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Characterization of phosphate and potassium solubilization, and antifungal activity of bacteria isolated from rhizosphere of *Allium ascalonicum* (L.) grown in Ninh Hai district, Ninh Thuan province, Vietnam

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Abstract

Bacteria in the rhizosphere of red onion plants grown in Ninh Hai district, Ninh Thuan province were isolated, tested for P, K solubility and antifungal ability. Bacteria were isolated and cross-cultured on two media, NBRIP containing apatite and modified Aleksandrov containing mica. Dissolved P was measured molybdate colorimetric method and dissolved K was determined based on turbidity through present of sodium tetraphenylborate. Antifungal performance was limited to selected lines and was performed by co-culture. Bacterial identification was based on two-way sequencing for 16S rDNA sequences. The five best strains out of a total of 33 isolates were TH8, TH9, TH10, VH3 and VH5, which were able to dissolve phosphate in the range of 29.4 – 54.4 mg/L P2O5, and dissolved in potassium in the range of 13.0 – 26.4 mg/L K2O, and were resistant to *A. niger* with inhibition efficiency from 57% – 62.6% after 7 days of co-culture. Two strains VH5 and TH8 were also capable of antagonizing *F. oxysporum* with the inhibition efficiency of 60.2% and 70.7%, respectively. The identification results showed that TH9, TH10 and VH5 were homologous to *Enterobacter* sp., TH8 was homologous to *E. coli*, and VH3 was homologous to *Novosphingobium* sp. *Enterobacter* and *Novosphingobium* had been reported to promote plant growth in addition to salt tolerance and resistance to heavy metal pollution, so these bacterial strains could be applied to the cultivation of red onions in Ninh Thuan.

Keywords: *Allium ascalonicum* (L.); Antifungal activity; Ninh Thuan province; *Novosphingobium*; Phosphate and potassium solubilizing; Plant growth promoting bacteria

1. Introduction

Alliums ascalonicum (L.), commonly known as red onion or shallot, is a popular spice plant. Red onion was also a medicinal plant in Vietnamese folk medicine with spicy taste, neutral properties, non-toxicity, blood-activating effects, stimulating sweating, diuretic, anti-inflammatory, anti-infective [1]. It could also be used as a remedy for toothache, fever, headache and edema besides the effects of safe pregnancy, bright eyes, and good for the internal organs [1]. Studies also showed that red onions had antioxidant and free radical scavenging, antifungal properties, and antibacterial potential against *Helicobacter pylori*, the causative agent of stomach ulcers. The extract of red onion also had anti-cancer and anti-inflammatory properties [2, 3]. In Vietnam, Ninh Hai district, Ninh Thuan province was one of the four key purple onion growing areas with a cultivation area of about 470 hectares. This locality had suitable climate and soil conditions for growing red onions for bulbs [4, 5]. Onion bulbs were large, firm, fragrant, and long-lasting, so they are popular with consumers. Therefore, growing purple onions was one of the long-standing traditional occupations of farmers there and brought a good income. Towards the production of purple onions according to VietGAP (Vietnamese Good Agricultural Practices) standards and facilitated access to export markets with the orientation of local leaders, organic farming was a priority option [4, 5].

