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Phenazine-1-carboxylic acid mediated anti-oomycete activity of the endophytic *Alcaligenes* sp. EIL-2 against *Phytophthora meadii*

Amith Abraham[a], Shaji Philip[a], Manoj Kurian Jacob[a], Sunilkumar Puthenpurackel Narayanan[b], C. Kuruvilla Jacob[a], Jayachandran Kochupurackal[c,*]

[a] *Rubber Research Institute of India, Kottayam, Kerala, India*
[b] *Institute of Biophysics and Physical Biochemistry, Centre of Magnetic Resonance in Chemistry and Biomedicine (CMRCB), University of Regensburg, D-93040 Regensburg, Germany*
[c] *School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India*

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ABSTRACT

The oomycete pathogen, *Phytophthora meadii*, causes various diseases in *Hevea brasiliensis* at different stages of its life cycle. The study reports the structural characterization of the active principle from the culture filtrate of *Alcaligenes* sp. EIL-2 (GenBank ID: HQ641257) offering antagonistic activity against *P. meadii*. Gas Chromatography Mass Spectroscopy (GC–MS) analysis showed the similarity of the compound with phenazine derivatives. The specific representations of FT-IR spectrum such as 3200 cm^{-1} (—OH stretching, —NH stretching and presence of aromatic ring), 1737 cm^{-1} (carboxylic acid), 2200–2400 cm^{-1} (conjugated dienes) and 1467 cm^{-1}, and 1422 cm^{-1} (C—N bonds) were an indicative of phenazine-1-carboxylic acid (PCA). The structure of the compound was further confirmed by ^{1}H NMR/^{13}C NMR spectroscopy, DEPT experiments, and two-dimensional NMR spectral studies, including ^{1}H–^{1}H COSY and ^{1}H–^{13}C HSQC as PCA with the molecular formula of $C_{13}H_8N_2O_2$. *P. meadii* was sensitive to purified PCA extract from the endophyte and a concentration of 5 μg/ml completely inhibited the mycelia growth. PCA also showed zoosporicidal activity against *P. meadii* zoospores. This is the first study of this kind where PCA from an endophyte of *H. brasiliensis* is being confirmed to carry antagonistic activity against *P. meadii*.

Introduction

Phytophthora is one of the most destructive genera of plant pathogens in temperate and tropical regions, attacking a wide range of host plants and often causing multiple diseases on different parts of the same host. It also causes damage to *Hevea brasiliensis* trees, and thereby adversely affects the production of natural rubber. *Phytophthora meadii* causes severe leaf fall, shoot rot, bark rot and growth retardation in rubber trees. Extensive defoliation during abnormal leaf fall (ALF) disease results in considerable losses of 38–56% rubber yield in different clones (Jacob et al. 1989). Plant diseases caused by *Phytophthora* sp. are mainly controlled by synthetic fungicides but many of these synthetic fungicides are not effective against oomycete pathogens and fungicide resistance has become a problem with some fungicides (Gisi and Cohen 1996). In this context, the development of new, effective and safe strategy for controlling *Phytophthora* disease in *H. brasiliensis* is necessary.

Biological control through the use of microbial antagonists can be an environmentally friendly and effective approach to control oomycete pathogens. Numerous studies have demonstrated that metabolites including antibiotics, enzymes and volatiles produced by antagonistic bacteria play key roles in the control of various plant pathogens (Whipps 2001). Natural antibiotics have been a subject of intense research for the past 70 years and natural antibiotics, together with their semisynthetic derivatives, form the foundation of modern antimicrobial therapy. The antibiotic mediated inhibition of plant pathogens through bio-control mechanisms is well documented and summarized in several review papers (Haas and Defago 2005; Weller et al. 2007).

The various antibiotics characterized from microbial agents such as gopalamicin (Nair et al. 1994), tubercidin (Hwang et al. 1994), a manumycin-type antibiotic (Hwang et al. 1996), oligomycin A (Kim et al. 1999a), streptimidone (Kim et al. 1999b),

* Corresponding author at: Mahatma Gandhi University, School of Biosciences, P.D. Hills, Kottayam, Kerala 686560, India. Tel.: +91 9446356612.
E-mail addresses: jayansbs@gmail.com, jayansbs@yahoo.in (J. Kochupurackal).

