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Antifungal activity of pyocyanin produced by *Pseudomonas aeruginosa* against *Fusarium oxysporum* Schlech a root-rot phytopathogenic fungi

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Abstract : The *Pseudomonas aeruginosa* produces secondary metabolites such as pyocyanin. The study aimed to production and characterization of pigment pyocyanin and to evaluate its antifungal potential against *Fusarium oxysporum* Schlech. causing root-rot diseases of agronomic crops. Fifteen bacterial strains were isolated from wild rhizosphere plants then identified based on biochemical and physiologic tests and MicroScan. Antifungal activity of *P. aeruginosa* isolates was screened using dual culture assay. The antifungal compound were extracted by chloroform extraction method then partially purified. Fourier Transform Infrared (FT-IR) was used to characterize the compound, then its structural was done using FTIR library. Pyocyanin antimicrobial activity was evaluated using light microscopy. On the basis of biochemical and physiologic tests and MicroScan, three isolates were identified as *P. aeruginosa*. Among these isolates, isolate HB9 exhibited the highest activity against *F. oxysporium*. Pigment production was achieved after 24 hrs of incubation with a color change to bluish green. UV-visible spectra and FT-IR study revealed characteristic of pyocyanin. It showed to inhibit the growth of *F. oxysporium* with the clear inhibition zone. Spores formation was inhibited and immature, also swelling hyphae were observed in pyocyanin-treated *F. oxysporum*. The properties of the pyocyanin indicated an important bioactive compound which has the ability to cease the reproduction of *F. oxysporum.*
Keywords: Pyocyanin, *Pseudomonas aeruginosa*, FT-IR, Antifungal activity, *F. oxysporum.*

Introduction

Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means[1] and is known to be a cheap and effective eco-friendly method for the management of crop diseases[2]. The use of biological control agents as an alternative to fungicides is increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Members of the genus *Pseudomonas* have been known for their potential to reduce the plant disease caused by fungal pathogens and they have gained considerable importance as potential antagonistic microorganisms[3]. Among these the bacteria that aggressively

colonize the root zone and promote plant growth are generally termed as plant growth promoting rhizobacteria (PGPR) *Pseudomonas* spp. is identified as an important organism with ability for plant growth promotion and effective disease management properties[4]. Their applicability as biocontrol agents has drawn wide attention because of the production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones[5].

The characteristic of *P. aeruginosa* is the production of soluble pyocyanin pigment, a water soluble blue green phenazine compound produced in large quantities. Pyocyanin has antibiotic activity against bacteria and fungi[6,7,8]. *P. aeruginosa* was found to produce phenazine pigment identified as pyocyanin[9]**.** The present study deals with biosynthesis, purification and characterization of pyocyanin pigments produced by *P. aeruginosa.* The purified pigment was used as bioactive compound to study the *in vitro* antagonistic activity against *F. oxysporium* causing root-rot diseases of agronomic crops.

Material And Methods

Microorganisms isolates

***P. aeruginosa*:** Isolation of *P. aeruginosa* was made from rhizhosphere of wild plants. The 10 gm rhizhosphere soil particles loosely adhering to the roots were gently teased out. The soil thus obtained was crushed in a sterile mortar and pestle and shaken with 100 ml of sterile distilled water for 10-20 min to obtain standard soil suspension. Isolation of *P. aeruginosa* was made by serial dilutions and pour plate method using the specific cetrimide agar [10] as a selective medium. One ml of soil suspension from aliquot dilutions (10^5 to 10^8) was aseptically added to sterile Petri plates containing twenty ml of sterile medium and incubated at 28±20C for 48 h. All well separated individual colonies with yellow green and blue white pigments were marked and detected by viewing under UV light. The single colonies were picked up with sterile loop and transferred to fresh King's B slants and the pure cultures so obtained were stored in refrigerator at 4ºC for further use. Identification of *P. aeruginosa* was done based on morphological, cultural, biochemical and physiological characteristics as suggested by [11]. A reference strain, ATCC27863 was also included in this study.

