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In vitro study of biocontrol potential of rhizospheric *Pseudomonas aeruginosa* against *Fusarium oxysporum* f. sp. *cucumerinum*

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Abstract

Fusarium wilt is an economically important disease of cucumber caused by the fungus *Fusarium oxysporum* f. sp. *cucumerinum* (*Foc*). It causes severe losses in the yield and quality of cucumber and is extremely difficult to control conventionally using chemical fungicides. Biological control offers an eco-friendly alternative to chemical pesticide for sustainable plant disease management. In this context, biocontrol activity of rhizosphere soil bacteria was investigated against *Foc* in vitro. Thirty-five rhizobacterial isolates were screened for antagonistic activity in dual culture, and isolate BA5 showed the highest antagonistic activity (58.33% mycelial growth inhibition) against *Foc*. Maximum fungal biomass reduction (90.20%) was found in King's B broth in shake flask culture. Cell-free culture filtrate and ethyl acetate crude extract inhibited mycelial growth of *Foc* by 56.66 and 25.0%, respectively. Further, the selected isolate produced siderophores, volatile compound(s), hydrocyanic acid, and protease. Siderophores and volatile compound(s) were involved in the isolate-induced antagonism. In addition, the isolate exhibited several plant growth-promoting traits, including phosphate and zinc solubilization, ammonia production, organic acid production, and in vitro biofilm formation. Based on the morphological, physiological, biochemical characteristics, and phylogeny analysis, the isolate BA5 was identified as *Pseudomonas aeruginosa*, and the 16S rDNA sequence was submitted in the NCBI GenBank under the strain name RKA5. Because of the novel antifungal and plant growth promotion potentials, the strain can be used as a promising biocontrol agent against the fungal pathogen *Foc*.

Keywords: Biological control, Antagonistic activity, *Fusarium*, Cucumber, *Pseudomonas aeruginosa*, *Fusarium oxysporum* f. sp. *cucumerinum*

Background

Plant diseases account for ~ 13% of the world's crop production lost, nearly equivalent to $220 billion lost every year (Kandel et al. 2017). Among the crop pests, phytopathogenic fungi are the most common and cause a wide range of diseases to economically important plants (Mehnaz et al. 2013). *Fusarium oxysporum*, for example, is an important fungal pathogen known to cause vascular wilt diseases in more than 100 different species (Lopez-Berges et al. 2012). *Fusarium oxysporum* f. sp. *cucumerinum* (*Foc*), a soil-borne pathogen, is the causal agent of vascular wilt disease in cucumber and causes significant yield loss (Al-Tuwaijri 2015). Cucumber (*Cucumis sativus* L.) is one of the most important economical crops (Ahmed 2010) and commercially cultivated in Bangladesh throughout the year. *Foc* invades cucumber at any stage of development and colonizes the vascular vessel. The visible symptoms of the disease include necrotic lesions, followed by foliar yellowing, wilting, vascular tissue damage, and finally plant death (Ahmed 2010). It can grow along the xylem vessel in plant tissues and survive in soil as chlamydospores or saprophytes over a year (Yang et al. 2014), making it extremely difficult to control.

Use of synthetic fungicides is challenged due to the accumulation of these compounds in the ecosystem and the development of resistant fungal strains (Mehnaz

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et al. 2013). Interactions between antagonistic microorganisms and plant pathogens are widespread in nature and can be utilized to control or reduce fungal diseases of crop plants (Fridlender et al. 1993). Bacteria are vital components of soil (Ahemad and Kibret 2014), and over 95% of them exist in or near the plant roots (Ji et al. 2014). Rhizobacteria obtain their foods from root exudates and provide essential nutrients and protection to the plants; hence, it was rightfully stated that the rhizosphere is the "hotspot" of microbial interactions (Raaijmakers et al. 2009). The phenomenon of using beneficial soil microorganisms for plant disease management is known as biocontrol, and the microorganisms are known as biocontrol agents (Kandel et al. 2017). The antagonistic activities of bacterial biocontrol agents can be attributed to (i) synthesis of hydrolytic enzymes that can lyse fungal cell walls (such as chitinase, glucanase, protease, and lipases), (ii) competition for nutrients and niches, (iii) siderophores and antibiotic production, and (iv) induced systemic resistance (Beneduzi et al. 2012). In addition to their biocontrol activity, rhizobacteria also directly promote plant growth and health through "phytostimulatory" and "biofertilizing" traits (Raaijmakers et al. 2009).

A number of soil bacterial strains have been exploited for their plant growth promotion and biocontrol potentials, particularly the genera *Bacillus* (Lee et al. 2017), *Pseudomonas* (Priyanka et al. 2017), and *Streptomyces* (Lu et al. 2016). The genus *Pseudomonas* possesses superior biocontrol properties because of their adaptive metabolism and their ability to produce a range of antifungal compounds (Trivedi et al. 2008). Examples of antifungal and secondary metabolites produced by *Pseudomonas* spp. include phenazines (Hu et al. 2014), 2,4-diacetylphoroglucinol (Zhang et al. 2016), pyoluteorin (Wu et al. 2011), pyrrolnitrin (Zhang et al. 2016), cyclic lipopeptides (Michelsen et al. 2015), siderophores (Sulochana et al. 2014), volatile compounds (Mannaa et al. 2017), hydrolytic enzymes (Solanki et al. 2014), and so on. Fluorescent pseudomonads, for example, *Pseudomonas aeruginosa* (Fatima and Anjum 2017), *Pseudomonas putida* (Yu and Lee 2015), and *Pseudomonas fluorescens* (Zhang et al. 2016), are well-known to protect plants from fungal infections.