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In that approach, the role of biological fertilizers and biopesticides based on the application of PGPR (Plant Growth-Promoting Rhizobacteria) was highlighted. PGPR were bacteria that live in the rhizosphere of plants. They had properties that directly promoted plant growth such as nitrogen fixation, phosphate and potassium solubilization, and phytohormone production. PGPR also promoted plant growth through indirect mechanisms that kept plants healthy and adaptive to environmental stress [6]. Chemical fertilizers were the main input of inorganic phosphorus (P) in agricultural soils, but its supply was limited due to immobilization. Phosphorus immobilization was a pH-dependent process. When phosphate fertilizers were applied to the soil, negatively charged inorganic phosphate easily reacted with iron and aluminum ions in acidic soils or calcium ions in alkaline soils to form relatively immobile compounds that became unavailable to plants [7, 8]. In terms of potassium (K), depending on the soil type, about 90 – 98% of this chemical element existed in the soil as minerals such as feldspar and mica, and most of this potassium was not available for absorption by plants [9, 10]. Many soils were initially rich in potassium but then became deficient due to improper fertilization, leaching and soil erosion [11]. Phosphate solubilizing bacteria (PSB) improved the bioavailability of P by releasing phosphate-soluble mineral compounds, dissolving Pi and lowering soil pH e.g. organic acids, protons, hydroxyl ions. Other mechanisms included releasing of extracellular enzymes such as phosphatases for organic P mineralization, and/or chelating P from compounds of phosphate and Fe^{3+}, Al^{3+}, or Ca^{2+} [12, 13]. Potassium solubilizing bacteria (KSB) were able to dissolve potassium in rocks, synthesized potassium minerals, and converted insoluble potassium into soluble forms of potassium available for plant absorption through production and excretion of some organic or inorganic acids and specific enzymes [14, 15]. Bacterial growth promoting bacteria on red onion plants had been isolated and characterized, including nitrogen fixation, phosphate solubilization, and IAA synthesis [16, 17]. Selected strains were also able to increase vigor and viability of purple onion seeds [16], increase plant height, number of leaves, number of bulbs, fresh and dry weight of bulbs [18], increase growth rate and tolerance index [19]. Besides, for the antagonistic ability of PGPR, the study of Fernandes et al (2018) had shown the ability to control wilt disease caused by *Fusarium* on red onions [18]. *Fusarium* is the largest genus of fungi causing wilt disease for many crops. *Fusarium* spp. causing root rot for onions have been reported in Asia, Europe, America and Africa with damage rates ranging from 10% to 70% depending on stage, season and cultivation technique [20-22]. Meanwhile, *Aspergillus niger* was a common seed, soil and airborne pathogen that causes onion black mold in the field, during transport and storage [23]. In India, the percentage of red onions infected with black mold in the market ranged from 25.5 to 56%. Black mold disease also caused damage in the field with the rate of 8.9 - 10.1% [22].

In Vietnam, rot disease caused by *Fusarium* spp. and tuber rot during storage caused by *A. niger* on red onion were also listed in 10 fungal diseases [24]. Meanwhile, research on red onions in the field of PGPR was not much. Therefore, the study was conducted with the main content of isolating and identifying bacteria strains with phosphate and potassium solubilizing properties, antifungal ability from the rhizosphere of red onions grown in Ninh Hai district, Ninh Thuan province, one of the key red onion growing areas of Vietnam.

2. Research materials and methods

2.1. Samples collection and preparation

The location of Ninh Thuan province was at 11°18’ – 11°10’ North latitude and 108°39’ – 109°14’ East longitude. It had a typical tropical monsoon climate with hot, dry, windy, and strong evaporation characteristics [25]. Twelve rhizosphere soil samples were taken in 12 onion fields in Nhon Hai, Thanh Hai and Vinh Hai communes, Ninh Hai district, Ninh Thuan province. Sample preparation of rhizospheric soil was according to “shaking method” (ID: 13_Turpault) [26]. This method did not target rhizoplane bacteria.

2.2. Isolation and collection of phosphate and potassium solubilizing bacteria

The rhizospheric soil samples were homogenized with dilution factor of 100 in sterilized distilled water. After shaking 200 rpm (rounds per minute) for 12 hours and depositing for 3 hours, the supernatant was collected and spread on NBRIP [27] or modified Aleksandrov [28] agar plates. Morphologically distinct bacterial colonies were selected for further purifications. Subsequently, the pure isolates would be streaked reciprocally between those two media to collect isolates having two abilities of phosphate and potassium solubilization. These isolates were preserved temporarily in agar slant tubes at 4°C.

2.3. Morphological characterization of bacterial isolates

Colony morphology including form, elevation, margin, surface and size were recorded after 24 hours of growth on LB agar plates at 28 ± 2 °C. Cellular size, shape and mobile were observed by light microscopy. Gram staining and “KOH String Test” was performed to differentiate two groups of bacteria [29].

2.4. Quantification of phosphate solubilization

A loop filled with the biomass of each bacterial strain was added to a test tube containing 5 mL of liquid LB medium and subjected to a 120 rpm (rounds per minute) shaker, at 28 ± 2 ℃. After 48 hours, 1 mL of the bacterial suspension was transferred to a flask containing 99 mL of liquid LB medium and cultured for 24 – 48 hours to increase bacterial density. The bacterial suspension adjusted to a turbidity corresponding to the 0.5 McFarland standard (1.5×10^8 CFU/mL) would be considered the standard concentration for the experiments [30].