Three *P. aeruginosa* cultures were subjected to MicroScan for confirmation. MicroScan® instrumentation (auto SCAN®-4 and WalkAway® System) (Siemens Healthcare Diagnostics Inc, USA) was used. Panels used were MicroScan Dried Gram Positive MIC/Combo, Dried Gram Positive Breakpoint Combo and Dried Gram Positive ID Type 2 or 3. Also, MicroScan Dried Gram Negative MIC/Combo panels and Dried Gram Negative Breakpoint Combo Panels were used. MicroScan panels were designed for use in determining agent susceptibility and/or identification to the species level of rapidly growing aerobic and facultative Gram positive cocci or aerobic and facultatively anaerobic Gram negative bacilli. The tests were performed as recommended by supplier guidelines [12]. The results of confirmation test provided a sense of the accuracy of laboratory method in identifying organism.

***F. oxysporium*:** was kindly provided by Dr. Eman S. Farrag, Agricultural Botany Department, South Valley University (SVU), Egypt [13]. *F. oxysporum* were maintained on potato dextrose agar (PDA) medium.

Antagonistic effect to *F. oxysporium*

The antagonistic activity of *P. aeruginosa* isolates against *F. oxysporum* was tested by dual culture technique [14]. Bacterial isolates were streaked at one side of Petri dish (one cm away from the edge) containing PDA. 9 mm mycelial disc from seven days old PDA culture of *F. oxysporum* was placed at the opposite side of Petri dishes perpendicular to the bacterial streak and incubated at 28±20C for 5-7 days. Petri dishes inoculated with fungal discs alone served as control. Three replications were maintained for each isolate. Observation on width of inhibition zone and mycelia growth of test pathogen was recorded and per cent inhibition of *F. oxysporum* growth was calculated by using the formula proposed by [15]. Per cent inhibition (I) = C-T/C ×100. Where, I = inhibition (%), C = colony diameter in control plate and T = colony diameter in treated plate.

Antagonistic compound production and extraction

Production: The three isolates of *P. aeruginosa* were inoculated into potato glycerol broth; nutrient broth and mineral salt medium were used. Sterilized media were inoculated with 1.0% of 18 hrs culture and incubated on a rotary shaker at 150 rpm, 35ºC for 72 hrs to produce pyocyanin.

Extraction: The broth culture was centrifuged at 10 000 rpm for 15 min at 4ºC (Hettich, UNIVERSAL 320 R). The pigment was extracted using chloroform (1:2) and the aqueous phase discarded. The pyocyanin was then extracted from the chloroform by adding 0.2 N HCl was added until the color change observed [16]. To the deep red acid solution 0.4 M borate-NaOH buffer (pH 10) was added until the color changed to blue and the blue coloured pyocyanin was again extracted into chloroform. This step was repeated 2 to 3 times, resulting in a clear blue solution of pyocyanin in chloroform. This chloroform fraction was then transferred into a 500ml vacuum rotary flask (IKA® RV 10, Switzerland) and concentrated in a vacuum rotary evaporator at 40°C (IKA® RV 10, Switzerland) [17].

Antagonistic compound production was assayed by extracting 5ml culture supernatant with 3ml chloroform. This was then re-extracted in 1ml 0.2N HCl which gave a red-coloured solution. The absorbance at Abs_{520nm} of the solution multiplied by a factor 17.072 was used to quantify the antagonistic compound (mg ml^{-1}) according to[18].

