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Full Length Article

Detection of Phenazines from UPMP3 Strain of *Pseudomonas aeruginosa* and its Antagonistic Effects against *Ganoderma boninense*

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Abstract

To identify the phenazine antibiotics from the selected UPMP3 strain of *Pseudomonas aeruginosa* and assessment of its antagonistic activity of *Ganoderma boninense*, three antibiotics phenazine (PHZ), phenazine-l-carboxylic acid (PCA) and pyocyanin (PYO) were extracted from the bacterial fermented broth using benzene and chloroform and detected through High-performance liquid chromatography (HPLC). The effects of three antibiotics on suppressing mycelial growth of *G. boninense* were assayed. The maximum amount of PHZ, PCA and PYO were recorded as 1.36, 9.62 and 15.48 µg/mL, respectively and showed *in vitro* antagonistic activity against *G. boninense*. Phenazine was found to be more effective than PCA and PYO to inhibit the mycelial growth of *G. boninense*. It was 100% for PHZ at 1.0 mg/mL, while 78.61% for PCA and 91.87% for PYO after 7 days of incubation. Phenazine, PCA and PYO were identified as first time from oil palm rhizospheric UPMP3 strain of *P. aeruginosa*. The results indicated that the identified PHZ, PCA and PYO compound had good antifungal activity against *G. boninense*, however the PHZ can be developed as an ideal biofungicide for the control basal stem rot disease of oil palm and helps to overcome the concerns about the residual effects of synthetic fungicides. © 2016 Friends Science Publishers

Keywords: Antagonistic effect; *Pseudomonas aeruginosa; Ganoderma boninense*; Phenazine; Phenazine-l-carboxylic acid

Introduction

Phenazines are chemically heterogeneous group of small organic molecules of microbial origin. It is produced at low concentrations and deleterious to the growth or metabolic activities of other microorganisms (Kavitha *et al.,* 2005). Antibiotics can contribute to microbial competitiveness and the suppression of plant pathogenic microorganism. Different species of *Pseudomonas* spp. are able to produce phenazine and phenazine derivatives, pyocyanin, phenazine-l-carboxamide, phenazine-l-carboxylic acid, 2, 4 DAPG and other antibiotics when grown aerobically in phosphate-poor medium (Watson *et al.,* 2005). *P. aeruginosa* secrets numerous proteins and secondary metabolites, many of which have biological effects against pathogens which cause various diseases (Gerene *et al*., 2003). Phenazines have been known as one of the most important metabolic products produced by *P. aeruginosa* (Blankenfeldt *et al*., 2004). These compounds have been studied intensively because of their broad spectrum antibiotic properties and roles in virulence, against different pathogenic bacterial and fungal species (Pierson and Pierson, 2010). Several strains of *Pseudomonas* produce phenazine-1-carboxylic acid, is a strong and broad range antifungal metabolite. Pyocyanin has antibiotic activity against a wide variety of microorganisms (Liang *et al.,* 2008).

Ganoderma boninense has been reported as pathogenic fungus responsible for basal stem rot disease of oil palm (Sariah *et al.,* 1994). Oil palm growers in Malaysia are facing major problem due to *G. boninense* for the commercial cultivation. In spite of the detection of phenazines from the oil palm rhizospheric strain of *P. aeruginosa* have not been studied; few attempts were made to investigate the antagonistic activity of *P. aeruginosa* against plant pathogenic fungi. Hence, the present study was undertaken to identify, phenazine, phenazine-1-carboxylic acid and pyocyanin from the oil palm rhozospheric strain of *P. aeruginosa* UPMP3 and assessment of its biological activity against *G. boninense*.

Materials and Methods

Bacterial Strain and Cultivation

UPMP3 strain of *Pseudomonas aeruginosa* was previously isolated from rhizospheric region of oil palm garden at

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United Plantations, Perak in Malaysia and its NCBI accession no. GQ183951 - *P. aeruginosa* strain UPMP3 (Zaiton *et al*., 2006). This bacterial strain was collected from Plant Protection Department, Universiti Putra Malaysia. *P. aeruginosa* UPMP3 was cultured on King's B agar medium for 24 h incubated at 30 ± 2°C to continue the extraction and identification of phenazine antibiotics.

