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# ISOLATION OF RICE ENDOPHYTIC BACTERIAL STRAIN VY81 AND STUDY ON ITS BIOACTIVE COMPOUND ANTAGONIZING THE PHYTOPATHOGEN *DICKEYA ZEAE*

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SUMMARY

The bacterial communities performing endophytic lifestyle have been proven to possess a number of characteristics useful to host plants and thus are considered as “plant probiotics”. Many probiotic bacteria were reported for antagonism against different plant pathogens, including bacteria, fungi, and nematodes. The use of endophytic bacteria as biocontrol agents would have great potentials, allowing reducing the use of agrochemicals and thus support a sustainable agriculture.

In this study, endophytic bacteria isolated from rice plants of IR4625 cultivar from Long An province, Vietnam were used for screening strains that have antagonistic activity against *Dickeya zeae* (*Dz*), the bacterium causing foot rot disease. The rice plants had foot rot disease symptoms, i.e. dark-brown foot with odor smell typical for bacterial infection. Strain VY81 was isolated from a surface sterilized rice stem sample adjacent to the foot rot area. The crude extract of strain VY81 showed significant antagonistic activity against *Dz*, with the inhibition zone of 14,25 mm ± 1,06 in diameter. Strain VY81 produced the compound antagonizing *Dz* at maximal level after 48 h cultivated in TSB medium. The activity was found mainly in the culture broth, just a small part was found intracellularly. The bioactive compound antagonizing *D. zeae* produced by strain VY81 was purified by HPLC and analyzed by mass spectrometry and NMR spectroscopy. The compound was identified as a quinoline alkaloid, the chemical formula is $C_{17}H_{21}ON$ with chemical name 2-(2-heptenyl)-3-methyl-4(1H)-quinolone. Comparative analyses of the 16S rDNA gene sequence revealed that strain VY81 belonged to the genus *Burkholderia*, most closely related to *Burkholderia cepacia* (99,77% sequence homology). The 16S rDNA sequence of strain VY81 was deposited at GenBank under accession number MW056196. Strain VY81 and its quinolone compound would have application potential for development of biocontrol product against the foot rot disease caused by *Dickeya zeae*.

**Keywords:** Biocontrol, *Burkholderia cepacia, Dickeya zeae*, endophytic bacteria, quinoline, rice foot rot disease

## INTRODUCTION

The genus *Dickeya* includes pectin-utilizing species, belonging to the family *Enterobacteriaceae*. *Dickeya* spp. causes disease in monocot and dicot plants (Samson *et al*., 2005; Ma *et al*., 2007). *Dickeya zeae* (*Dz*, former *Erwinia chrysanthemi* pv. *zeae*) has been identified as the cause of rice foot rot, corn stalk rot, banana and ornamental plant soft rot in various regions of the world (Pu *et al*., 2012; Bertani *et al*., 2013; Zhang *et al*., 2014; Kumar *et al*., 2017; Hu *et al*., 2018). Corn stalk rot has been reported in the US, Brazil, France, Italy, Senegal, Cuba, Egypt, Mexico, India, Korea, Iran, Japan, China and Thailand (Li *et al*., 2020). Rice foot rot disease has mainly occurred in southern China resulting in ~ 10 to 30%, even 60% losses ofrice yield. The disease threatens other rice growing regions in Southeast Asian and European countries (Bertani *et al*., 2013; Hu *et al*., 2018). Banana soft rot disease caused by *Dz* has been found in Ivory Coast, Jamaica, Panama and Martiniue (Samson *et al*., 2005; Hu *et al*., 2018). In China, soft rot disease has become serious in over 6000 ha of banana plantation from 2009 to 2012 (Lin *et al*., 2010; Zhang *et al*., 2014). The natural host range of *Dz* has been extended to hyacinth and lily (Jafra *et al*., 2009; Hu *et al*., 2018). The main virulence factors of *Dickeya* spp. are cell wall degrading enzymes that lead to soft rot. This phytopathogen can be spread out through water, stays on weeds and plant residues, leading to difficulty in controlling the disease (Samson *et al*., 2005). In Vietnam, *Dz* mainly attacks rice and dragon fruit trees with symptoms such as stem rot, green wilt (Nguyen Van Hoa *et al*., 2015; Tran Hung Minh *et al*., 2016). Rice foot rot was first reported in Tieu Can, Tra Vinh in 2000, and then it spread rapidly throughout the Mekong Delta provinces. The infected rice plants had rotten roots and stalks that turned dark brown with foul odor, leading to the loss of a clump or the whole field (Tran Hung Minh *et al*., 2016).