Purification and characterization of antagonistic compound

Purification: Among the 3 *P. aeruginosa* isolates examined, HB9 was selected for extraction and characterization of the active compound based on its potential to exhibit higher inhibitory activity. One liter of 24 h old HB9 culture was used for extraction as mentioned above. The chloroform fraction, blue in colour due to the presence of blue coloured pyocyanin was concentrated and purified by silica gel column having 3 cm diameter and 60 cm length (Magnum, India). The column was packed with silica having a mesh size 100-200 and equilibrated using chloroform-methanol solvent system in the ratio 1:1 and the concentrated pyocyanin fraction was loaded into the column. Chloroform-methanol solvent system in the ratio 1:1 was used as the mobile phase to separate pyocyanin. The blue colored pyocyanin fraction was then collected into a 250ml conical flask, protected from light, then concentrated in vacuum rotary evaporator at 40°C (IKA® RV 10, Switzerland). Dried pyocyanin was dissolved in 500 µL dimethyl sulfoxide (DMSO) and stored at 4°C till use.

Antifungal activity: The antifungal activity of the purified pyocyanin was determined by well diffusion technique. Different concentrations of purified pyocyanin such as 5, 10, 20 and 30 mg l^{-1} obtained by adding double distilled water. An aliquot of 50 µL of these concentrations each was tested against *F. oxysporum* in triplicates. The percentage growth inhibition was calculated as mentioned above. Wells containing 50 µL DMSO alone were used as control. For study effect of pyocyanin on hyphal morphology and spores, the fungal growth located at the edges was monitored microscopically.

Identification of the purified compound

UV-Visible Spectroscopy measurement: The purified pyocyanin was subjected to spectroscopic analysis. UV-visible spectrophotometer and a maximum absorption were recorded by UV T-1800. 1ml aliquot of the pigment was subjected to absorption maxima in the wave length range from 200 to 400 nm.

Fourier Transform Infrared (FT-IR) Spectroscopy analysis: The pigment was subjected to FT-IR Spectroscopy analysis on a FTIR 4600^{TM} spectrophotometric instrument (Easton, MD, USA) in Kbr disc. Two milligrams of the sample was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded using Spectrum Manager ^{TM} V. 2 software in the range 4,000–400 cm^{-1} in FTIR spectroscopy at a resolution of 1 cm^{-1}. FTIR results were searched online library database.

Results

Isolation and identification of *P. aeruginosa*

All 15 isolates of *Pseudomonas* **(Figure 1)** were subjected for colony characterization, Gram staining and motility test. The Gram-negative rods, motile which produced characteristic pigments were subjected to biochemical tests **(Table 1)**. Three isolates showed uniform biochemical characteristics of *P. aeruginosa*. The results were compared with the type strain *P. aeruginosa* ATCC 27863 to confirm its phenotypic identity. Each identified isolate was coded as HF9, HF12 and HF13 **(Table 1 and Figure 1)** then stored in 15% glycerol at -80ºC. After the three isolates were tested for validation testing using MicroScan, no errors were found.

Table 1. Biochemical properties of Pseudomonas isolates.

Properties	**Test strain:**	**Reference strain:**
	HF9, HF12, HF13	**ATCC27863**
Colony Morphology	Small, raised, circulates to oval colony	Small, raised, circulates to oval colony
Cell shape and arrangement	Single small rods	Single small rods
Motility	+	+
Gram reaction	Negative	Negative
Endospore	Not formed	Not formed
Indole test	-	-
Cytochrome oxidase test	+	+
Arginine dihydrolase test	+	+
Sudan black test	-	-
Fluorescent pigment production	+	+
Lecithinase production	+	+
TSI test	Alkaline with H_2S production	Alkaline with H_2S production
Catalase test	+	+
Citrate test	+	+
Growth at 4°C	-	-
Growth at 42°C	+	+