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rhamnolipid B (Kim et al. 2000a), daunomycin (Kim et al. 2000b), fistupyron (Igarashi et al. 2000), phenylacetic acid (Hwang et al. 2001), aerugine (Lee et al. 2003), thiobutacin (Lee et al. 2004) and staurosporine (Park et al. 2006) have potent anti-fungal or anti-oomycete activity for the control of economically important plant pathogens. Anti-oomycete compounds are reported from various microbial sources. The bacterium, *Serratia marcescens*, produced a novel macrocyclic chlorinated lactone termed oocydin A that demonstrated selective toxicity towards the various *Phytophthora* spp. (Strobel et al. 1999). Antagonistic compounds from microbial endophytes, which live in the intercellular spaces of living plant tissues, are a relatively untapped source of microbial metabolites (Bacon and White 2000). In the present study, we have characterized the bioactive compound from the antagonistic bacterial endophyte of *H. brasiliensis* (Abraham et al. 2013) against *P. meadii*. Phenazine-1-carboxylic acid (PCA) was isolated and purified from the culture filtrates of the isolate. The chemical structure of the PCA was determined by GC/MS, FT-IR and NMR spectroscopic analyses. The *in vitro* anti-oomycete activity of PCA against *P. meadii* was also evaluated.

Materials and methods

The antagonistic bacterial endophyte against P. meadii

Endophytic bacterium, *Alcaligenes* sp. EIL-2 (GenBank ID: HQ641257), isolated from leaf tissues of *H. brasiliensis* was selected for the study. The isolate showed the highest percentage of growth inhibition against *P. meadii* (available from the culture collection of the pathology division, Rubber Research Institute of India) in dual culture plate and in *H. brasiliensis* (Abraham et al. 2013).

Production, extraction and purification of antagonistic compound

Alcaligenes sp. EIL-2 grown in nutrient broth for 24 h at 28 ± 2 °C was used as inoculum at 1% concentration in physiological saline for potato dextrose broth. The culture was incubated for 3 days at 28 ± 2 °C on a rotary shaker at 150 rpm and was centrifuged at 8000 rpm for 20 min to recover the cell-free supernatant. The culture filtrate was extracted with diethyl ether (1:1, v/v) in a separating funnel and the ether extract was concentrated by a vacuum evaporator (Buchi, Switzerland) at 40 °C followed by separation in a flash column chromatography of 80 mm × 30 mm column containing 100–200 μm silica gel (Merck, Germany). The solvent used was hexane:ethyl acetate (8:2, v/v) at a linear flow of 10 ml/min and fractions were collected as aliquots of 10 ml. The antagonistic activity of each fraction towards *P. meadii* was assayed using petri plates containing potato dextrose agar with filter paper discs (0.5 cm in diameter carrying 100 μg of each concentrated fraction) kept at a distance of 1.0 cm away from the rim of the plate. A disc of agar culture of *P. meadii* was removed with a 5 mm cork borer and placed in the center of the plate. The plates were incubated at 28 ± 2 °C for 5 days and the mycelia growth was observed.

Reverse phase high performance liquid chromatography (RP-HPLC) was performed for the fine purification of the compound using semi-preparative C-18 (10 mm × 250 mm) VYDAC column (VYDAC, USA). Elution was done with linear gradient of buffer A (95% water, 5% acetonitrile and 0.1% TFA) and buffer B (95% acetonitrile, 5% water and 0.08% TFA) at a flow rate of 2 ml/min with detection at 250 nm by a UV detector. The samples were eluted with a three-step linear gradient of solvent A from 100 to 0% over 40 min, 20 min hold, followed by a return to 100% A over 5 min and then held at 100% A for 10 min. The fractions of interest were collected and evaporated to dryness *in vacuo*. The antagonistic activity of the purified sample was checked by filter paper disc assay as mentioned above and further used for characterization studies.