One milliliter of standard concentration bacteria was inoculated into a 50 mL flask containing 20 mL of liquid NBRIP medium and shaken at 120 rpm. The time points for suspension collection for soluble phosphate quantitation were 5, 10, 15 and 20 DAI (days after inoculation). Two milliliters of suspension were collected and centrifuged at 12,000 rpm in 5 minutes to obtain the supernatant for the next colorimetric analysis. The analysis was based on phosphomolybdate colorimetric method and the process description by Thanh and Tram [29].

2.5. Quantification of potassium solubilization

Method for the determination of potassium was turbidity measurement in the presence of sodium tetraphenylborate, a known potassium precipitating reagent. The reagent consists of 1,115 mL distilled water, 0.075 mL of screening agent solution 1 (solution 1: 4 g $CuSO_4.5H_20$ and 10 g tartaric acid dissolved in 700 mL H_2O, added 180 mL concentrated sulfuric acid and allowed to cool then settled to 1,000 mL with distilled water), 0.18 mL help and cover agent solution 2 (Solution 2: 65 g EDTA disodium and 140 g NaOH dissolved and settled to 1,000 mL with distilled water), and 0.13 mL turbidity agent solution (solution 3). Solution 3 was made by added 0.4 mL of sodium hydroxide (7.0 g of NaOH dissolved in 100 mL of distilled water) to 1000 mL of sodium tetraphenylboron (65.0 g of sodium tetraphenylboron dissolved and settled to 1,000 mL with distilled water) then shaken up, and filtered to clear the solution [31].

One milliliter of standard concentration bacteria was inoculated into a 50 mL flask containing 20 mL of modified Aleksandrov liquid medium and shaken at 120 rpm. The time points for suspension collection for soluble phosphate quantitation were 7, 14 and 21 DAI. Two milliliters of suspension were collected and centrifuged at 12,000 rpm in 5 minutes to obtain the supernatant for the next colorimetric analysis. Sample and reagent volumes were 0.5 and 1.5 mL, respectively. The absorbance of the sample was measured by spectrophotometer at 685 nm wavelength.

The pH of the culture solution was also measured at 21 DAI based on numerous studies suggesting that organic acid production was one of the mechanisms for potassium solubilization.

2.6. Investigation of antifungal ability

The antifungal activity investigation was limited to strains with good results for phosphate and/or potassium solubility using double inoculation method [32]. Two indicator fungal strains, *Aspergillus niger* and *Fusarium oxysporum*, were being stored at the microbiology laboratory of Saigon University. These indicator fungal strains were cultured on PDA (Potato Dextrose Agar) medium within 5 days to collect fungal discs (5 mm diameter). An indicator fungal disc was placed in the center of each PDA plate and two bacterial streaks were inoculated on either side. Plates were incubated at room temperature for 3 – 7 days then formation of the antifungal zones were observed. Inhibition efficiency of molds by bacteria were calculated according to formula H (%) = (R – r)/R × 100, where R was the radius of the fungus colony in the negative control treatment, r was the radius of the fungus colony on the experimental plate containing the bacterial strain. Hyphae of the molds in the treatments after the experimental period were also observed morphologically through optical microscopy.

2.7. Identification of selected bacteria

This experiment was limited to the bacterial strains that have the best results in the above tests. Selected bacterial DNA was isolated and amplified by primer pairs 27F and 1492R as described by Tam and Diep [32]. An amount of 10 µL of each PCR product was separated and visualized in 1% agarose gel using standard electrophoresis procedure. The satisfactory PCR products were sequenced 16S rRNA gene by 1st BASE Pte Ltd, Singapore (bi-directional sequencing). The sequencing results were processed by BioEdit software version 7.2 then compared with the reference sequences of the 16S rRNA genes contained in the GenBank of National Center for Biotechnology Information (NCBI) using Nucleotide BLAST tool. Query and reference sequences were used to learn the phylogenetic tree by Neighbor-Joining method with bootstrap 1,000 with the support of MEGA 11 software.

2.8. Experiment design and data analysis

The quantitative experiment was arranged in a completely randomized design with 3 replications. Negative controls were performed in a similar manner to treatments that did not use bacterial suspensions but instead with sterile culture media. One-factor analysis of variance and Duncan's test with the value α=0.05 with the support of IBM SPSS Statistics software version 20.0.