Application of biological control agents in the fight against microbial phytopathogens (bacteria and fungi) has become attractive in terms of reducing the use of agrochemicals (O'Brien 2017). Jafra *et al*. (2009) reported that *Rahnella aquatilis* and *Erwinia persicinus* had high antagonistic activity against *Dz* infected hyacinth. Recently, Li *et al*. (2020) demonstrated that *B. subtilis* A2 isolated from root of the guzmania rondo tree had a suppressing effect on stinging rot of host plant. Other bacterial strains, i.e. *Pseudomonas fluorescens* SC3, *P. parafulva* SC11 and *Bacillus velezensis* 3–10 isolated from healthy root and stem of cabbage, ginger, banana, rice, as well as rhizosphere soil were also shown to be new potential biological control agents against *Dz* (Li *et al*., 2020).

In Vietnam, only a few researches on biological antagonistic activity of microbial strains against *Dz* causing rice foot rot have been published. Tran Vu Phuong and Phung Thi Thanh Thao (2015) reported three strains *Bacillus* sp. B57, B54 and B128.2 that were able to inhibit *Erwinia chrysanthemi* with the inhibitory zone of 8.14 mm, 7.57 mm and 7.52 mm in radius, respectively. In another study, Tran Hung Minh *et al.* (2016) investigated the effects of bacteriophages on 14 strains of *E. chrysanthemi* isolated from rice rot roots. The study has identified 35 bacteriophages parasitizing 14 strains of *E. chrysanthemi*, among those 8 bacteriophages showed multi-host infection. Of special interest was the bacteriophage ΦEchKG8b that showed the most efficiency in controlling the disease under net house conditions (Tran Hung Minh *et al*., 2016).

In this study, we isolated rice endophytic bacteria and selected strains that have antagonistic activity against *Dz* for application in controlling the foot rot disease. The endophytic strain VY81 isolated from stem of a rice plant with foot rot disease was studied in details since it had prominent antagonistic activity against *Dz*. The taxonomic position of the strain was identified based on 16S rDNA sequence comparative analyses and the *Dz* inhibiting bioactive compound produced by the strain was purified and its chemical structure was determined.

MATERIAL AND METHODS

## Materials

Rice plants (cultivar IR4625) were harvested in Spring-Summer season 2018 in Long An province for the isolation of endophytic bacteria. The strain *Dickeya zeae* DZ2Q was kindly provided by Professor Vittorio Venturi (International Center for Genetic Engineering and Biotechnology, Trieste, Italy).

## Isolation of rice endophytic bacteria

Rice plants were washed carefully under tap water and the upper stems were cut into segments of 500 - 1000 mg. The stem samples were surface sterilized by submerging successively in 75% ethanol for 2 min, then in 50% hypochlorite (7% of active Cl) for 2 min and again in ethanol 75% for 1 min. Between each of these surface sterilization steps and at the end of the treatment procedure, the samples were rinsed carefully with sterile water (5 times). Efficiency of the surface sterilization procedure was controlled by plating 0.1 mL of the final wash as well as a piece of the sterilized roots on Tryptic Soy Agar (TSA) plates and checked for bacterial growth in the next 72 h. These plates were used as epiphytic controls for selectively picking endophytic colonies from the root samples (Bertani *et al*., 2016).

To release the endophytic bacteria, the surface sterilized stems were macerated in 10 mL of PBS 1× solution by using sterile mortar and pestle, then the suspension was diluted with PBS 1×. Aliquotes of 50 µL from different dilution levels were plated on 1/5 TSA plates and incubated at room temperature for 5 days. Single colonies showing distinct morphology in comparison to the epiphytic controls were selected and purified again by streaking on 1/5 TSA plates, then stored at −80°C in 18% glycerol/PBS for further experiments.