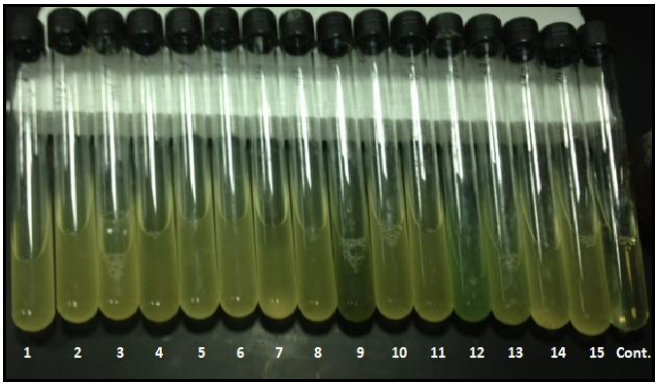


Fig. 1. Growth of all *Pseudomonas* isolates cultured at nutrient broth showed pigment production in 9, 12 and 13 isolates.

Antifungal activity

The results of the dual culture technique indicated that the 3 isolates of *P. aeruginosa* inhibited growth of *F. oxysporum* with growth inhibition *ranging* from 62.6 to 94.1%. A maximum inhibition was showed by HF9 *P. aeruginosa* isolate. Differences in growth inhibition related to pyocyanin quantities produced by each isolate.

Pyocyanin production and extraction

Pigment production was accomplished after 24 h of incubation. It was demonstrated in shades of bluish green color due to the release of pyocyanin into the medium. The extent of pyocyanin production of all isolates of *P. aeruginosa* was influenced by medium type **(Figure 2)**. All isolates of *P. aeruginosa* produced detectable levels of pyocyanin in all media supplemented with glycerol. Maximum pyocyanin production occurred when cultured on potato glycerol broth medium. The extraction of pyocyanin from the broth using chloroform yielded a deep blue colored chloroform-pyocyanin mixture. Pyocyanin was produced by using potato glycerol broth for 24 hrs. Presence of pyocyanin was confirmed by adding 0.2N HCl which lead to developing pinkish red color.

Among the 3 isolates, pyocyanin production was higher in HB9 compared HB12 and HB13. Of all the isolates, the HB9 produced the highest concentration (18.2 mg ml^{-1}) of pyocyanin followed by the HB12 isolate (14.9 mg ml^{-1}).

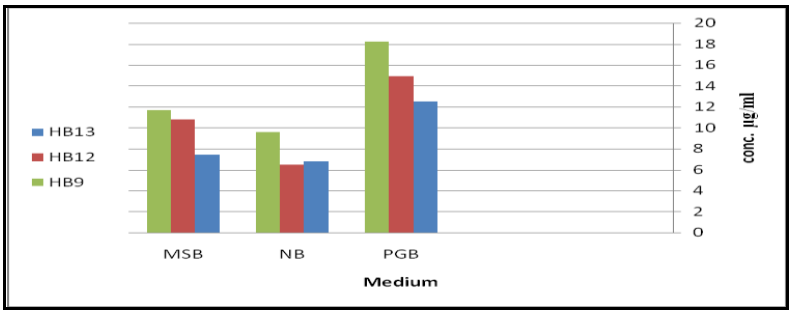


Fig. 2. Pyocyanin production by *P. aeruginosa* isolates cultured at different media.

Purification and activity of the antagonistic compound

Extracted pyocyanin was partially purified by column method and the fractions were collected by using anelute chloroform. The antifungal activity of purified pyocyanin was found towards *F. oxysporium* as root-rot pathogen. It showed to inhibit the growth of *F. oxysporum* with the clear inhibition zone **(Figure 3B)**. The purified pyocyanin inhibited the growth of *F. oxysporum* above a concentration of 10 mg ml^{-1}. At 5 and 10 mg ml^{-1}, no zone of inhibition was observed. At 15 mg ml^{-1}, the zones were less than 14 mm. The control wells containing DMSO did not show any inhibitory activity. Light microscope investigation revealed that the pyocyanin induced morphological abnormalities in fungal structures. It had effect on spore germination and hyphal morphology after 24 h of incubation at room temperature. Conidial spores formation was inhibited and immature, also swelling hyphae were observed in pyocyanin-treated *F. oxysporum* **(Figure 3 A1)**.