Structure elucidation of antagonistic compound

Spectroscopic studies were carried out for elucidating the structure of the purified antagonistic compound. The absorbance maximum of the compound was determined by scanning its dilute solution in diethyl ether against pure diethyl ether on UV–vis Spectrophotometer (Shimadzu, Japan). The purified compound was analyzed on GC–MS (Perkin Elmer Clarus 600) and Electron Impact (EI) mass spectra of the parent ions were recorded. The spectrum of the compound was compared with the mass spectra from the National Institute of Standards and Technology (NIST) Library. The Fourier transform infrared (FT-IR) spectrum was recorded in an FT-IR spectrophotometer with attenuated total reflectance (ATR) facility (Bruker, Germany). The spectrum was measured from 4000 to 600 cm^{-1}. A 71.4 mM (8 mg/ml) solution of the purified compound in deuterated chloroform ($CDCl_3$) was used for NMR experiments. The data were obtained at 30 °C using a Bruker 400 MHz spectrometer (Bruker Biospin, Switzerland) with a 5 mm probe. The spectra were referenced using tetramethylsilane (TMS) as internal standard. The one-dimensional (1D) ^1H, ^{13}C, DEPT 45, DEPT 90 and DEPT 135, and the two-dimensional (2D) $^1H/^1H$ COSY and $^1H/^{13}C$ HSQC spectra were used for the complete assignment.

Detection of in vitro antimicrobial activity

The antimicrobial activity of the purified compound produced by antagonist *Alcaligenes* sp. EIL-2 was determined by poisoned agar technique as follows. Concentrations of 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 3 μg/ml, 4 μg/ml, 5 μg/ml, 6 μg/ml, 7 μg/ml and 8 μg/ml of purified compound dissolved in diethyl ether were incorporated in PDA plates. Plates without the addition of compound other than solvent served as controls. Agar cultures of *P. meadii* were taken with a 5 mm cork borer and placed in the center of the plate. Plates were incubated at room temperature for 5 days. Radial growth of *P. meadii* was measured and growth inhibition of the isolate was calculated by dividing colony diameter in amended plates by that in the non-amended control plates and expressed in percentage.

The activity of purified compound to lyze zoospores of *P. meadii* was also evaluated. For sporulation, white oat broth was inoculated with a 2-day-old fresh mycelium and incubated in the dark for 2 days. The mycelia were then washed in sterile water and exposed to light for 24 h to promote sporangial growth. The motile zoospores were liberated from sporangia by giving a cold shock (1 h at 4 °C), followed by a 1 h equilibration at room temperature. The zoospores were separated from the solution by filtering through 2 layers of cheesecloth and the suspension calibrated using a haemocytometer. An aliquot (0.5 ml) of zoospore suspension (1×10^4 zoospores/ml) was dispensed in the microtube containing 0.5 ml of sterile potato dextrose broth amended with purified compound (5 μg/ml) as test solution. Tubes without the addition of purified compound other than potato dextrose broth and zoospore suspension were served as control. There were three replicate for each test and control solution. After incubation for 2 h at 28 ± 2 °C, an aliquot (20 μl) of the zoospore suspension from test and control solution was added to a glass slide and the behaviour of the zoospores was observed microscopically under a light microscope. Test and control zoospore suspensions were also inoculated onto PDA plates and incubated at 28 ± 2 °C for 5 days.

Results

Antagonistic compound from bacterial endophyte

Alcaligenes sp., the antagonistic bacterial endophyte EIL-2 (GenBank ID: HQ641257) selected among 252 endophytic isolates of