## DNA extraction, PCR amplification, sequencing and phylogenetic analysis

The bacterial genome DNA was extracted using the mini column Bacterial DNA Kit (Omega, USA). Amplification of 16S rRNA gene was performed with primer pairs 27F and 1492R (Weisburg *et al*., 1991). Prior to sequencing, the PCR products were purified with QIAquick PCR purification Kit (Qiagen, Germany). The sequencing was performed on an ABI 3110 Avant Applied Biosystems sequencer (ABI, USA). The 16S rDNA sequences were compared with related sequences available on the GenBank by using the BLAST Search tool. The alignment of sequences was performed by using CLUSTAL_X program, version 1.8 and a phylogenetic tree was reconstructed using the neighbor-joining method (Saitou, Nei, 1987). Topography of the reconstructed tree was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

## Determining antagonistic activity against *Dickeya zeae*

*Dickeya zeae* strain DZ2Q was cultured in Tryptic Soy Broth (TSB) (Himedia, India), shaken 160 rpm/min at 30°C for 24 h and used as testing pathogen. TSA was sterilized, let to cool down to below 50°C, then pre-grown culture of *Dz* was added (1%, vol/vol), gently shaken and poured into Petri dishes. To screen for the *Dz* antagonizing activity, the endophytic bacterial (EB) strains were cultured on TSA 1/2 plates. Agar discs with EB colonies (diameter 5 mm) were placed on the Petri dishes containing the *Dz*, the distances between the agar disks were ~ 3 cm (Jiménez-Esquilín *et al*., 2005).

Crude extracts or extracted fractions obtained from HPLC were examined on *Dz* inhibitory activity by agar-well diffusion method (Magaldi *et al*., 2004). Aliquotes of 50 µL of sample were dripped into 5 mm diameter wells created on agar plates containing *Dz*. The plates were incubated for 24 h at 30ºC and *Dz* antagonism was determined by the size of the clearance zone (ΔD) formed around the agar wells according to the following equation:

$$\Delta D = D - d$$

where: D is the diameter of the antibacterial zone (mm); d is diameter of the agar well (mm).

**Extraction of the *Dz*-inhibiting bioactive compound from strain VY81**

Culture of strain VY81 was centrifuged at 4000 rpm for 20 min to remove cell biomass. Four different solvents, i.e. ethyl acetate, butanol, ethanol and n-hexane were tested for the extraction of the bioactive compounds. The solvent (each of the four) was added to the culture broth in a 1: 1 ratio and mixed (3 replicates). Afterward, the solvent and water phases were separated and the crude extracts were recovered from the solvent by evaporation in vacuum evaporator at 30°C. In addition, ethanol was used to extract bioactive compounds from the cell biomass. The ethanol and cell biomass mixture was centrifuged and the supernatant was subjected to vacuum evaporation. The crude extracts were then re-dissolved in 15% acetonitrile ($CH_3CN$) and tested for anti-*Dz* activity. The experiment was carried out with culture broth of strain VY81 at different growth time (1-7 day) (Roitman et al., 1990).

**Purification and structural determination of the *Dz*-inhibiting bioactive compound from strain VY81**

The crude extract was transferred to gel chromatography using C18 column (Agilent, USA). $CH_3CN/H_2O$ was used as mobile phase in gradient elution of 15%, 30%, 45%, 60%, 80% and 100% (2× repetitions). The fractions were then tested for anti-*Dz* activity. The fraction with the best activity was then analyzed by HPLC using Eclipse Plus C18 column (150 × 4.6 mm; 3 µm) (Agilent, USA), with $CH_3CN$ concentration increased from 15 - 85% in 35 min at a rate of 1.2 mL/min. Each fraction (1 min/fraction) was collected and tested for anti-*Dz* activity. The peak corresponded to the fraction with the best inhibitory activity was collected, dried by lyophilization. Chemical structure of the compound was analyzed by mass spectrometry (Agilent, USA) and nuclear magnetic resonance spectroscopy (NMR) (Bruker, USA).