Extraction and Purification of Phenazine, Pyocyanin and Phenazine-l-carboxylic Acid

Phenazine, pyocyanin and phenazine-l-carboxylic acid were extracted by the modifications of the method described by Chang and Blackwood (1969) and Linda *et al.* (1990). They were separated into their respective fractions after first extraction by acidifying the culture with an equal volume of benzene and chloroform. To extract PHZ, PYO and PCA the bacterium was grown on King's B broth (KMB) at 30°C on an incubator shaker at 150 rpm for 2 days. The cells were collected by centrifugation at 3500 rpm for 7 min, 8000 rpm for 10 min and 6500 rpm for 5 min for PHZ, PYO and PCA, respectively. After then the pellets of each was suspended in 5 mL of Pigment Production Medium (PPM) broth and re-incubated on the incubator shaker at 30°C for 4 days at 150 rpm. The cultures were adjusted to pH 2.0 with concentrated HCl and extracted with an equal volume of benzene and chloroform. The benzene and chloroform layer was subjected to evaporation in rotary evaporator at 40°C. After evaporation the residues were resuspended in 1 mL of absolute methanol and filtrate by 0.22 µm membrane filter for each. After filtration the phenazine and pyocyanin extract was kept at -20°C and used for identification through HPLC. Accordingly, Phenazine-1-carboxylic acid was extracted with an equal volume of benzene. Then the benzene phase was pooled, dehydrated with Na_2SO_4 and evaporated to dryness using rotary evaporator. PCA antibiotic present in the residue were dissolved by gentle agitation for 8 to 12 h in 5 mL of aqueous 5% (w/v) $NaHCO_3$ by incubator shaker 50 rpm. The bicarbonate solution was extracted with an equal volume of benzene, acidified to pH 2.0 with concentrated HCl. Then PCA was recovered in benzene layer and dried with evaporation. After evaporation the residues were resuspended in 1 mL of absolute methanol. The PCA was applied in benzene to a Sep-Pak C18 cartridge and eluted with chloroform. The eluted PCA extract was kept at -20°C and used for identification through HPLC.

Preparation of Standard Curve

Pure phenazine (Sigma-Aldrich, USA), PCA (Nacalai Tesque, Japan), and pyocyanin (Sigma-Aldrich, USA) were used as standard in this experiment. A series of dilutions (0, 5, 10, 20 and 50 µg/mL) of each antibiotic and mixture standard were prepared and chromatographed separately to determine the retention time for phenazine, PCA and pyocyanin. The signal of the compounds was monitored at 250, 262 and 280 nm for identification of better peak resolution of each antibiotic. The retention times of the solutes were determined from three different determinations. Peak identification was based on retention time of the standard, and detector response was measured in terms of peak area. Standard curve of each antibiotic was drawn using concentrations of standard versus responses in peak area, respectively and amount was determined in micrograms (µg). The stock solutions were stored at 4°C until analysis in HPLC.