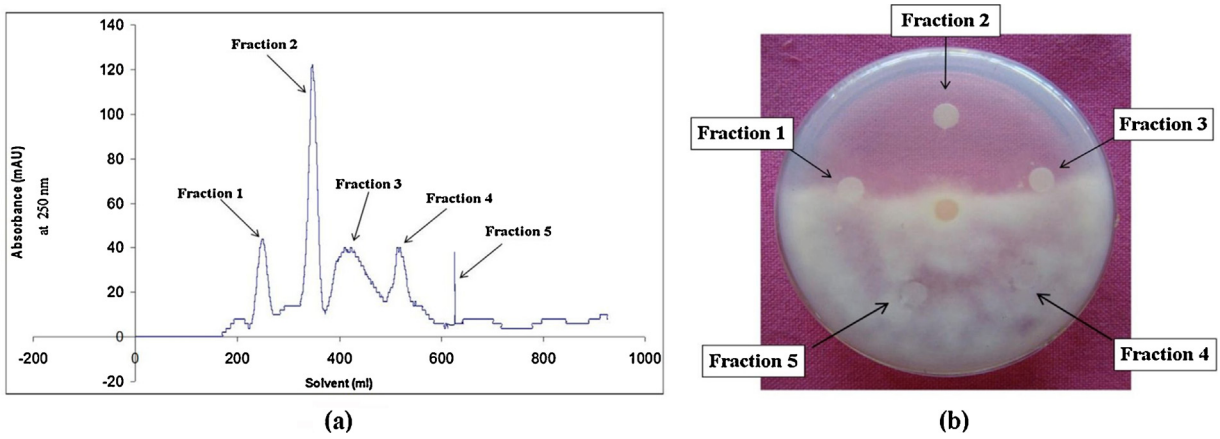

Fig. 1. (a) Flash column chromatogram of diethyl ether extract of broth supernatant from an antagonistic bacterial isolate *Alcaligenes* sp. EIL-2. Fractions separated in silica column marked in chromatogram. (b) Antagonistic activity of compounds separated by flash column chromatography. Each fraction incorporated in filter paper discs in PDA plates. Fraction 2 showed growth inhibition of *P. meadii*.

H. brasiliensis (Abraham et al. 2013) was cultured in potato dextrose medium. The supernatant after extraction with diethyl ether was fractionated by flash column chromatography into five fractions (Fig. 1a). 100 μg of each concentrated fraction was subjected to *in vitro* antagonistic activity and only one fraction (fraction 2) showed inhibition of *P. meadii* growth (Fig. 1b). Fraction 2 was further purified by reverse phase HPLC and the major compound obtained at a retention time of 36.20 min was collected. The activity of the purified sample was confirmed by its inhibitory action against *P. meadii*.

Structure elucidation of antagonistic compound

UV–vis spectrum analysis of the purified compound showed maximum absorbance at 254 and 360 nm. The GC–MS spectrum showed a weak peak at $m/z \sim 224$ (molecular ion peak), a strong peak at m/z 180 and a series of less abundant fragment peaks (Fig. S1). The GC–MS and NIST Library search proposed the antagonistic compound as carboxylic acid derivative of phenazine with a molecular weight of 224 Da. The IR spectrum showed characteristic absorption bands at 3200 cm^{-1}, 2664 cm^{-1}, 1737 cm^{-1}, 1597 cm^{-1}, 1522 cm^{-1}, 1467 cm^{-1} and 1422 cm^{-1} (Fig. S2).

The structure of the antagonistic compound was further confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy, DEPT experiments, and various two-dimensional NMR spectral studies, including ^{1}H–^{1}H COSY and ^{1}H–^{13}C HSQC. In the ^{1}H NMR spectrum 7 peaks in 7.30–9.00 ppm region were assigned to 7 aromatic protons and peak at 15 ppm was assigned to the carboxylic acid proton (Fig. S3). There were 13 well-resolved signals in the ^{13}C NMR spectrum of the compound (Fig. S4). The 12 peaks between 124.97 and 144.12 ppm indicated the presence of 12 aromatic carbons in the structure. The peak at 165.93 ppm indicated the presence of a carbonyl carbon. In the Distortionless Enhancement by Polarization Transfer (DEPT), the DEPT 45, DEPT 90 and DEPT 135 spectra of the antagonistic compound showed that the 7 peaks are in phase (Fig. S5). The DEPT 45 indicated the seven protonated carbon in the structure of antagonistic compound. DEPT 90 showed seven singly protonated (CH) carbons in the structure. The spectrum of DEPT 135 eliminated the possibility of any CH_2 group in the structure.