The objectives of this study were to explore the biocontrol potentials of local rhizosphere soil bacteria against the cucumber wilt pathogen *Foc* and to identify and characterize the prominent biocontrol bacterial isolate for antagonistic, enzymatic, and plant growth-promoting traits.

Materials and methods

The fungal pathogen

Fusarium oxysporum f. sp. *cucumerinum* (*Foc*), the causative agent of Fusarium wilt in cucumber, was obtained from Professor Dr. Md. Rezuanul Islam, Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh. The fungal pathogen was grown on potato dextrose agar (PDA) plates incubated at 27 ± 2 °C for 5 days. The fungal cultures were stored in PDA slants at 4 °C for further use.

Isolation of rhizobacterial strains

Six soil samples were collected from the rhizosphere of five different crop/vegetable plants, namely mustard (*Brassica campestris*), pea (*Pisum sativum*), bathua (*Chenopodium album*), lentil (*Lens culinaris*), and radish (*Raphanus sativus*), grown in agricultural fields located near the Islamic University, Kushtia, Bangladesh. Soil bacteria were isolated from the samples by serial dilution technique. Briefly, 5 g of soil sample was suspended in 45 ml of sterile distilled water and shaken at 120 rpm on a rotary shaker for 10 min. The soil mixture was diluted 1:10 ratio with distilled water up to 10^{-7}. An aliquot of 100 μl from 10^{-4} to 10^{-7} dilutions was distributed in tryptone soya agar (TSA) plates and gently spread with a sterile glass rod spreader. The plates were incubated at 30 ± 2 °C for 2 days, after which morphologically distinct colonies were subcultured onto the same medium in another plate to isolate single colonies. The purified bacterial isolates were maintained in Eppendorf tubes in tryptone soya broth (TSB) containing 20% glycerol at − 80 °C (Han et al. 2015).

In vitro mass screening for antagonistic activity

In vitro screening for antagonistic activity was performed by dual culture technique on PDA plates. Briefly, PDA medium was prepared and poured (20 ml) in sterile Petri dishes. A 5-mm agar disc of an actively growing culture of *Foc* was placed in the center of each plate. Each isolate was streaked 3 cm away from the agar disc towards the edge of the Petri dish. In the control plate, no bacterial isolate was inoculated. Plates were parafilmed and incubated at 27 ± 2 °C for 5 days until the fungal mycelia reached the edge in the control plates. Mycelial growth inhibition towards the direction of the bacterial isolate was indicative of antagonistic activity. Percentage (%) of radial mycelial growth inhibition was calculated according to Ji et al. (2013).

Quantitative evaluation of antagonism of the selected isolate

One milliliter (A_{600} = 0.2) culture broth of the selected isolate, i.e., isolate BA5, and a 5-mm disc of an actively growing culture of *Foc* were inoculated in 50 ml broth medium in 250 ml conical flasks and incubated at 27 ± 2 °C for 48 h on a rotary shaker. Five different media (potato dextrose, King's B, tryptone soya, nutrient, and tryptone yeast extract broth) were used. Broth inoculated only with *Foc* served as

control. Reduction in fungal biomass in co-culture compared to control was determined (Trivedi et al. 2008).

Antagonism due to volatile compound(s)

A bacterial lawn of isolate BA5 was prepared on TSA plate, and after incubation for 24 h, the lid was replaced by a plate containing an agar disc (7 mm diameter) of *Foc* grown on PDA. The two plates were sealed together with parafilm. Control plates were prepared similarly without the bacterial isolate in the bottom plate. Such sealed sets of Petri dishes were incubated at 27 ± 2 °C, and the observations were recorded at intervals of 24 for 72 h. The mycelial growth inhibition (%) of the fungus was determined (Trivedi et al. 2008).

Evaluation of the effect of cell-free culture filtrate

Isolate BA5 was grown on nutrient broth medium in 250-ml conical flask at 30 ± 2 °C on a rotatory shaker at 100 rpm. Culture broth after 24 and 48 h of incubation was centrifuged at 10,000 rpm at 4 °C for 10 min, and cell-free culture filtrate (CFCF) was obtained by passing the supernatant through 0.22 μm pore size syringe filter. PDA plates were prepared, and a mycelial disc of an actively growing culture of *Foc* was placed in the center of each plate. Two wells (5 mm) were made with sterile cork borer 3 cm away from the center and aliquoted with 100 μl of CFCF. Plate in which wells were aliquoted only with nutrient broth served as control. Plates were incubated at 27 ± 2 °C for 5 days. Mycelial growth inhibition (%) was measured as described above.