Identification of PHZ, PCA and PYO

The HPLC system was developed using a separation module (Alliance, Waters e2695) and a Multi λ Photo diode array detector (Waters e2998). To determine the presence and concentration of phenazine, PCA and pyocyanin antibiotics, samples were analyzed according to a modification of the methods described by Watson *et al.* (2005). A 150 x 4.6 mm Ultracarb 5 µm ODS (30) column and a 30 X 4.6 mm Ultracarb 5 µm ODS (30) guard column were used. Throughout this study, water-trifluoroacetic acid (100:0.04, v/v) i.e. solvent A and acetonitrile-water-trifluoroacetic acid (90:10:0.04, v/v/v) i.e. solvent B were used as the mobile phase. This mobile phase was filtered through Whatman Nylon membrane filter (0.2 µm) using a filtration syringe system. Elution was as follows: Solvent A was maintained for 15 min and then changed to 90% A and 10% B. This mixture was used for 10 min. A linear gradient to 70% A and 30% B in 15 min was then applied. These conditions were maintained for 5 min (until 45 min from onset). Finally, solvent composition was changed to 64% A and 36% B and maintained until the end (65 min from the starting time). Running was monitored at 250, 262 and 280 nm with a bandwidth of 4 nm in all cases. Sample solution was injected along with mixture of standard solution. Antibiotics were identified and quantified irrespective of peak area of samples and calculation was carried out irrespective of antibiotic standard curve by comparison of retention time and UV absorbance at an optimum point of the standard. All concentrations were reported on per unit weight basis and each sample was analysed in triplicate. Results of each antibiotic PHZ, PCA and PYO was analysed and quantified as µg/mL.

Antagonistic Effect of PHZ, PCA and PYO against *G. boninense*

Pure culture of *G. boninense* was obtained from Malaysian Palm Oil Board (MPOB). It was maintained and stored at the laboratory of plant pathology, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia and cultured on Malt Extract Agar (MEA, Merck) medium. The antagonistic effects of PHZ, PCA and PYO against *G. boninense* were assayed by the agar plate dilution method. Six different concentrations of each compound i.e. 0.1 mg/mL, 0.3 mg/mL, 0.5 mg /mL, 0.7 mg/mL, 0.9

mg/mL and 1.0 mg/mL were used. MEA medium was prepared and allowed to cool at 50˚C. Then 20 mL of the medium was poured on the sterilized petriplate for each compound. The different concentrations of compound were supplemented with the medium in different petriplates and mixed with gentle agitation. After solidifying the medium, 7 days old culture of *G. boninense* was cut into 5 mm cork borer and placed at the centre of the medium. MEA plate with *G. boninense* and without compound served as a control. The plates were incubated at 28±2˚C in dark growth chamber for 7 days. The mycelial growth and inhibition zone was recorded after incubation. Each of these dilution agar plates was tested against *G. boninense* in order to establish the minimum inhibitory concentrations of PHZ, PCA and PYO.

Statistical Analysis

All experiments were performed as completely randomized design (CRD). Three replications were considered for each treatment and repeated twice. Data were analysed using statistical analysis system and means were statistically compared using LSD test. The significance level was set up at $p \leq 0.05$.

Results

Detection of PHZ, PCA and PYO in Culture Conditions

The presence of PHZ, PCA and PYO were detected in respect to different color production during extraction and the incubation period of bacterial growth. It was observed that the color of the culture medium was changed to blue green or green during the incubation period, which indicated the production of organic compounds by this strain (Fig. 1). Therefore, the extraction phases of the different antibiotics produced different distinct colors. As for PHZ and PCA produced bright orange and lemon yellow color respectively in benzene fraction. While, PYO produced the blue color in chloroform fraction (Fig. 2).

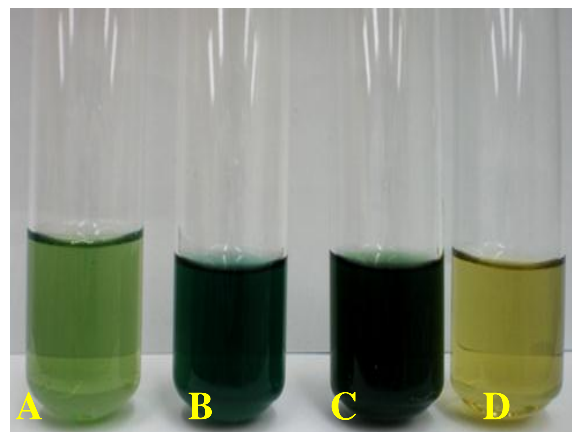

Fig. 1: Different colour productions in culture medium from UPMP3 strain of *P. aeruginosa* after 4 days incubation period. A= light green, B=blue green, C= deep green, D= control