3. Results

3.1. Morphological characteristics of isolated bacteria

There were 40 bacterial strains which were isolated from NBRIP medium and 27 bacterial strains which were isolated from modified Aleksandrov medium. After cross-culture, there were 33 bacterial strains capable of growing on both media, including 15 strains isolated from Nhon Hai commune, 10 strains from Thanh Hai commune, and 8 strains from Vinh Hai commune. Bacterial strains grew on two types of media after 24 – 96 hours of culture. Most colonies were round, smooth, translucent or transparent. Some strains of bacteria produced clear soluble zones on turbid media due to insoluble P/K minerals (Figure 1). On LB agar medium, major morphological characteristics of colonies were circular (84.8%), entire (57.6%), convex (90.9%), milky white or ivory white in color (90.9%) and diameters of colonies ranged from 0.5 to 2 mm (93.9%). Through microscopic observation, the main shapes were short-rod and spherical, accounted for 30% of each type. Gram-negative bacteria accounted for 63.6% and most of the cells were motile (93.9%).

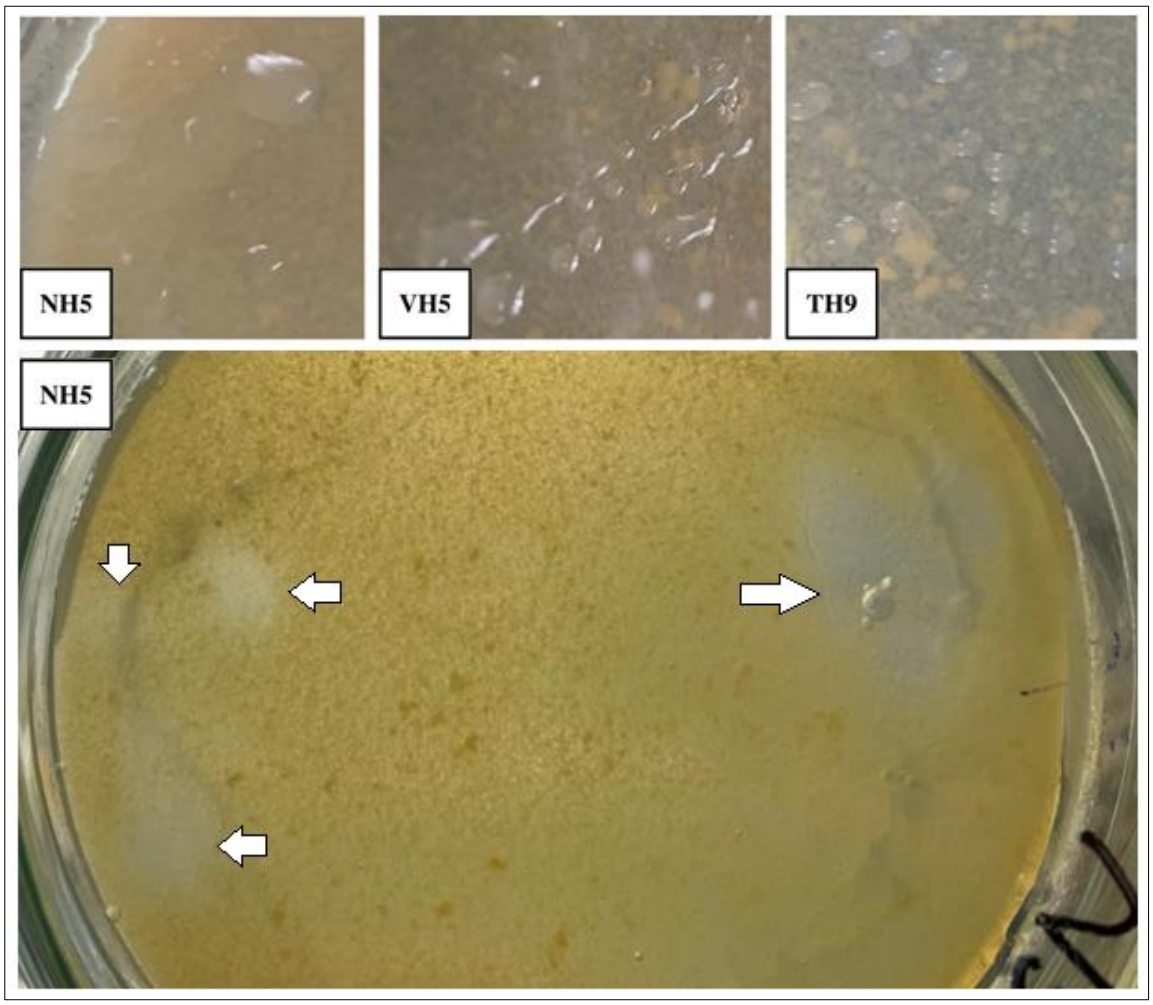
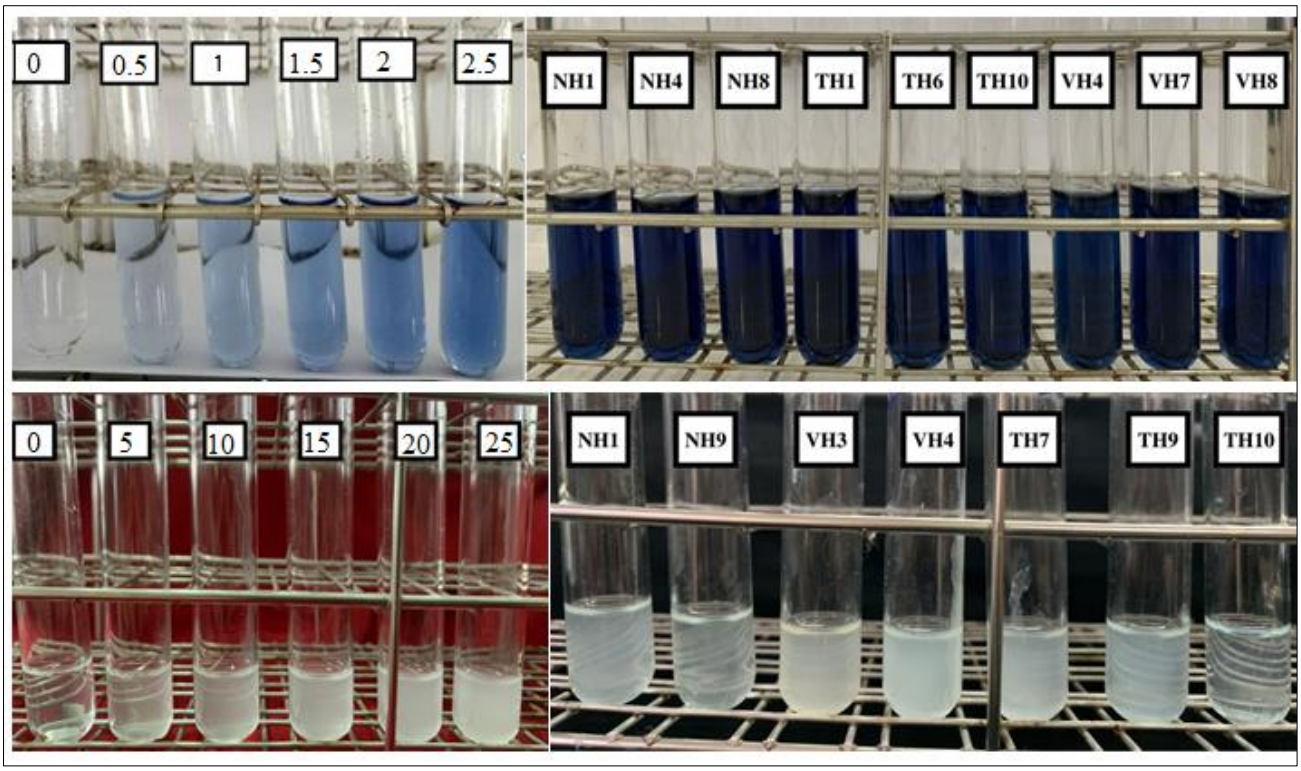