Evaluation of organic solvent-aided crude extract activity

The effect of organic solvent-aided crude extract in fungal growth inhibition was carried out as described previously (Islam et al. 2012). The crude antifungal substance was recovered from the culture broth of isolate BA5 by solvent extraction (ethyl acetate and chloroform). The extracts were dried, weighed, dissolved in methanol, and stored at 4 °C. Antifungal activity of the resulting crude compound(s) was evaluated in agar well diffusion assay.

Characterization of antagonistic and enzymatic properties

Hydrocyanic acid (HCN) production was tested as described previously (Trivedi et al. 2008). Siderophore(s) and their chemical nature were examined as described in Yeole et al. (2001). Involvement of siderophore in antifungal activity was evaluated according to the method of Kumar et al. (2002). Cyclic lipopeptide (CLP) surfactant production was assessed according to De Bruijn and Raaijmakers (2009). Proteolytic activity was screened in nutrient agar plates supplemented with 3% skim milk powder (Han et al. 2015). Assay for cellulase production was done according to Kasana et al. (2008), and extracellular amylase production was screened on starch agar plates (Deb et al. 2013).

Characterization of plant growth promotion traits of the selected isolate

Phosphate solubilizing activity was qualitatively detected in Pikovskaya's agar (PKV) medium (Kumar et al. 2005). The solubilizing efficiency was calculated using the following formula: solubilizing efficiency (% S.E.) = $(Z - C)/C \times 100$; Z = solubilization zone (mm) and C = colony diameter (mm). The solubilizing zone around the colony was calculated by subtracting colony size from the total size. Zinc solubilizing activity was carried out in a modified PKV agar medium (Bapiri et al. 2012). Organic acid production was assessed using PKV agar medium with bromothymol blue indicator (Kumar et al. 2012). Nitrogenase activity was detected in Norris glucose nitrogen-free medium. Indoe-3-acetic acid (IAA) production was determined by the method reported by Bric et al. (1991). Isolate BA5 was grown on LB broth supplemented with 5 mM L-tryptophan and incubated at 30 ± 2 °C for 48 h. The culture broth was centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatant was collected. The supernatant (2 ml) was mixed with two drops of O-phosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) (Gordon and Weber 1951). The appearance of a pink color in the supernatant confirmed the production of IAA. Assay for ammonia production was performed as discussed in Trivedi et al. (2008). In vitro biofilm formation was carried out as described by Zhou et al. (2012).

Identification of the selected isolate

Morphological, physiological, and biochemical characterization

Morphological and biochemical tests were performed as described in Benson's Microbiological Applications Lab Manual (Benson 2002).

The ability of isolate BA5 to grow at different temperature was carried out by inoculating the isolate on TSA (pH 7.0) medium and incubating the plates at varying temperature, viz. 4, 25, 37, 42, and 50 °C. Growth was evaluated either as positive (+) or negative (–). The capability of the isolate to tolerate different osmotic pressure was performed by culturing in different concentration of sodium chloride. Nutrient broth medium (pH 7.0) was prepared (in 50-ml conical flasks) supplemented with 0.5, 1, 3, 5, 7, and 9% NaCl (*w*/*v*) and inoculated with the isolate. The presence of growth was evaluated by observing turbidity after an incubation period of 24 and 48 h at 30 ± 2 °C. Growth in different pH was observed by inoculating the isolate in nutrient broth medium of varying pH (4.0, 5.0, 7.0, 9.0, 10.0, and 11.0) at 30 ± 2 °C for 24–48 h. The pH of the broth was adjusted with 1 N NaOH/HCl with the help of a pH meter.

The ability of isolate BA5 to utilize a range of organic compounds as the sole source of carbon and energy was determined in modified Koser citrate medium (Koser 1923). In the basal medium, di-ammonium hydrogen orthophosphate was used in place of sodium-ammonium phosphate, and various organic compounds were added in place of sodium citrate. In addition, sodium chloride was added at 5 g/l concentration. The basal medium, without the carbon sources, was autoclaved at 121 °C, 15 psi for 15 min. Each of the carbon sources was dissolved in sterile distilled water, filter sterilized, and added to the basal medium at 0.3% final concentrations, except phenol, which was added at 0.025% (Stanier et al. 1966). Three test tubes with the same carbon source and one tube without the carbon source (control) were inoculated with isolate BA5 and incubated at 30 ± 2 °C. The inoculated test tubes were scored after 24, 48, and 72 h. The growth was recorded as "+" (positive, growth) or "–" (negative, no growth).

Molecular identification of isolate BA5

Extraction of genomic DNA, PCR, and sequencing

Genomic DNA was extracted by phenol: chloroform:iso-amyl alcohol method following the protocol described in He (2011). PCR was performed from the genomic DNA by using 16S rDNA bacterial universal primer set of 27F (5-AGA GTT TGA TCC TGG CTC AG-3) and 1492R (5-GGC TAC CTT GTT ACG ACT T-3). The purified PCR product was sequenced in 4-capillary ABI 3130 genetic analyzer from Applied Biosystems.