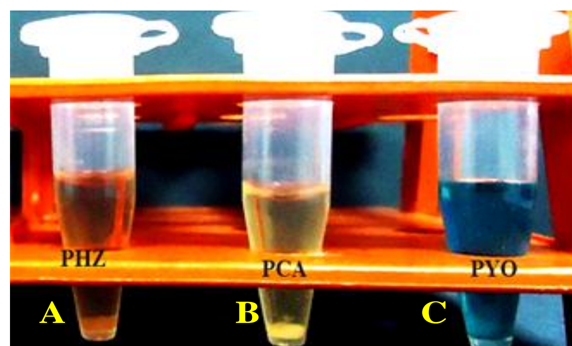

Fig. 2: Different colour indicates different antibiotics in benzene and chloroform fraction during extraction phase. A= Extract of phenazine (bright orange color), B= Extract of PCA (lemon yellow colour) and C= Extract of pyocyanin (blue color)

Purification and Identification of PHZ, PCA and PYO

Phenazine, PCA and PYO from cultures of *P. aeruginosa* UPMP3 were extracted employing well known HPLC methods. Furthermore, HPLC analysis was carried out with the mixture of standards (Fig. 3), along with individual standard at 250 nm, 262nm and 280 nm wavelength which showed no other peaks than those corresponding to the expected peaks.

Standards were separately run on HPLC to obtain the spectra of the antibiotics under different running conditions. The highest sensitivity was obtained choosing the wavelength at maximum absorbance for each antibiotic. The absorptions were recorded at 250 nm for PHZ and PCA, on the other hand 262 nm for PYO. In contrast, for PHZ and PCA, a poor absorbance was observed at 262 and 280 nm. While, in the case of PYO, poor absorbance was recorded at 250 and 280 nm. These wavelengths were used to monitor the chromatography and peak areas were used in the calibration curves of the respective antibiotics. The peaks of standards PHZ, PCA and PYO, were completely separated with retention times of 41.590, 39.740 and 34.863 min at 250 nm (Fig. 3), 262 nm and 280 nm wavelengths, respectively.

The HPLC responses (peak areas) of PHZ, PCA and PYO were observed to have highly positive correlations with the respective standard concentrations. The retention times of PHZ, PCA and PYO were detected from the standard as well as from samples at 41.896, 39.764 at 250 and 34.895 min at 262 nm respectively (Figs. 4, 5 and 6).

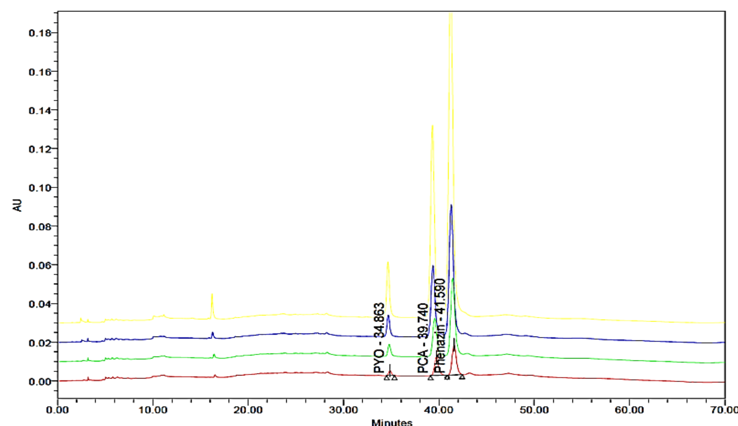

Fig. 3: Chromatographic separation of phenazine (PHZ), phenazine -1- carboxylic acid (PCA) and pyocyanin (PYO) from standard mixture at 1000 µg/mL concentration with various retention times at 250 nm wave length