The COSY spectrum of the antagonistic compound showed that the H(3) proton is directly coupled to H(2) and H(4) protons. The H(9) proton was directly coupled with H(8) and H(10) protons. The H(10) proton was directly coupled with H(9) and H(11) protons (Fig. S6). The corresponding correlations were labelled in Fig. 2a. The $^{1}H/^{13}C$ Heteronuclear Single Quantum Coherence (HSQC) spectrum gave correlation between the carbon and its attached protons (Fig. S7). The protons attached to each carbon atoms in the structure, H(2)/C(2), H(3)/C(3), H(4)/C(4), H(8)/C(8), H(9)/C(9), H(10)/(10) and H(11)/C(11), are labelled in Fig. 2b. The count of protons and carbons from ^{1}H to ^{13}C NMR spectra revealed the presence of 8 protons and 13 carbons, which supported the molecular formula of phenazine-1-carboxylic acid ($C_{13}H_8N_2O_2$) (Table 1).

In vitro antimicrobial activity

Purified phenazine-1-carboxylic acid showed antagonistic activity against *P. meadii*. The percentage inhibition of *P. meadii* mycelia growth increased with increasing concentration of phenazine-1-carboxylic acid. The minimum inhibitory concentration of phenazine-1-carboxylic acid against *P. meadii* was optimized as 5 μg/ml (Table 2, Fig. 3). To evaluate zoospore-lytic activity, zoospore suspensions of *P. meadii* with and without phenazine-1-carboxylic acid were prepared and examined under a light microscope. At the concentration of 5 μg/ml of phenazine-1-carboxylic, zoospore behaviour was distinctly changed, compared with the untreated control. The zoospore shape was completely altered by phenazine-1-carboxylic acid and lysis of entire zoospore populations occurred. In control solution, zoospore shape was intact and emergence of new germ tubes was observed (Fig. 4). Zoospores failed to germinate when suspension amended with Phenazine-1 carboxylic acid was incubated on PDA plates. In control plates, zoospores were germinated and the mycelia fully covered the PDA plates after incubation.

Table 1
Chemical shift values in ^{1}H and ^{13}C NMR spectrum of antagonistic compound from the bacterial isolate *Alcaligenes* sp. EIL-2.

Atom position	$^{\delta}H$ (ppm)	$^{\delta}C$ (ppm)
1	–	124.9
2	8.54	135.1
3	8.06	130.2
4	8.99	137.4
5	–	144.1
6	–	143.4
7	–	140.1
8	8.36	130.1
9	8.00	131.7
10	8.02	133.2
11	8.29	127.9
12	–	139.8
13	15.61 (carboxylic proton)	165.9 (carboxylic carbon)

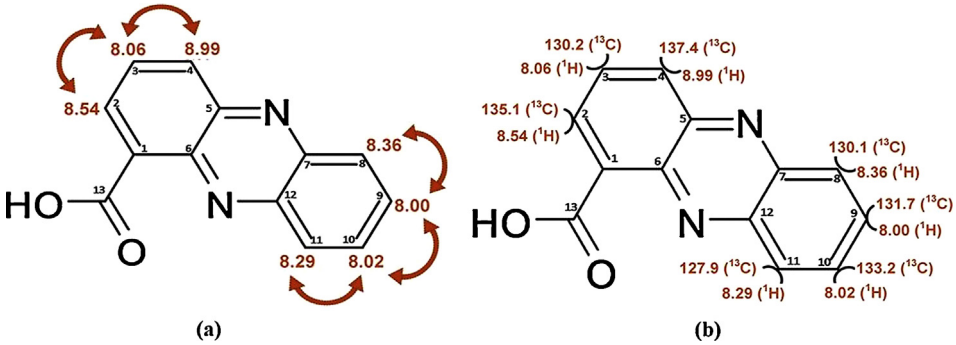

Fig. 2. (a) Directly coupled protons predicted from the NMR–COSY spectrum of antagonistic compound. (b) Correlation between the carbons and its attached protons predicted from the NMR–HSQC spectrum of antagonistic compound.