Sequence analysis and phylogeny interpretation

The obtained sequence was compared for similarity with sequences present in the gene database bank by using the BLASTn program in the GenBank of NCBI (National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The higher similarity sequences of 16S rRNA gene of type strains were retrieved and aligned with the 16S rRNA gene sequence of isolate BA5 in ClustalW program and subjected to a phylogenetic tree construction in MEGA7 (Kumar et al. 2016) with 1000 bootstrap replications, and evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987).

Statistical analysis

All experiments were conducted in triplicate, and data were presented as means ± standard deviations (mean ± SD) where appropriate. Data were statistically analyzed by one-way ANOVA and two-tailed *t* tests using Microsoft Excel™ 2013. Intergroup differences were considered to be statistically significant when $P \leq 0.05$ and highly significant when $P \leq 0.001$. Graphs were prepared in scientific 2D graphing software, GraphPad Prism.

Results and discussion

Mass screening for antagonistic activity

A total of 35 bacterial isolates were obtained from rhizosphere soils of five different crop/vegetable plants by serial dilution technique. In vitro screening for antagonistic activity was carried out in dual culture on PDA plates. Among the 35 isolates, five isolates showed different degrees of mycelial growth inhibition of *Foc* (Fig. 1a). Isolate BA5 (isolated from rhizosphere soil of bathua, *Chenopodium album*), was the most promising antagonist (58.33% mycelial inhibition, significant at $P \leq 0.001$) (Fig. 1a, and b) and selected for further investigations.

In vitro dual culture test is one of the key tests used for preliminary screening of biological control agents. Antagonistic effects are usually confirmed by the formation of inhibition zones between the bacteria isolates and the fungal isolates (Ji et al. 2014) or by measuring the percent of radial mycelial growth inhibition towards the bacterial isolates (Lee et al. 2017).

Quantitative evaluation of antagonism in different media

Antagonistic activity of the prominent isolate BA5 was also screened in broth-based dual culture. Fungal biomass was considerably reduced in broth media inoculated with isolate BA5 compared to the fungus only (Fig. 1c). Significant reduction of *Foc* biomass was found in King's B broth (90.20%), nutrient broth (86.38%), and potato dextrose broth (75.92%) compared to the respective fungus-only controls. According to Trivedi et al. (2008), in vitro broth-based dual cultures offer a better method for evaluation of the antagonistic efficiency of the biocontrol agents as the liquid medium may provide a better environment to allow the antagonistic activities from all possible interacting sites.

Antagonism due to volatile compound(s)

Volatile compounds such as ammonia and hydrogen cyanide are produced by a number of rhizobacteria and are reported to play an important role in biocontrol. Isolate BA5 produced antifungal volatile compound(s) (VOCs), as evident from the growth inhibition of *Foc* in sealed Petri dishes. Radial mycelial growth was significantly inhibited (31.11%) compared to control (Table 1, Fig. 1d). In addition, aerial mycelial growth was also reduced due to the effect of volatile metabolites. Raza et al. (2016) demonstrated the role of VOCs produced by *P. fluorescens* WR-1 in biocontrol activities. Kandel et al. (2017) and Lee et al. (2017) also reported VOCs mediated antifungal activities recently.

Antifungal activity of cell-free culture filtrate

Cell-free culture filtrate (CFCF) exhibited significant antifungal activity against *Foc*. Maximum mycelial growth inhibition (54.16%) was found with CFCF from 48-h-old