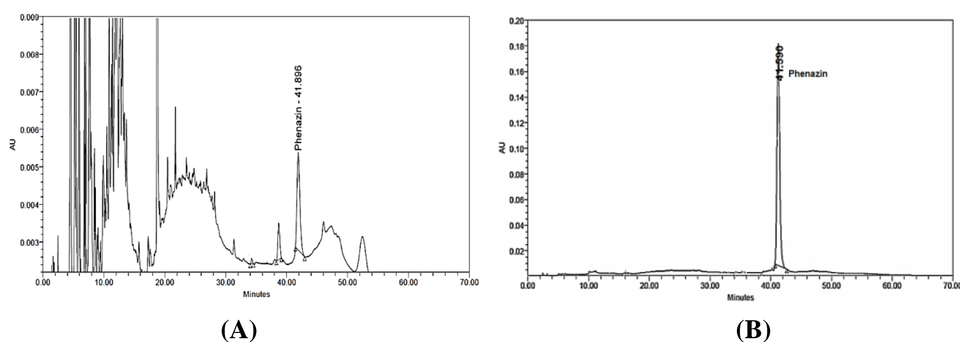

Fig. 4: Chromatographs of phenazine peaks with retention time for the sample (A) and standard (B) at 250 nm

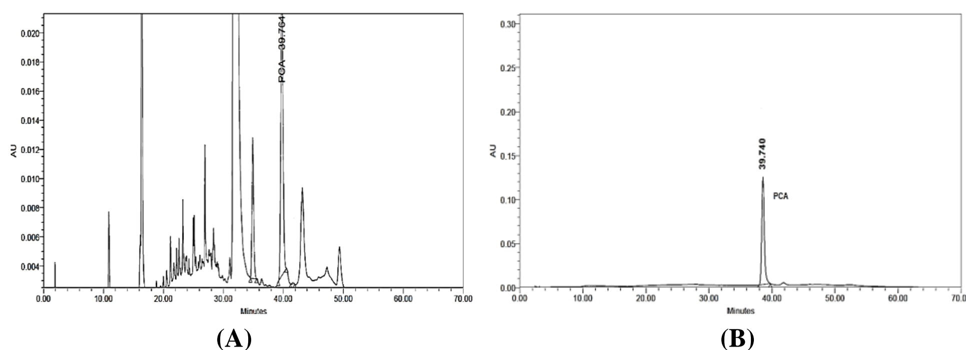

Fig. 5: Chromatographs of PCA peaks with retention time for the sample (A) and standard (B) at 250 nm

These three antibiotics were quantified from the respective standard curve. The maximum amount of PHZ, PCA and PYO were calculated as 1.36 µg/mL, 9.62 µg/mL and 15.48 µg/mL, respectively (Table 1).

Antagonistic Effect of PHZ, PCA and PYO against *G. boninense*

The antifungal activity of the three antibiotics PHZ, PCA and PYO against *Ganoderma* were tested by the agar plate dilution methods. The six different concentrations, 0.1 mg/mL, 0.3 mg/mL, 0.5 mg /mL, 0.7 mg/mL, 0.9 mg/mL and 1.0 mg/mL of each antibiotic were supplemented in the medium to evaluate their effectiveness on the mycelial growth of *G. boninense* as compare to control (Fig. 7A, B and C).

The result revealed that among the different concentrations 1.0 mg/mL was found to be the best concentration to inhibit the mycelial growth of *G. boninense* for all of the tested antibiotics. The inhibition percentage was increased gradually with the increase of antibiotic concentration. The maximum percent (100%) growth inhibition was recorded in PHZ at 1.0 mg/mL, and followed by PYO (91.87%), PCA (78.61%), respectively (Fig. 8).