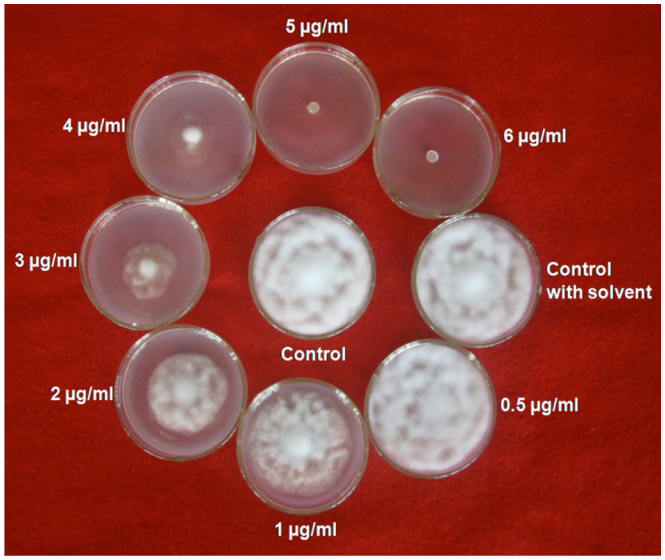

Fig. 3. Growth inhibition of *P. meadii* on PDA plates incorporated with different concentrations of phenazine-1-carboxylic acid.

Table 2
Percent growth inhibition of *P. meadii* in different concentrations of phenazine-1-carboxylic acid.

Concentrations of phenazine-1-carboxylic acid (µg/ml)	Percent inhibition of *P. meadii* mycelia growth over control
0.5	00.0 ± 0.00
1	12.6 ± 0.43
2	33.7 ± 0.57
3	54.8 ± 0.42
4	77.4 ± 0.21
5	100 ± 0.00
6	100 ± 0.00
7	100 ± 0.00
8	100 ± 0.00

Values are the means ± standard error of three replicates.

Discussion

Diseases caused by the oomycete, *P. meadii*, are one of the greatest threats to *H. brasiliensis* cultivation. Increasing concerns over environmental problems due to spraying of fungicides and emergence of resistant *Phytophthora* supported the need for alternative pathogen control methods. Investigation on microbial metabolites is gaining greater momentum in the agrochemical industry as a source for the development of new products. Fungicides of microbial origin have been demonstrated to be not only specifically effective on the target organisms but also inherently biodegradable (Yamaguchi 1996). Endophytic bacteria have recently been a focus of interest as bio-control agents and a source of bioactive metabolites (Strobel et al. 2004). Such bacteria are indigenous to most plant species, colonizing the tissue locally or systemically (Hallman et al. 1997). In this study, *Alcaligenes* sp. EIL-2 was isolated from *H. brasiliensis* and selected as the potent endophyte offering maximum inhibition to the growth of *P. meadii*. The antagonistic compound from the culture supernatant of *Alcaligenes* sp. EIL-2 was efficiently extracted and purified through various downstream processes.

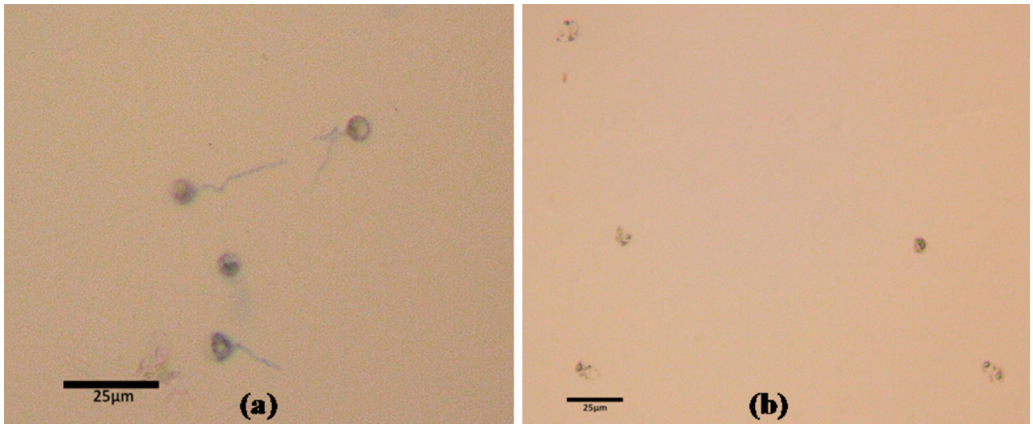

Fig. 4. Effect of phenazine-1-carboxylic acid on zoospores of *P. meadii*. (a) Zoospore germination in control. (b) Lysis of zoospores during interaction with phenazine-1-carboxylic acid.