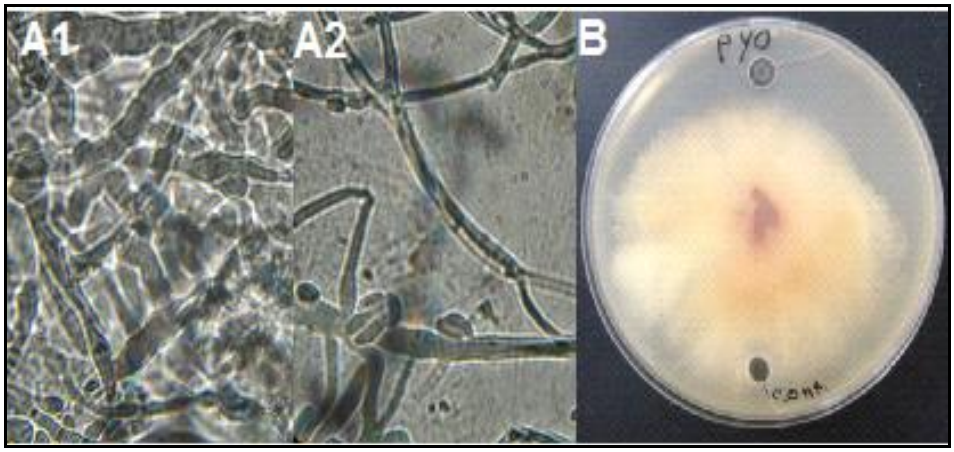


Fig. 3. Antagonistic activity of purified pyocyanin (50 µL containing 15 mg ml^{-1} pyocyanin) against *F. oxysporum*.

Identification of the antagonistic compound

Partially purified pyocyanin was subjected to UV-visible spectrophotometer for spectral analysis.UV-vis analysis result revealed a maximum absorbance about 278 nm, indicative of pyocyanin molecule **(Figure 4)**. The FT-IR analysis shows a number of vibrational bands, which corresponds to the various functional groups present in pyocyanin molecules (**Figure 5-left and Table 2)**. Online library database search indicated that molecule is 5-Methylphenazin-1-one (poycyanin) (**Figure 5-right)**.

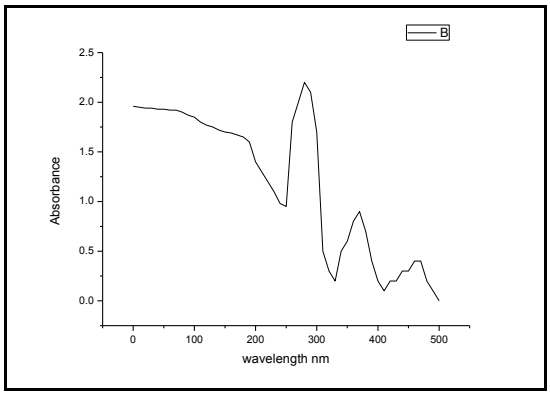
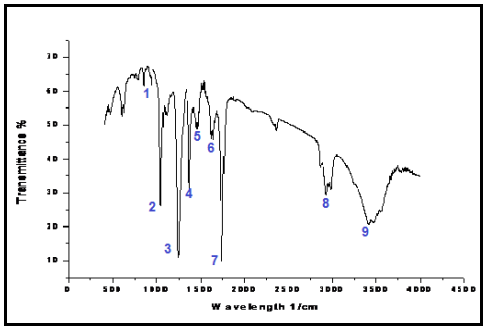


Fig. 4. UV absorption spectra of partially purified pyocyanin showing λmax around 278nm.



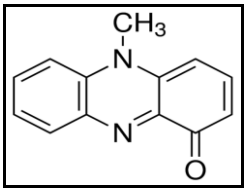


Fig. 5. Fourier-transform infrared spectra of partially purified pyocyanin (left), showed number of peak with vibration mode and wavelength as indicated in Table 1 (Left). Structural formula of 5-Methylphenazin-1-one as suggested by online library database search (Right).