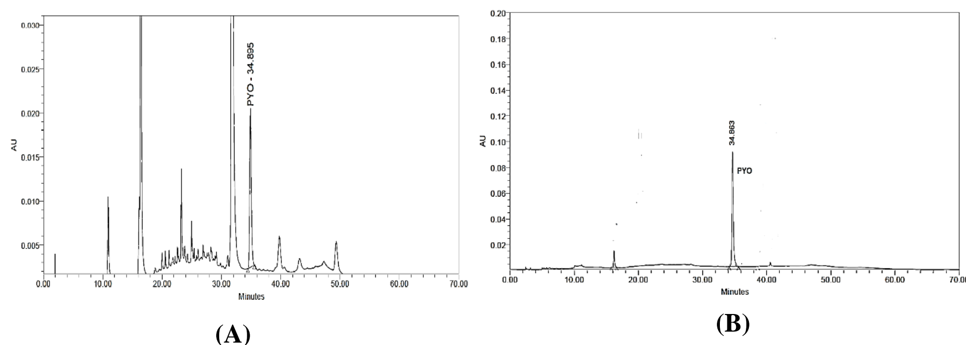

Fig. 6: Chromatographs of pyocyanin peaks with retention time for the sample (A) standard (B) at 262 nm

Table 1: Detection of PHZ, PCA, and PYO from UPMP3 strain of *P. aeruginosa*

Antibiotics		HPLC parameters				
		Retention time	Wavelength (nm) 250	262	280	Amount (µg/mL)
PHZ	Peaks	41.896	250	-	-	1.36
Standard PHZ		41.590	250	-	-	1000
PCA	Peaks	39.764	250	-	-	9.62
Standard PCA		39.740	250	-	-	1000
PYO	Peaks	34.895	-	262	-	15.48
Standard PYO		34.863	-	262	-	1000

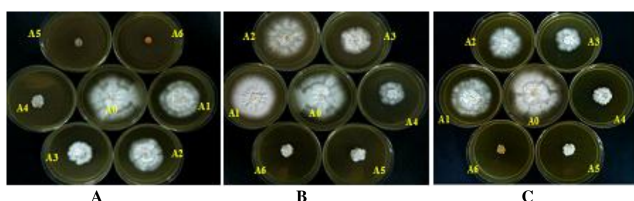

Fig. 7: Effect of mycelial growth inhibition of phenazine (A), PCA (B) and pyocyanin (C) at different concentrations against *G. boninense* after 7 days of incubation. A0 = Control. A1 = 0.1 mg/mL, A2 = 0.3 mg/mL, A3 = 0.5 mg/mL, A4 = 0.7 mg/mL, A5 = 0.9 mg/mL and A6 = 1.0 mg/mL

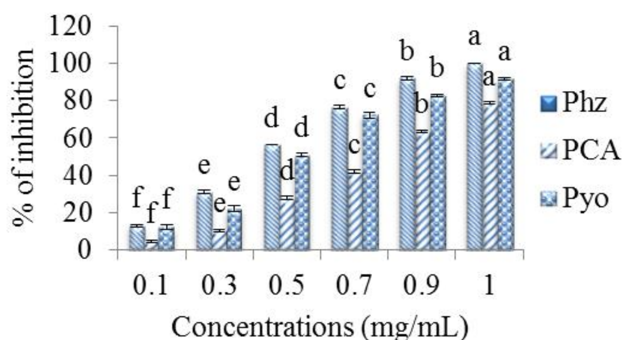

Fig. 8: Percent growth inhibition of *G. boninense* at different concentrations of phenazine, PCA and pyocyanin after 7 days of treatment. Values followed by the same letter are not significantly different according to LSD test at P≤0.05 level. Each value is the mean of 3 replications. Vertical bars represent standard error

From the above result it was observed that all the three antibiotics PHZ, PCA and PYO have a significantly positive effect to inhibit the mycelial growth of G. *boninense*. Antibiotic PHZ was found to be more effective than PCA and PYO.

Discussion

The rhizospheric bacteria, different species of *Pseudomonas* sp., are very important sources for the detection of antimicrobial compounds and their practical use as biopesticides (Cazorla *et al*., 2006). UPMP3 strain of *P. aeruginosa* produced organic compounds during the incubation period which was detected by the color change of the culture medium. The production of blue, orange and lemon yellow color in liquid KBA medium in different incubation period indicated the presence of different compounds PYO, PHZ, and PCA. Similarly, Saosoong *et al*. (2009) reported that the blue green solution in KA liquid medium of *P. aeruginosa* (TISTR 781) and the crude extraction eluted with dichloromethane and methanol showed yellow or yellow green and blue color indicated phenazine and pyocyanin production.