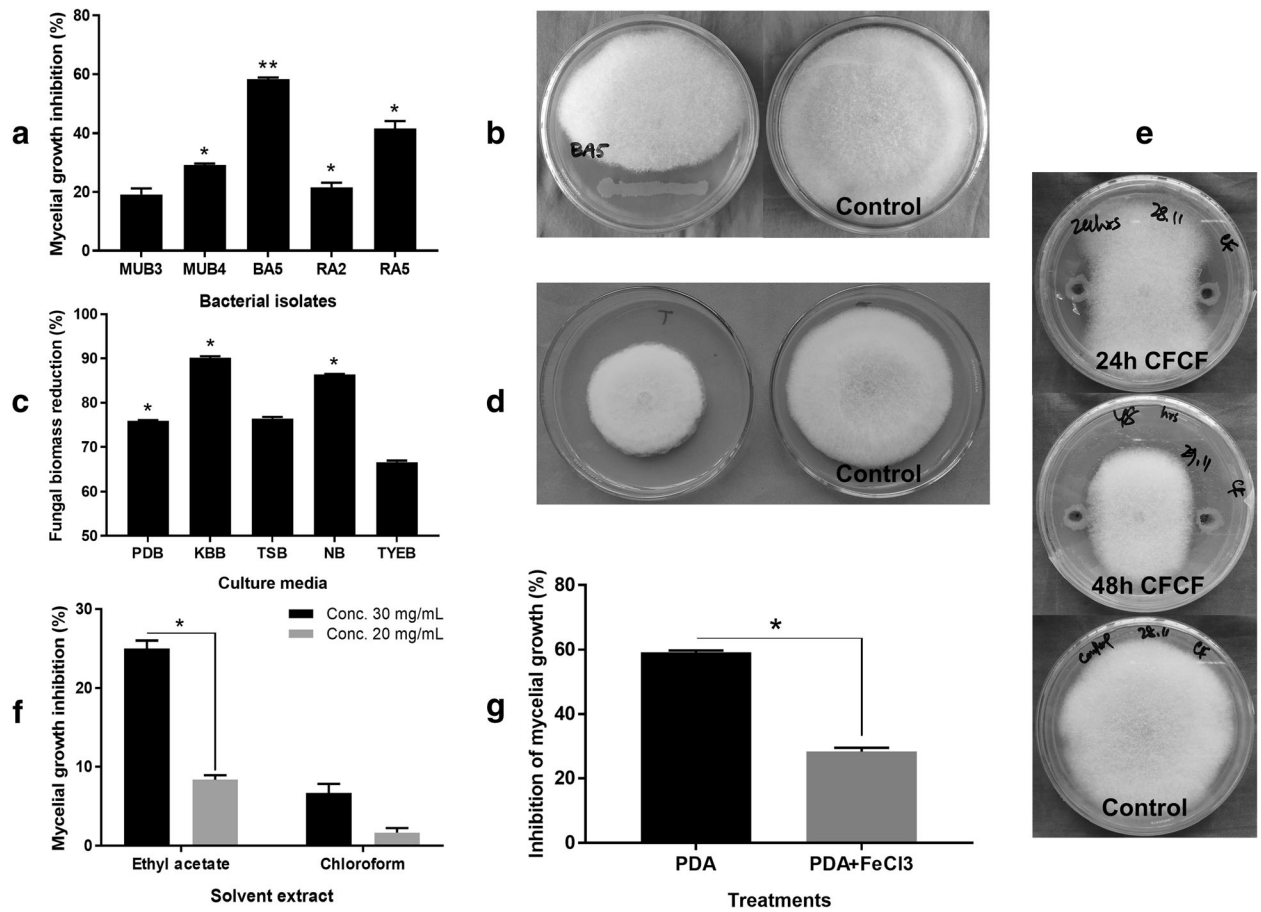

Fig. 1 Antagonism of isolate BA5 against *Fusarium oxysporum* f. sp. *cucumerinum* (*Foc*). **a** Antagonistic activity of soil bacterial isolates against *Foc* on potato dextrose agar (PDA), **b** antagonistic activity of isolate BA5 against *Foc* in dual culture on PDA after 5 days of culture at 27 ± 2 °C, **c** biomass reduction of *Foc* in broth-based dual culture with isolate BA5 in different media, **d** mycelial growth inhibition of *Foc* due to production of volatile compound(s), **e** *Foc* mycelial growth inhibition by cell-free culture filtrate from BA5 in dual culture on PDA, **f** antifungal activity of the solvent-aided crude substance of isolate BA5 against *Foc*, and **g** siderophore-mediated antagonism of isolate BA5 against *Foc*. Vertical bars represent standard deviations. The number of asterisks indicates the level of significance: a single asterisk (*) as significant ($P \leq 0.05$) and double asterisks (**) as highly significant ($P \leq 0.001$)

culture broth followed by (45.83%) with CFCF from 24-h-old culture broth (Table 1, Fig. 1e). Li et al. (2011) showed that CFCF of *Streptomyces globisporus* JK-1 inhibited mycelial growth of *Magnaporthe oryzae*.

Antifungal activity of crude bioactive compound(s)

The resulting crude extracts of both ethyl acetate and chloroform solvents were brownish in color, sticky, and readily dissolved in methanol. Both solvent extracts showed mycelial growth inhibition of *Foc* in a concentration-dependent manner (Fig. 1f). However, 30 mg/ml ethyl acetate extract showed significant (25.0%) mycelial growth inhibition compared to 20 mg/ml concentration. Upon further incubation, fungal mycelia became powdery and brittle. Chloroform extract crude substance showed insignificant mycelial inhibition of *Foc*. Evaluation of crude compound(s) for bioactivity is the prerequisite for further purification and identification of the antifungal metabolites. It is often regarded as one important preliminary screening for structural and functional characterization of bioactive compound(s). Kumar et al. (2005) purified a broad-spectrum antifungal compound from the ethyl acetate crude extract of *P. aeruginosa* PUPa3.

Antagonistic and enzymatic characteristics

Antagonistic and enzymatic properties of isolate BA5 were examined by various tests (Table 1). A remarkable change in the color of filter paper from yellow to light brown suggested the moderate HCN production in isolate BA5 (Fig. 2a). HCN is a broad-spectrum antimicrobial compound involved in biological control of root diseases by many plant-associated fluorescent pseudomonads (Ramette et al. 2003). Dharni et al. (2012) also reported a *P. aeruginosa* SD12 with the ability to produce HCN.