RESULTS AND DISCUSSION

**Isolation of rice endophytic bacteria and screening for *Dz*-inhibiting activity**

Total 70 EB strains were isolated from different plant parts (root/stem/leaf) of the rice cultivar IR4625 planted in Long An in Spring-Summer season 2018. Strain VY81 was isolated from surface sterilized stem, adjacent to the rot area. The strain possessed prominent activity against *Dz* (Figure 1A) as shown in the screening experiment. Strain VY81 had round (diameter of 2 – 3 mm), slightly convex, uniform, glossy, light yellow colonies when grown in TSA after 48 h at 30 °C. Cells were of 1,4 – 2,2 × 0,5 – 0,6 µm in size, non-motile as observed under phase-contrast and scanning electron microscope (Figure 1B), Gram-negative bacillus. Comparative analyses of 16S rRNA gene sequence indicated that strain VY81 belonged to the genus *Burkholderia*, most closely related to *Burkholderia cepacia* (99.78% sequence homology) (Figure 1C). The nearly full length of 16S rRNA gene sequence of strain VY81 was deposited at GenBank under accession number MW056196.

*Burkholderia* spp. have been reported to be plant EB, providing various benefits to the host plants such as modulating growth and stress, related phytohormones and nitrogen fixation (Doty *et al*., 2016). In addition, *Burkholderia* spp. that grow in the rhizosphere are also capable of decomposing toxic pollutants and/or inhibiting phytopathogens via production of variety of secondary metabolites such as antibiotics, enzymes (Suárez-Moreno, 2012). Potential applications of *Burkholderia* spp. in agriculture to promote plant growth and control pathogens have been reported recently (Paungfoo-Lonhienne *et al*., 2014; Bernabeu *et al*., 2015).

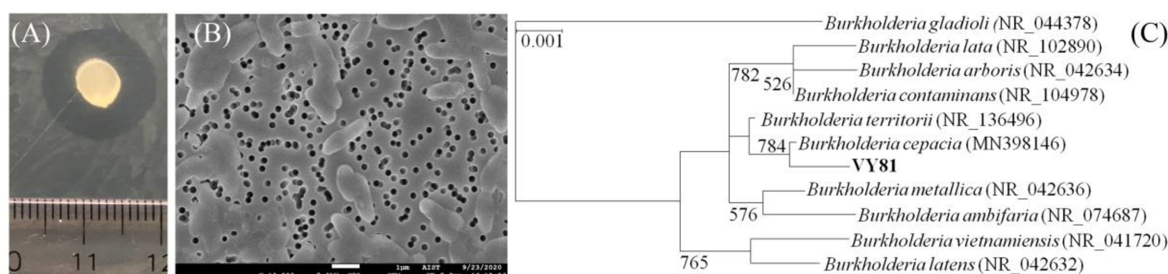


**Figure 1.** Antibacterial activity against *Dz*, cell morphology and taxonomic position of the rice endophytic bacterium VY81. (A) *Dz* inhibition zone (agar plug diffusion method); (B) Cell morphology under scanning electron microscope (SEM), magnification of 10,000×; (C) Taxonomic position based on 16S rDNA sequence comparison of strain VY81 with related species.

## Extraction and purification of *Dz*-inhibiting bioactive compound

All crude extracts from culture broth of strain VY81 using three solvents n-hexane, ethyl acetate and butanol showed antagonistic activity against *Dz* (Figure 2A). It is shown that ethyl acetate and butanol had similar extraction efficiency, whereas n-hexane produced a crude extract with ~ 40% lower antagonistic activity. The crude extract from cell biomass of strain VY81 with ethanol had low *Dz*-inhibitory effect, at the same level of the culture broth extracted by n-hexan, indicating that the target compound accumulated at higher concentration extracellular in the culture broth. Ethyl acetate was selected to perform extraction of the *Dz*-inhibiting compound since it has higher volatility than butanol, i.e. is more convenient of being removed by evaporation. Tests of crude extracts from culture broths of different ages also indicated that 48 hours was the best time for harvesting the target compound from culture of strain VY81 (Figure 2B).