Figure 1 Colonies and halo zones of some strains growing on modified Aleksandrov medium

3.2. Quantitative results of phosphate and potassium solubilization

After the survey, the average results of phosphorus and potassium solubility of 33 bacterial strains ranged from 29.4 – 54.4 mg/L P_2O_5 and 13.0 – 26.4 mg/L K_2O respectively. The best phosphate solubilizing bacterial strains were mainly

isolated from Nhon Hai (NH) and Thanh Hai (TH) communes while the best potassium solubilizing bacterial strains were mainly isolated from Vinh Hai (VH) commune. The best time to dissolve minerals also varies between strains of bacteria. The best P-solubilization occurred at 5 DAI or 10 DAI while the best K-solubilization occurred at 14 DAI (Figure 2). The pH of the culture solutions at 21 DAI changed significantly from 7.5 (negative control) to 4.8 - 5.6.



Quantitative assay of dissolved-P at 5 DAI (above) and Quantification of dissolved-K at 21 DAI (below)

Figure 2 Standard concentration ranges (ppm) and reaction results of some experimental strains

Some of the best strains and the highest dissolved mineral values were shown in Table 1. In which, there were five strains NH15, TH7, TH8, TH9 and TH10 with the best P-solubilization at 5 DAI while five strains NH8, NH12, NH13, NH14 and TH4. Strains VH3, VH4, VH5, VH6 and VH7 had the best capability of K-solubilization at 21 DAI.

Table 1 Highest mineral solubility value of some prominent bacterial strains

P-solubilization (mg /L P2O5)				**K-solubilization (mg /L K2O)**	
Strains	**At 5 DAI**	**Strains**	**At 10 DAI**	**Strains**	**At 21 DAI**
NH15	45,55 $^{b-g}$	NH8	42,05 $^{b-e}$	VH3	41,48 ^{ab}
TH7	55,50 $^{a-e}$	NH12	46,35 $^{a-e}$	VH4	43,19 ^{a}
TH8	56,25 $^{a-e}$	NH13	47,75 $^{a-e}$	VH5	38,90 ^{b}
TH9	59,45 ^{ab}	NH14	52,35 ^{ab}	VH6	35,05 ^{c}
TH10	51,30 $^{a-g}$	TH4	58,00 ^{a}	VH7	43,96 ^{a}

In the same column, values followed by the same letter were not statistically significant according to Duncan's test

3.3. Fungal inhibition ability of selected bacterial strains

Five bacterial strains were selected based on the best phosphate and potassium solubility including TH8, TH9, TH10, VH3, and VH5. All 5 strains were resistant to *Aspergillus niger* with 57.0% to 62.6% yield after 7 days of co-culture.

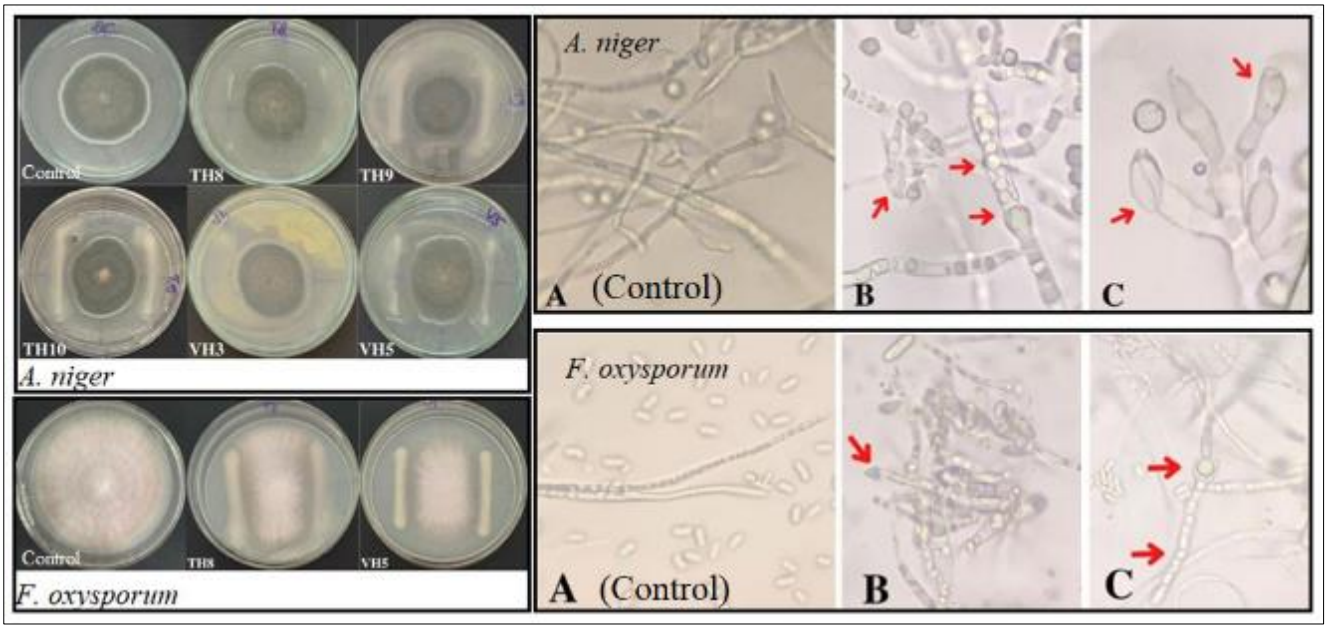
Meanwhile, only two strains TH8 and VH5 exhibited ability of resistance to *Fusarium oxysporum*, with an efficiency of 70.7% and 60.2%, respectively (Table 2).