Table 1 Antifungal, antagonistic, enzymatic, and plant growth promoting activity of isolate BA5

Test	Result
Antifungal activity	% mycelial growth inhibition (± SD)[a]
Volatile compound(s)[b]	31.11 ± 0.57**
24 h CFCF[c]	45.83 ± 2.08*
48 h CFCF[d]	54.16 ± 0.57**
Antagonistic properties	Activity
HCN production[e]	+
CLP surfactant production[f]	–
Siderophore production	+
Enzymatic properties	
Protease	+
Cellulase	–
Amylase	–
Plant growth promotion properties	
Phosphate solubilization[g]	+
Zinc solubilization[h]	+
Organic acid production	+
Nitrogenase activity	–
IAA production	–
Ammonia production	+
In vitro biofilm formation	+

[a]Effect of volatile compound(s) and cell-free culture filtrate (CFCF) from isolate BA5 on mycelial growth inhibition of *Foc.* [b]Mycelial growth was measured after 72 h of culture in sealed Petri dish. [c & d] CFCF obtained from 24- and 48-h-old culture broth of isolate BA5, respectively. * and ** significant at $P \leq 0.05$ and $P \leq 0.001$ level, respectively. [e]*HCN* hydrocyanic acid. [f]*CLP* cyclic lipopeptide. The test medium was supplemented with insoluble [g]tri-calcium phosphate and [h]zinc oxide, respectively. "+" indicates positive activity and "–" indicates no activity

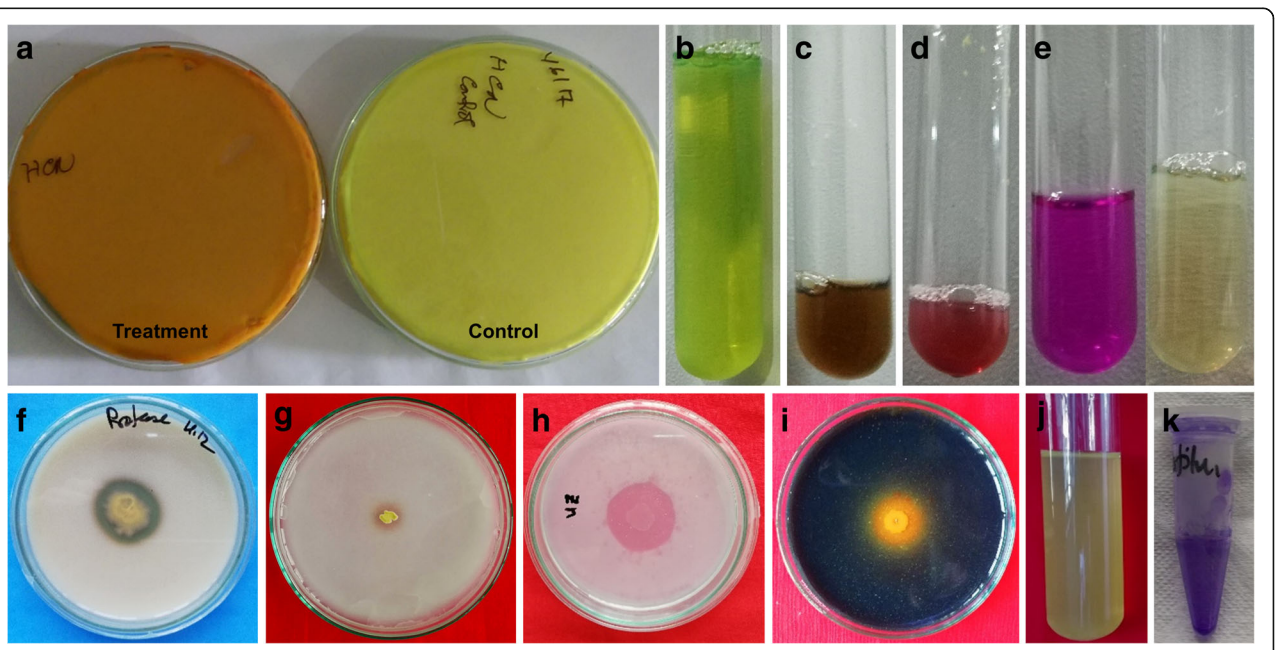

Fig. 2 Antagonistic, enzymatic, and plant growth-promoting potentials of isolate BA5. **a** HCN production, **b** siderophores obtained as cell-free culture filtrate, **c** $FeCl_3$ test, **d** tetrazolium salt test, **e** Vogel's chemical test, **f** protease activity, **g** phosphate solubilizing activity, **h** zinc solubilizing activity, **i** organic acid production, **j** ammonia production, and **k** biofilm formation in plastic Eppendorf tube

Siderophore production and chemical nature of siderophore were confirmed by chemical and spectrophotometric assays. The CFCF obtained by centrifuging the 72-h-old culture broth was light green to yellowish green (Fig. 2b). Formation of dark orange to light brown color of the CFCF after addition of 2% aqueous $FeCl_3$ solution was confirmative for siderophore production (Fig. 2c). Isolate BA5 produced two types of siderophores. In tetrazolium salt test, the appearance of a red color indicated the production of hydroxamate-type siderophore (Fig. 2d), and the absorption maximum of the iron-siderophore complex at 450 nm in UV-Vis spectrophotometer further confirmed the hydroxamate nature of the siderophore. Carboxylate-type siderophore was confirmed in Vogel's chemical test. Addition of the CFCF to the alkaline phenolphthalein solution made the light pink color of the solution disappeared instantly (Fig. 2e); however, the carboxylate nature of siderophore was not confirmed in the spectrophotometric assay.