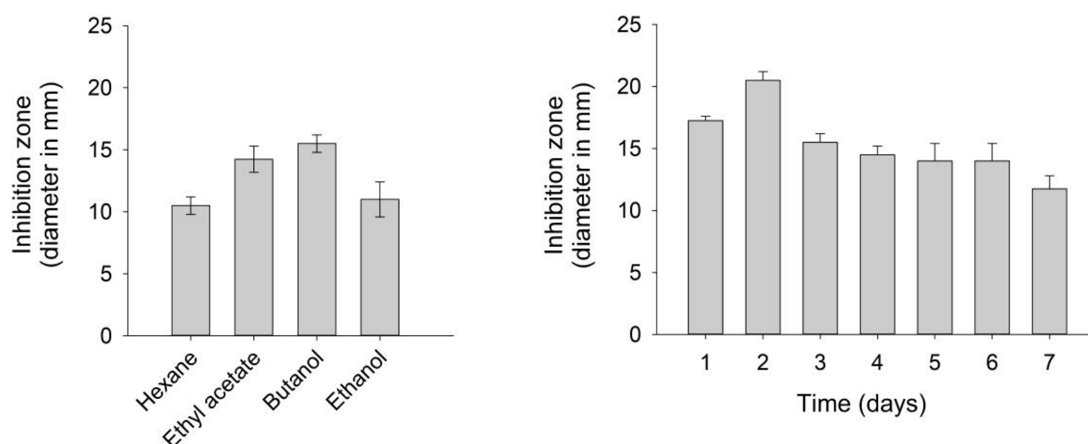


**Figure 2.** Extraction of bioactive compound from VY81 strain. (A) The extraction efficiency of the different solvents. (B) Anti-*Dz* activity at different culture times.

Strain VY81 was grown in TSB for 48 h and culture broth was treated with ethyl acetate, the crude extract was then recovered after evaporating the solvent and re-dissolved in 15% ACN. The first purification step was performed using C18 gel chromatography column, eluted

with different concentrations of $CH_3CN$ (15%, 30%, 45%, 60 %, 80% and 100%). The highest *Dz*-inhibiting activity was observed at the 60% $CH_3CN$ fraction.

This fraction was then selected for the next purification step using C18 HPLC. The highest *Dz*-inhibiting activity was observed at fraction 17, therefore, the single HPLC peak at 16.2 – 16.8 min was collected. This experiment was repeated 100 times to obtain 20 mg pure compound for further analyses.

**Determination of chemical structure of the *Dz*-inhibiting compound from strain VY81**

Compared with previously published researches, Dz-inhibiting compound produced by strain VY81 has a characteristic UV absorption spectrum (Figure 3A), similar to that of compound 2-(2-heptenyl)-3-methyl-4-quinolinol (chemical formula $C_{17}H_{21}ON$) (Hashimoto, Hattori, 1967) (Figure 3B). The double branching in the 320 - 340 nm region is characteristic for the 4-quinolinol derivatives (Hashimoto, Hattori, 1967). In 1990, Roitman *et al*. reported on compounds 2-(2-heptenyl)-3-methyl-4(1H)-quinolone with UV spectrum similar to that of Hashimoto and Hattori (Roitman*et al*., 1990).

The purified target compound was lyophilized to remove the solvent and was then analyzed for chemical structure by electrospray ionisation mass spectrometry (ESI-MS) and $^1$H-NMR and $^{13}$C-NMR. Mass spectrometry analysis showed that the *Dz*-inhibiting compound produced by strain VY81 had m/z of 255.8 $[M + H]^+$. Meanwhile, the nuclear magnetic resonance spectrum analysis showed that the compound had chemical formula $C_{17}H_{21}ON$ and chemical name 2- (2-heptenyl) -3-methyl-4 (1H) - quinolone (HMQ), similar to the compound (Figure 4B) reported by Roitman *et al*. (1990).