The HPLC analysis of the extracted compounds PHZ, PCA and PYO were found to be the highly positive correlations with the respective standard concentrations. The retention times of PHZ, PCA and PYO were detected from the standard as well as from samples 41.896 and 39.764 min at 250 and 34.895 min at 262 nm, respectively. Kavitha *et al.* (2005) reported that HPLC analysis of antibiotics production from *P. chlororaphis* PA23 was obtained three peaks with a retention time of 18.3 min, 20.3 min and 22.8 min at 248 and 367 nm which showed the presence of three antibiotics viz., 2-OH-PHZ, PCA and 2-OH-PCA. The purified antibiotics were quantified from the respective standard curve of PHZ, PCA and PYO which were recorded as 1.36 µg/mL, 9.62 µg/mL and 15.48 µg/mL, respectively. Similarly, Kerr *et al.* (1999) obtained 1.13 µg/mL and 1.26 µg/mL purified pyocyanin from the two strains of *P. aeruginosa* N11 and D23 in BHI agar medium, respectively by HPLC analysis. Concerning the production of PCA on YM media by 4 isolates of *P. aeruginosa* and the HPLC

results indicated that *P. aeruginosa* isolate 109, 108, 107 and 105 produced 12.25 µg/mL, 11.5 µg/mL, 7.5 µg/mL and 10.7 µg/mL PCA, respectively (El-Sayed *et al*., 2008).

In this study the antifungal effects of the phenazines were found highly positive against the pathogenic fungus *G. boninense*. Phenazine was more effective than PYO and PCA to inhibit the mycelial growth of *G. boninense*. The growth inhibition was recorded maximum percentage in PHZ at 1.0 mg/mL concentration and followed by PYO and PCA, respectively. *P. aeruginosa*, has been found to be an effective biocontrol agent of root pathogens due to the production of antifungal phenazine compounds. *P. chlororaphis* PA23 produced phenazine which inhibited the mycelial growth of *Pythium aphanidermatum* (37.77%) at 200 mL concentration over untreated control (Kavitha *et al.,* 2005). *P. aeruginosa* PNA1, isolated from the rhizosphere of chickpea, inhibited the *in vitro* mycelial growth of different phytopathogenic fungi by the production of phenazine-1-carboxylic acid (PCA). The result of other studies revealed that 100% inhibition of spore germination of both *Botryodiplodia theobromae* and *Alternaria solani* were recorded by antibiotic phenazine under *in vitro* condition. Similarly, the growth of sclerotia of *Macrophomina phaseolina, Rhizoctonia solani* and *Sclerotium rolfsii* were also completely arrested by phenazine derivatives (de Souza *et al.,* 2003). *P. aeruginosa* ID 4365 produced PCA which showed inhibitory activities against *Aspergillus -niger* NCIM1025, *Fusarium oxysporum* NCIM1008, *Sclerotium rolfsii* NCIM 1084, *Colletotrichum falcatum* and several other phytopathogens (Makarand *et al.,* 2007). Lee *et al.* (2003) was also reported that minimal inhibitory concentration of PCA produced by this strain for *S. rolfsii* NCIM 1084 was found to be 29 mg/L. The inhibitory action of pyocyanin was proposed that, during respiration, pyocyanin becomes reduced and univalently reduces oxygen to the toxic superoxide radical. Many effects of pyocyanin possess *P. aeruginosa strain* zag2 shows antagonistic activities against fungi. It has been reported that a lower concentrations of pyocyanin act as inhibitors (Hassanein *et al.,* 2009). Rane *et al.* (2008) also reported the biocontrol activity of pyocyanin against *C. falcatum*, *F. oxysporum,* and *Sclerotium rolfsii.* In conclusion, although encouraging results were achieved from using phenazines to control *G. boninense in vitro,* studies must be done to confirm their efficacy *in vivo.* The formulation of the antibiotics and the best method of application in the field need to be investigated.

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