The role of siderophores in biocontrol has extensively been studied previously (Solans et al. 2016). Siderophores can inhibit the growth of soilborne fungi by reducing the amount of ferric ions available to rhizosphere microflora. It has also been stated that colonization of the rhizosphere, production of antibiotics, and their antagonistic activity of *P. aeruginosa* are presumably due to the production of the siderophores (Sulochana et al. 2014). Hydroxamate siderophores are common among the bacterial community (Yeole et al. 2001 and Dharni et al. 2012); however, carboxylate siderophores have not been reported very often. Tian et al. (2009) reported that *Pseudomonas* sp. G-229-21 could produce high-affinity carboxylate-type siderophores under low iron conditions.

Siderophores are not produced in the presence of iron (Kumar et al. 2002). Mycelial growth inhibition rate was significantly ($P \leq 0.05$) reduced (28.33%) in $FeCl_3$ (100 μg/ml) supplemented plates compared to in no $FeCl_3$ supplemented PDA plates (59.16%) (Fig. 1g). This is suggestive that siderophore was one of the key antifungal metabolites in the isolate BA5-induced antagonism.

Petri dish-based qualitative assays revealed that isolate BA5 produced protease but not amylase and cellulase. A clear zone on skim milk agar was evident for strong protease activity, measuring 7 mm halo zone after 3 days of culture at 30 ± 2 °C (Fig. 2f). Proteolytic activity has also been reported in *Pseudomonas* spp. in several studies (Dharni et al. 2012 and Zhou et al. 2012).

Plant growth promotion characteristics

Several plant growth-promoting properties were evaluated in vitro (Table 1). A clear zone surrounding the BA5 colony on PKV agar medium (Fig. 2g) indicated the phosphate solubilizing activity of the isolate. The diameter of the halo zone was 3 mm, and the phosphate solubilizing efficiency (S.E.) was 62.5%. Zinc solubilizing activity was indicated by the formation of a clear halo zone (8 mm; S.E. 47.05%) surrounding the colony on modified PKV agar medium supplemented with insoluble ZnO (Fig. 2h). Organic acid production was evident by the change of the color of bromothymol blue indicator from blue to orange-yellow (Fig. 2i) due to a decrease in the pH of the growth medium. No growth on Norris nitrogen-free glucose medium suggested the absence of nitrogenase activity in the isolate BA5. The presence of a light yellow color after the addition of Nessler's reagent to peptone water culture of isolate BA5 indicated the production of ammonia (Fig. 2j). The absence of pink/red color upon addition of Salkowski reagent to the culture supernatant indicated no IAA production by the isolate BA5. The trace of crystal violet in the Eppendorf tube (Fig. 2k) was indicative of in vitro biofilm formation by isolate BA5, suggesting its potential colonization ability in plant roots.

Phosphorus is one of the key mineral nutrients required for the growth and yield of agriculturally important crops. Phosphate solubilizing bacteria solubilize mineral phosphate in nature by secreting organic acids and/or enzymes (Paul and Sinha 2017). Change in the color of the bromothymol indicator from blue to yellow-orange was suggestive that phosphate solubilization by isolate BA5 was probably due to the production of organic acid(s). Phosphate solubilizing *Pseudomonas* sp. was previously reported from rhizoplane of rice in Bangladesh (Islam et al. 2007). Zinc solubilization in *P. aeruginosa* and *P. fluorescens* has been reported by Bapiri et al. (2012). Several workers have described biofilm formation in plastic Eppendorf tubes (Zhou et al. 2012).

Identification of the selected isolate

The morphological, biochemical, and physiological characteristics of isolate BA5 (Table 2) were typical properties of species *Pseudomonas aeruginosa* (Stanier et al. 1966 and Liu 1952). When the morphological, biochemical, and physiological data were submitted in the ABIS online bacterial identification tool (Costin and Ionut 2017), the studied characteristics showed 92% similarity with *P. aeruginosa* (100% accuracy). Finally, the systematic affiliation of the isolate was confirmed by16S rRNA gene sequencing. The amplified PCR product of the 16S rRNA gene showed a band approximately at 1.5 kb. A 710-bp 16S rDNA partial sequence of isolate BA5 was subjected to compare using BLAST and suggested a close relationship with *P. aeruginosa* (99% similarity). Phylogenetic analysis indicated that isolate BA5 formed a clade with reference *P. aeruginosa* strain LFII sequences at a bootstrap value of 94% (Fig. 3). The 16S rDNA sequence was submitted in the NCBI GenBank under the strain name RKA5, and an accession number (MG786551) was received.