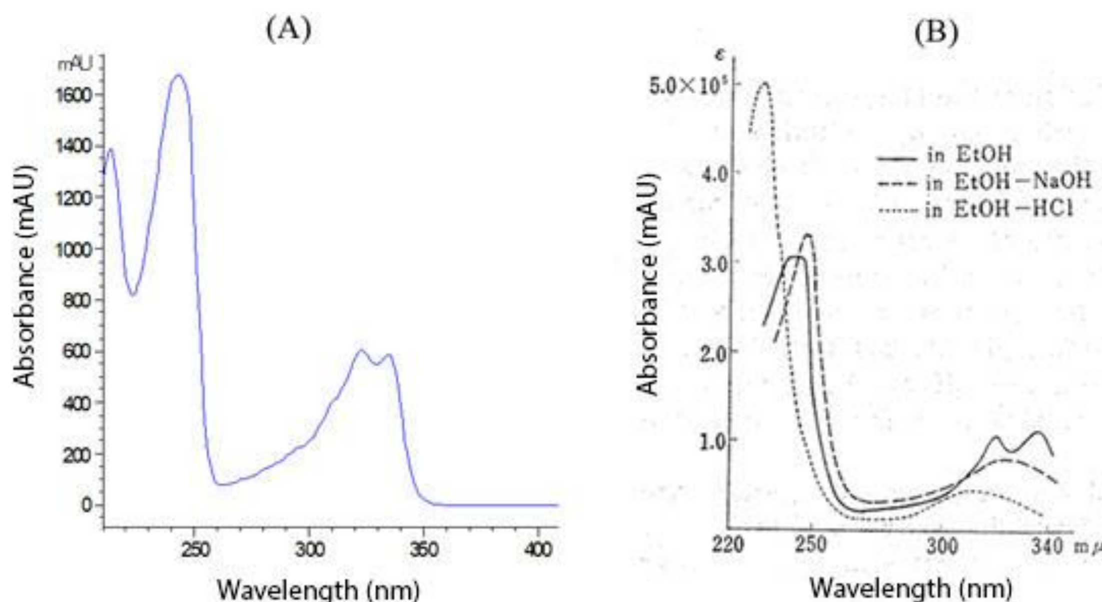


**Figure 3.** UV absorption spectra of anti-*Dz* bioactive compound from VY81 strain (A) and 2- (2-heptenyl) -3-methyl-4-quinolinol (B) (Hashimoto & Hattori, 1967).

The compound 2-(2-heptenyl)-3-methyl-4-quinolinol was extracted for the first time with acetone from *Pseudomonas pyrrocinia* in 1967. Mass spectrometry, UV spectroscopy and NMR spectroscopy showed that this compound has a molecular weight of 255, chemical formula $C_{17}H_{21}ON$, chemical structure as shown in Figure 4A (Hashimoto, Hattori, 1967). The

results of extraction from the culture of *Pseudomonas cepacia* strain RB425 (later *Burkholderia cepacia* RB25) isolated from lettuce roots by chloroform/ethyl acetate/benzene also showed similar results (Homma *et al.*, 1989). This alkaloid compound exhibits antagonistic activity against many fungal and bacterial plant pathogens, including *Pyricufaria oryzae*, *Rhizoctonia solani*, *Pythium ultimum*, *Furasium oxysporum*, *Verticillium dahliae*, *Gaeumannomyces graminis*, *Cochliobolus miyabeanus* (Homma *et al.*, 1989). This compound is a derivative of compound 2-(2-heptenyl)-3-methyl-4(1H)-quinolone extracted from *P. cepacia* LT4.12-W (now is *B. cepacia* LT4.12W) isolated from apple leaves using acetone and methanol solvents (Roitman *et al.*, 1990). The crude extract was analyzed by antiphase HPLC C18 column with the mobile phase $CH_3CN/H_2O$ (3:2, v/v). The obtained bioactive compound was purified by HPLC cyanosilica column with the mobile phase CHCl/hexane (1:1). The resulting compound is colorless crystal, $^{13}C$-NMR, δ10.6 (C3-Me), 13.9(C7'), 22.2 (C6'), 31.3 (C5'), 32.2 (C4'), 35.6 (Cl'), 115.6 (C3), 117.3 (C8), 123.0 (C6), 123.3 (C3'), 123.6 (ClO), 125.8 (C5), 131.1 (C7), 135.5 (C2'), 139.2 (C9), 147.9 (C2), 178.0 (C4). The UV spectrum was similar to that of Hashimoto and Hattori, 1967 (Figure 3B) (Roitman *et al.*, 1990). The physical constants of the bioactive compound produced by VY81 strain were similar to that of this compound, so the active ingredient extracted in this study was 2-(2-heptenyl) -3-methyl-4(1H)-quinolone.

