussion

Prokaryotic and eukaryotic interactions are ubiquitous and have important medical and environmental significance, such as probiotic bacteria in the animal intestines, *Rhizobium*-legume symbiosis in the rhizosphere, and rhizosphere microorganisms as biocontrol agents. Despite this, a paucity of data exists in the mechanisms of bacterial and fungal

encounters (Peleg et al. 2008). The present study endeavors to elucidate the mode of action of growth inhibition and alterations of morphology in a eukaryotic phytopathogen *A. cochlioides* induced by a major antimicrobial compound 2,4-diacetylphloroglucinol (DAPG) producing prokaryotic biocontrol bacterium *P. fluorescens* ECO-001 through visualizing organization of F-actin in the affected hyphal cells using rhodamine-conjugated phalloidin. This study has revealed the underlying mechanism of *A. cochlioides* growth inhibition by DAPG which is generally expressed through excessive branching and curling of the hyphae. Our investigation aided by CLSM suggested that excessive branching and curling of AC-5 occurred by DAPG was due to marked disruption of cytoskeletal F-actin network in the hyphal cells. Although antimicrobial activities of DAPG to fungi, bacteria, nematodes, protists and peronosporomycetes have been documented in many reports (Isnansetyo et al. 2003; de Souza et al. 2003), yet very little is known about its mode of action. This is the first report on disruption of cytoskeleton of a eukaryotic microorganism by the DAPG. In addition to DAPG, *P. fluorescence* is known to produce several other antimicrobial metabolites including HCN and phenazines, therefore, a further study using DAPG-deficient mutants would elucidate whether other minor antimicrobial compounds produced by ECO-001 can also affect actin cytoskeleton in concert with the DAPG. Disruption of F-actin and ultrastructures in hyphae and zoospores of *A. cochlioides* by the secondary metabolites of a biocontrol bacterium *Lysobacter* sp. SB-K88 has recently been reported (Islam 2008a).

The arrangement and location of filamentous and non-filamentous actin are under strict control within a given cell (Revenu et al. 2004). The acetylphloroglucinol such as DAPG produced from *P. fluorescens* is linked with control of plant root diseases by this biocontrol bacterium (Kamei and Isnansetyo 2003; Chin-A-Woeng et al. 1997). In this study, the synthetic DAPG showed almost similar growth inhibition and excessive branching in AC-5 hyphae as shown by ECO-001. In the affected hyphae, organization of actin filaments were disorganized or accumulated as patches (Figs. 2m, 3i, k) or changed into heavy bars (Fig. 2i) (Szymanski et al. 1999). The size of the plaques of actin became smaller than those of untreated control (Figs. 2, 3). However, at advanced stage of interactions with ECO-001 or higher doses of DAPG, both forms of actin were almost eliminated from the hyphal cells (Figs. 2k, 3c, g). Results obtained by CLSM demonstrated a time and dose-dependent disruption of F-actin cytoskeleton in the hyphae exposed to DAPG. Our findings suggest that DAPG exerts its toxic activity likely through disruption of F-actin cytoskeleton of the pathogenic Peronosporomycetes because when a DAPG-induced excessively branched hyphae were transferred to a new PDA medium, the polar growth of hyphae returned to the normal (data not shown). Furthermore, the observed progression of F-actin disruption in *A. cochlioides* hyphae by DAPG was similar to those observed in *Saprolegnia farax* hyphae by latrunculin B (Heath et al. 2000) and defective trichome morphogenesis in *Arabidopsis thaliana* by cytochalasin D (Szymanski et al. 1999). A wide range of antimicrobial activity of DAPG produced from biocontrol bacterium *P. fluorescens* has been shown in many reports (Bender and Rangaswamy 1999; de Souza et al. 2003; Bangera and Thomashow 1999; Ayyadurai et al. 2006). For example, DAPG causes different stages of disorganization in hyphal tips of *Pythium ultimum* var. *sporangiiferum*, (de Souza et al. 2003) and also induces bulb formation in fungal mycelia (Ayyadurai et al. 2006). The dramatic morphological alterations caused by DAPG in *P. ultimum* might also be linked to the disruption of F-actin in the hyphal cells.

Bacteria and unicellular eukaryotes, such as yeasts, filamentous fungi and peronosporomycetes, are found together in a myriad of environments and exhibit both synergistic and antagonistic interactions (Hogan and Kolter 2002). The cytoskeleton is considered as a target of biotic interactions (Takemoto and Hardham 2004). This study suggested that DAPG produced by ECO-001 has direct effect on growth and development of *A. cochlioides* through disruption of cytoskeletal F-actin network. Several marine natural products such as okadaic acid (Fiorentini et al. 1996) and pectenotoxin-6 (Leira et al. 2002) have also shown disruption of actin cytoskeleton in the cells. Our results suggested that DAPG exerts its toxic activity likely through disruption of F-actin cytoskeleton *A. cochlioides* hyphal cells. Isolation and identification of potent and specific actin disrupting agents from the bacterial antagonists would lead interesting molecules as agrochemicals against the phytopathogenic peronosporomycetes. The precise mechanism of DAPG-induced disruption of the actin cytoskeleton as well as downstream signal transduction pathways affected by this bacterial metabolite should therefore, be studied in order to elucidate the molecular basis of its toxicity.

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