The GC–MS and NIST Library search proposed the antagonistic compound as carboxylic acid derivative of phenazine with a molecular weight of 224 Da. The peak at *m*/*z* 180 of MS indicated the presence of a phenazine moiety in the molecule. The free acid showed only a weak molecular ion, with the bulk of the ion current being carried by the base peak fragment ion (*m*/*z*, 180; M-CO2). The peak at 180 represented loss of the carboxyl group. The base peak at 180 reflected the tendency of the *m*/*z* 179 moiety to capture hydrogen to complete the phenazine nucleus. (Fig. S1). The specific representations of FT-IR spectrum in the range 3200 stand for —OH stretching and —NH stretching besides the representation of aromatic ring. The presence of carboxylic acid was confirmed by the representation at 1737 cm^{-1}. The range 2200–2400 cm^{-1} stands for conjugated dienes. The bands at 1467 cm^{-1} and 1422 cm^{-1} were indicative of C—N bonds (Fig. S2). Phenazine-1-carboxylic acid (PCA) has fused aromatic ring with conjugated dienes and two C—N bonds along with carboxylic side chain. GC–MS and FT-IR studies lead us to propose a possible chemical structure of the compound such as phenazine-1-carboxylic acid. The counts of proton and carbon from ^{1}H to ^{13}C NMR spectra revealed the presence of 8 protons and 13 carbons (Table 1), which supported the molecular formula of phenazine-1-carboxylic acid ($C_{13}H_8N_2O_2$).

Phenazines have been recognized for their antibiotic properties for over 150 years (Mavrodi et al. 2006; Rusman et al. 2013). Phenazines are inhibitory to a wide range of plant pathogenic fungi and have a well-characterized mechanism of bacterial plant disease control (Chin-A-Woeng et al. 2003). The phenazines produced by *Pseudomonas fluorescens* strain 30–84 are responsible for inhibition of *Gaeumannomyces graminis tritici* (Ggt), the take-all fungal pathogen of wheat. Both PCA and PCN were involved in the control of *Pythium myriotylum*, the causative agent of root rot of coco yam (Tambong and Hofte 2001). Antibiotic activity of phenazines was also shown against *Rhizoctonia solani*, *Gibberella avenacea*, *Alternaria* spp. and *Drechslera graminea* (Gurusiddaiah et al. 1986). Phenazine-1-carboxylic acid (PCA) exhibited a broad-spectrum antimicrobial activity. *Micrococcus luteus*, *Staphylococcus aureus*, *Klebsiella planticola* and *Candida albicans* were the microorganisms that showed greater susceptibility and the estimated MIC value was 9.3 μg/ml. The genera *Cochliobolus*, *Cortium*, *Gaeumannomyces*, *Rhizoctonia*, and *Trametes* were belonged in most PCA sensitive fungi (1–10 μg/ml) (Gurusiddaiah et al. 1986). However, no phenazine derivative has so far been reported as the active principle offering antibiosis against *P. meadii*. The present study optimized the minimum inhibitory concentration of phenazine-1-carboxylic acid against *P. meadii* as 5 μg/ml (Table 2 and Fig. 3). Zoospores have been accepted as the principal dispersive agents of oomycetes. Therefore, zoospore movement and encystment are important factors in the pathogenicity of oomycete pathogens such as *Phytophthora* spp. ALF disease caused by *P. meadii* starts from spores and zoospores cause new infections and spreading of the disease in favorable conditions. So the sporicidal activity of the phenazine-1-carboxylic acid against *P. meadii* zoospores may be used for the reduction of zoospore inocula and hence for the reduction of new infections.

Conclusion

We report for the first time that phenazine-1-carboxylicacid mediated antagonistic activity of *Alcaligenes* sp. EIL-2 against *P. meadii*, the ALF causing pathogen of *H. brasiliensis*. The present studies indicated that the oomycete *P. meadii* was highly sensitive to phenazine-1-carboxylic acid and even low concentrations of phenazine-1-carboxylic acid could effectively control the growth of *P. meadii* mycelia and zoospores. The factors affecting the production of Phenzine-1-carboxylic acid by *Alcaligenes* sp. EIL-2 and the mechanism by which the compound inhibits the growth of *P. meadii* are other interesting areas of future research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2014.06.002.

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