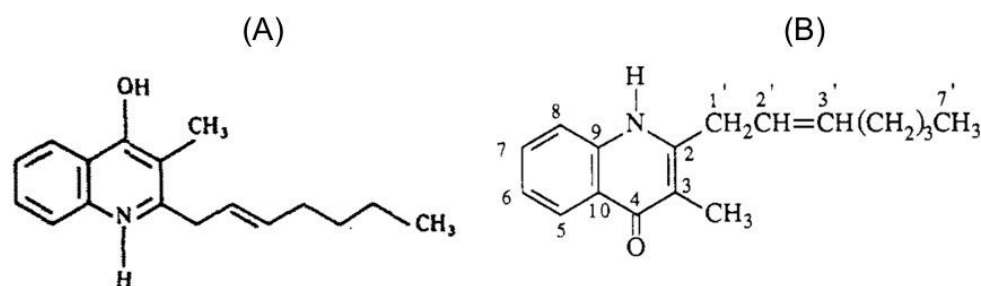


**Figure 4.** Chemical structure of (A) 2-(2-heptenyl)-3-methyl-4-quinolinol (Hashimoto & Hattori, 1967) and (B) 2-(2-heptenyl)-3-methyl-4(1H)-quinolone (Roitman *et al.*, 1990).

HMQ belongs to the quinoline alkaloids group of heterocyclic aromatic compounds with many important biological activities. Many alkaloid quinoline compounds have been discovered from natural sources, including several families of the plant kingdom, as well as from animals and microorganisms (Kshirsagar *et al.*, 2015). Quinine was the first quinoline alkaloid compound isolated from the quinquina phloem (*Cinchona* spp.) in 1820 and used as a substitute for crude phloem in malaria treatment (Shang *et al.*, 2018). Quinoline alkaloids containing pyrrole extracted from *Streptomyces* spp. were inhibitory to tumors and cancer cell lines. Quinoline alkaloids and their derivatives were widely used in medicine and agriculture, notably anti-malarial drugs (Quinine, Quinidine, Chloroquine, Mefloquine, etc.), antiviral (Saquinavir, etc.), anti-cancer (Camptothecin, Irinotecan, Topotecan, Gefitinib, etc.), antipsychotic drugs (Aripiprazole, Brexpiprazole, etc.), antiglaucoma (Cartiolol) and cardiotonic (Vesnarin) (Selvan *et al.*, 2011; Afzal *et al.*, 2015; Tiwary*et al.*, 2015; Patel *et al.*, 2017).

The endophytic bacterium *B. cepacia* VY81 isolated in this study is evidently capable of biosynthesizing quinolone, which effectively inhibits *Dickeyazeae*. The EB strain and its quinolone-derivative compound are supposed to

have potential applications in biological control of the rice foot rot disease caused by *Dz*. Using natural compounds that antagonize phytopathogens has become an indispensable trend in sustainable agricultural development. The results of this study also showed that plant endophytes are potential sources to search for bioactive compounds supporting organic agriculture.

## CONCLUSION

Endophytic bacterial strain VY81 isolated from rice stem exhibited high antagonistic activity against *Dickeya zeae*, the phytopathogen causing foot rot disease. Comparative analyses of 16S rRNA gene sequences allowed classifying strain VY81 into the genus *Burkholderia*, the most closely related species was *B. cepacia* (99.77% sequence homology). The nearly full length 16S rRNA gene sequence from strain VY81 was deposited at GenBank under accession number MW056196. The *Dz*-inhibiting compound produced by strain VY81 was purified and chemically determined as $C_{17}H_{21}ON$ with chemical name of 2-(2-heptenyl)-3-methyl-4(1H)-quinolones. The endophytic bacterial strain VY81 and its quinolone derivative product would have potential applications in producing microbial products for controlling the foot rot disease caused by *Dz*.

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