Table 2 Information on mineral solubility and antifungal results of five selected bacterial strains

Strains	P-solubilization* (mg /L P2O5)	K-solubilization* (mg /L K2O)	Fungal inhibition efficiency	
			Aspergillus niger	Fusarium oxysporum
TH8	41.4	14.3	62.6%	70.7%
TH9	54.4	17.4	59.6%	-
TH10	51.6	16.5	57.0%	-
VH3	30.2	26.4	58.0%	-
VH5	37.7	25.9	58.9%	60.2%

(*): Average value of the samples (at 5 to 20 DAI for P-solubilization; at 7 to 21 DAI for K-solubilization). (-): Inability to antagonize fungi.

Observation under the microscope showed that the fungal strains *A. niger* and *F. oxysporum* under the influence of antagonistic bacteria strains had morphological and structural abnormalities such as hyphae heads were enlarged, septum were disappeared, cell walls were thickened, spores were degenerated or deformed (Figure 3).



(A) Mycelia and spores in the control (normal); (B, C) Abnormal mycelia in morphology and structure

Figure 3 Bacterial strains and fungal inhibition (left) and microscopic morphology of mycelia (right) of *A. niger* (above) and *F. oxysporum* (below) under the influence of antagonistic bacteria

3.4. Identification results of selected bacteria

The five strains that give good PCR results through electrophoresis were TH8, TH9, TH10, VH3 and VH5. Except for TH10 with reliable sequencing results of only 427 nucleotide (nt), the 16S rRNA gene sequencing results of the remaining 4 strains ranged from 1110 to 1380 nt. Query Coverage reached 93 – 100% and Percent Identity reached 85.11 – 99.63%. The following Table 3 presented the names of some reference strains included in the NCBI database with highly homologous 16S rRNA gene sequences compared with the query sequences.

The identification of phylogenetic relationships by MEGA 11 software had contributed to the identification of 5 selected bacterial strains. Thereby strains TH9 (T9-964nt) and TH10 (T10-427nt) were identified as *Enterobacter* sp. shared a node with bootstrap 91. TH8 (T8-1380nt) and *Enterobacter soli* (NR117547.1) shared a common node. All formed a small cluster that shared a node with VH5 (V5-1110nt) in the first cluster containing all strains identified as *Enterobacter*. Meanwhile, VH3 (V3-1351nt) shared a node with *Novosphingobium* sp. (JQ806458.1) with bootstrap 100

forming the second cluster, *Novosphingobium* (Figure 4). The evolutionary distances shown in the scale bar and the bootstrap percentages were shown at the nodes of the tree.

Table 3 Identification results of five selected strains by Blastn tool in the NCBI database

Strains (query sequence)	Query Cover	Per. Ident	Accession	Reference strain names
TH8 (T8-1380nt)	100%	99.50%	NR_117547.1	*Enterobacter soli* ATTC BAA-2102 strain LF7
	100%	99.49%	NR_119276.1	*Klebsiella pneumoniae* subsp. ozaenae strain ATCC 11296
TH9 (T9-964nt)	93%	85.27%	JF911353.1	*Klebsiella oxytoca* strain EPAn27-1
	93%	85.11%	MK834717.1	*Enterobacter* sp. strain TBMAX79
TH10 (T10-427nt)	98%	95.72%	HE984303.1	*Enterobacter* sp. strain MP7
	98%	94.54%	KU292621.1	*Enterobacter* sp. VH-28
VH3 (V3-1351nt)	99%	99.63%	MN232170.1	*Novosphingobium* sp. strain PR81
	99%	99.11%	JQ806458.1	*Novosphingobium* sp. SaMR4
VH5 (V5-1110nt)	99%	99.55%	KT183542.1	*Enterobacter* sp. D1SM198
	99%	99.55%	OM5702571.1	*Enterobacter cloacae* strain ZA14