Table 2. Vibration frequency assignment for the peaks observed in the FTIR spectra of pyocyanin.

Peak No	Vibration mode	Wavelength cm $^{-1}$
1	C-H aromatic	989
2	C-O (ν)	1047
3	C-N	1250
4	CH_3 (δ)	1374
5	O – C – H (δ), CH_2 (δ)	1487
6	C=C aromatic	1681
7	C=C aromatic	1742
8	CH_3 (ν)	2984.3
9	O-H	3414
(δ) bending vibrations, (ν) stretching vibration		

Discussion

Biological control has acquired vigor in the recent years due to the emergence of fungicide resistance in pathogens besides increased health concerns for the producer and the consumer. *Pseudomonas* spp. have been studied mainly because of their widespread distribution in soil, their ability to colonize the rhizosphere of host plants and ability to produce a wide range of compounds inhibitory to a number of serious plant pathogens [19,20]. Pyocyanin a water soluble bio-active compound produced by *P. aeruginosa*, has the capacity to arrest the electron transport chain of the fungi and exhibit the antifungal activity[21]. The organism isolated from wild plants rhizosphere was designated as HF9 strain and confirmed as *P. aeruginosa* based upon the preliminary examination, biochemical test and MicroScan [22,12]. This strain was selected and used for the study by checking the potency of the strain HF9 by determining the antifungal activity using dual culture technique against *F. oxysporum*, from which among all the strain HF9 was every effective and further used for other studies.

Pyocyanin compound was produced and extracted by using Pseudomonas broth and a green shade color of the solution was obtained, extracted by adding chloroform which separated a blue color compound. The change in color of the pigment to deep pink observed upon addition of chloroform and 0.2N HCl confirmed the presence of pyocyanin[6]. Extracted biologically compound was further partially purified by using silica gel in a column bed and a single fraction of light blue color was obtained by eluting with chloroform solvent.

UV-Visible Spectroscopy analysis of separate compound showed a maximum absorbance around 278 nm, which indicative of pyocyanin molecule. These results also obtained by [21,6] for *P. aeruginosa* clinical isolate and *P. aeruginosa* ATCC. Later FT-IR Spectroscopy analysis of pyocyanin from *P. aeruginosa* culture showed aliphatic chain stretching and bending (2984.3, 1374 cm^{-1}), aromatic compound C=C stretching vibrations (1681, 1742 cm^{-1}), C-O stretching (1047 cm^{-1}), stretching of C-N (1250 cm^{-1}), banding vibration of O–C–H, CH_2 (1487 cm^{-1}). These bands correspond to the various functional groups present in pyocyanin [23, 24]. Online library database search indicated that molecule is 5-Methylphenazin-1-one (poycyanin). These results agreed with those previously reported by [25,26]. This compound was further analyzed for MIC activity. The results were correlated with the work of [27,28]. This research work showed that *P. aeruginosa* HF9 isolate had the capacity to inhibit the growth of *F. oxysporum* and this our results confirmed that isolate of *P. aeruginosa* JY21 was selected as biocontrol agent due to their producing pyocyanin highly antagonistic against several phytopathogens fungi i.e., Alternaria sp., Pythium sp., Phytophthora infestans, Rhizoctonia solani and Sclerotium sp. [29]. This *P. aeruginosa* strain can be economically used to control phytopathogens as a bio-control agent.

Conclusion

The purified pigment has bioactive properties and is especially active against fungal pathogens. The clinical strain *P. aeruginosa* could be used to produce the pigment in large quantities and simple purification method makes the culture a promising one in the field of agricultural application. Further strain improvement might be performed to increase the pigment production and apply it as a fungicidal in crop protection or as a biopesticide.

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