Table 2 Morphological, physiological, and biochemical characteristics of isolate BA5

Test	Result
Morphological properties	
Colony morphology[a]	
Shape/form	Round
Size	Large
Surface	Smooth
Elevation	Unbondate
Margin	Entire
Opacity	Opaque
Fluorescent	Positive
Degree of growth	Profuse
Cell morphology	
Gram reaction	Gram (−)
Shape	Rod
Motility	Motile
Physiological properties	
Growth at temperature(°C)	
4	−
25	+
37	+
42	+
50	−
Growth at osmotic pressure	
NB + 0.5% NaCl[b]	+
NB + 1% NaCl	+
NB + 3% NaCl	+
NB + 5% NaCl	+
NB + 7% NaCl	−
Growth at pH	
4	−
5	+
7	+
9	+
10	+
11	−
Growth in selective media	
Endo agar	−
MacConkey agar	+
Mannitol salt agar	−
Cetrimide agar	+
Utilization of organic compounds as sole source of carbon and energy	
Carbohydrates sugar derivatives	
D(+)-Glucose	+
D(−)-Fructose	+
L(+)-Arabinose	−
D-Xylose	−
L(+)-Rhamnose	−
D(+)-Mannose	−
Sucrose	−
Trehalose	+
Maltose	−
Gluctose	−
Inulin	−
Lactose	−
Starch	−
CMC[c]	−
Polyalcohols and glycols	
D(−)-Mannitol	+
D-Sorbitol	−
Meso-inositol	−
Glycerol	+
Organic acids	
Potassium acetate	+
Na-K tartrate	−
Tri-sodium citrate	+
Alcohols	
Methanol	−
Ethanol	+
Iso-amyl alcohol	+
Amino acids	
Glycine	−
L-Asparagine	+
L-Tryptophan	−
Miscellaneous	
Phenol	−
Tween 20	+
Biochemical properties	
Oxidase	+
Catalase	−
Methyl red	−
Voges-Proskauer	−
Methylene blue	+
Indole production	−
Citrate utilization	+
Urea hydrolysis	−
Nitrate reduction	+
H_2S production	−

Table 2 Morphological, physiological, and biochemical characteristics of isolate BA5 *(Continued)*

Test	Result
Casein hydrolysis	+
Starch hydrolysis	–
Fermentation and acid production from	
Glucose	+
Fructose	–
Xylose	+
Sucrose	–
Lactose	–
Starch	–
Mannitol	+
Sorbitol	–
Meso-inositol	–
Glycerol	+

[a]In King's B agar medium after 24 h of culture at 30 °C. [b]*NB* nutrient broth. [c]*CMC* carboxymethyl cellulose. "+" positive for growth and/or activity. "–" no growth or activity

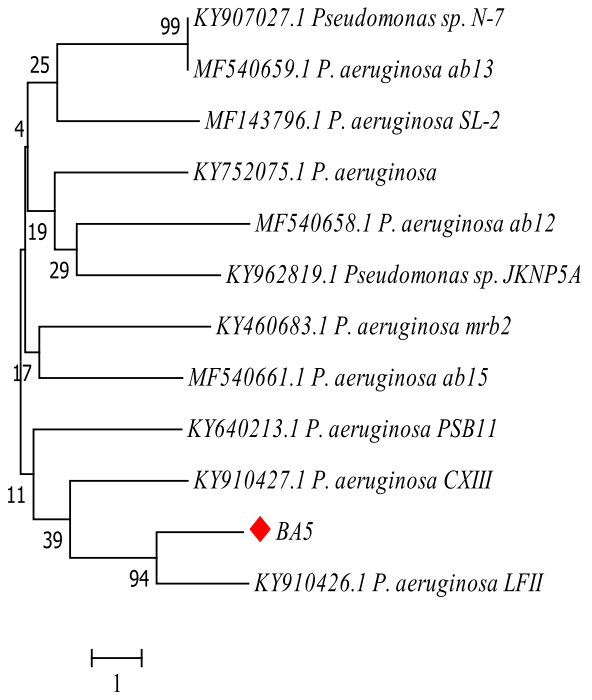

Fig. 3 Neighbor-joining phylogenetic tree showing relationships between isolate BA5 (red rectangular) and several other strains of *Pseudomonas aeruginosa* based on their 16S rRNA gene sequences. The tree was constructed in MEGA7 using the neighbor-method with 1000 bootstrap replicates

Conclusion

An attempt was made to isolate rhizobacteria with strong antagonistic activity against the cucumber wilt pathogen *Foc*. The isolate BA5 was a prominent antagonist against the pathogen and was able to produce various antagonistic compounds, including siderophores and VOCs, as well as it showed plant growth promotion potentials in vitro. The findings suggest that the selected isolate has the potential to be used as a biocontrol agent in the management of Fusarium wilt in cucumber. Nevertheless, field trial is needed to determine the disease suppression efficiency of the isolate in the natural soil environment.

Abbreviations

CFCF: Cell-free culture filtrate; CLP: Cyclic lipopeptide; *Foc*: *Fusarium oxysporum* f. sp. *cucumerinum*; HCN: Hydrocyanic acid; IAA: Indoe-3-acetic acid; KBB: King's B broth; NB: Nutrient broth; PDA: Potato dextrose agar; PDB: Potato dextrose broth; PKV agar: Pikovskaya's agar; TSA: Tryptone soya agar; TSB: Tryptone soya broth; TYEB: Tryptone yeast extract broth; VOCs: Volatile compound(s)

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MRI and MAI conceived and designed the experiments. MAI performed the experiments. ZN and MAI analyzed the data. MAI and ZN wrote the paper. MRI, MKA, and NAB contributed to the critical review and editing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors consent to publish this article in the Egyptian Journal of Biological Pest Control.

Competing interests

The authors declare that they have no competing interests.

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