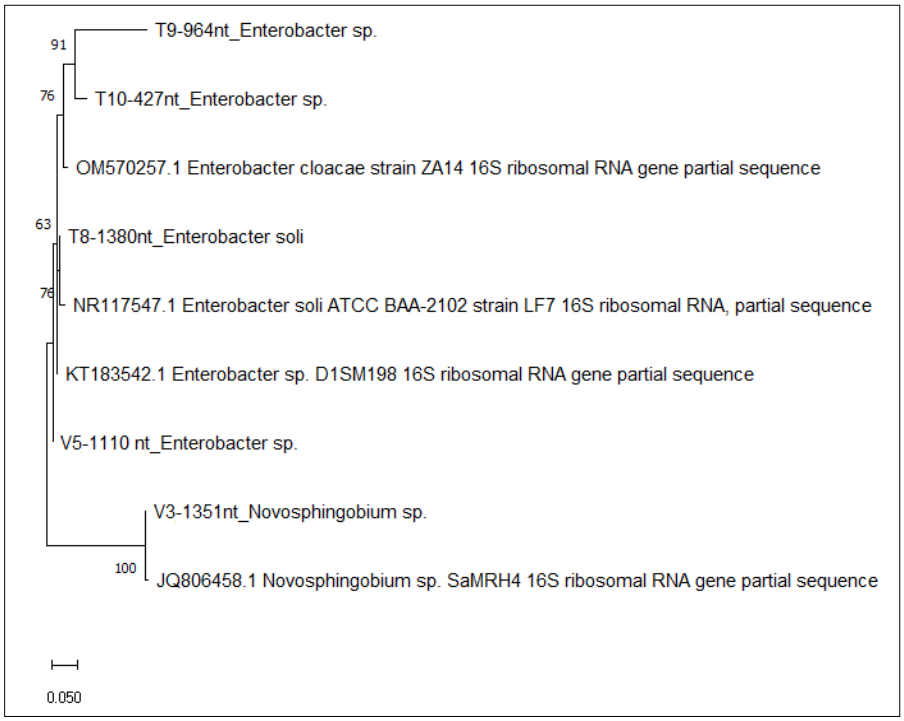


Figure 4 The phylogenetic tree showed the relative positions of five selected bacterial strains ith reference strains in the Gen Bank

4. Discussion

The morphological characteristics of colonies and cells, and Gram staining of 33 strains of rhizospheric bacteria of red onion in this study were similar to those reported on bacteria of plants grown in Vietnam and onions in particular [17]. It was noteworthy that Gram-negative bacteria had short rod shapes and difficulty distinguishing colony morphology

when the bacteria were grown on media containing insoluble phosphorus and potassium [29, 34, 35]. The time taken by *in vitro* culture to obtain the highest amounts of soluble P and K and a common mechanism of P and K dissolution through organic acid production that lowered pH had been reported in Vietnam and in the world [36-40].

Strains of *Enterobacter* such as *Enterobacter* sp. D1SM198, *E. cloacae* ZA14, *E. soli* LF7 and *Novosphingobium* sp. strain SaMRH4 (Figure 4) derived from plants or rhizospheric soils had good effects on soil structure or soil contaminated with heavy metals (NCBI, https://www.ncbi.nlm.nih.gov/) [41]. *Novosphingobium* was a Gram-negative bacillus genus with the ability to promote plant growth that had been isolated from the rhizosphere of many crops such as rice (in Russia), *Citrus* plants under salinity stress, Pokkali rice in saline soil [42-45]. Saline and drought conditions were also two characteristics of onion growing land in Ninh Hai district, Binh Thuan province, Vietnam. As for *Enterobacter* bacteria, many species in this genus had been reported for phosphate solubilization (*E. aerogenes*) [50], nitrogen fixation, phosphate solubilization and IAA production (*Enterobacter ludwigii* N6b) [17]. With the ability to antagonize the fungus *Fusarium* causing wilt in onions and the fungus *Aspergillus niger* causing spoilage in onion storage, selected *Enterobacter* strains such as TH8 and VH5 had outstanding growth-promoting potential, in addition to increasing P and K nutrition.

5. Conclusion

There were 33 strains of bacteria with capability of P and K solubilization had been isolated from the rhizosphere of red onion grown in Ninh Hai district, Ninh Thuan province. The five best strains were TH8, TH9, TH10, VH3 and VH5, which were able to dissolve phosphate in the range of 30.2 – 54.4 mg/L P_2O_5, and dissolved in potassium in the range of 14.3 – 26.4 mg/L K_2O, and were resistant to *A. niger* with inhibition efficiency from 57% – 59.6% after 7 days of co-culture. Particularly, strains VH5 and TH8 were also capable of antagonizing F. *oxysporum* with the inhibition efficiency of 60.2% and 70.7%, respectively. The identification results showed that TH9, TH10 and VH5 were homologous to *Enterobacter* sp., TH8 was homologous to *E. soli*, and VH3 was homologous to *Novosphingobium* sp.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest.

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