

THE TRAITS OF THE PLANT GROWTH PROMOTING ACETIC ACID BACTERIUM, *NGUYENIBACTER VANLANGENSIS*

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Received: 1 January 2019; Accepted for publication: 7 May 2019

Abstract. To prove the role of acetic acid bacteria as an additional plant growth promoter, the two strains of *Nguyenibacter vanlangensis* (TN01LGI^T and VTH-AC01), the family *Acetobacteraceae*, were examined for nitrogen fixing ability, phosphorus and zinc solubilizing capability, phytohormone auxin production, and inducing activity of an expression of encoding the pathogenic-related protein gene. The two strains were nitrogen fixers due to the observation of acetylene reduction, solubilized phosphorus and zinc in the presence of Ca₃(PO₄)₂ or ZnO (1 %, w/v, respectively), and produced phytohormone auxin, *i.e.* indole acetic acid (IAA) in the presence of L-tryptophan. The indirect effect to the plant growth was also recognized, since the two strains induced an expression of *pr-1* gene, an encoding pathogenic-related protein gene of *Arabidopsis thaliana* PR1::GUS. The two strains were plant growth promoters by direct and indirect mechanisms. The species is therefore an additional plant-growth promoting acetic acid bacterium.

Keywords: *Nguyenibacter vanlangensis*, Plant Growth Promoting Bacteria, *Acetobacteraceae*.

Classification numbers: 1.1.2, 1.4.5.

1. INTRODUCTION

Plant growth promoting bacteria (PGPB) are defined as the free-living soil, rhizosphere, rhizoplane, and phyllosphere bacteria that are, under some conditions, beneficial for plants [1-3]. They are capable of promoting plant growth by directly supplied substances for the metabolism of the plants through different mechanisms such as biological nitrogen fixation, phytohormone production, and mineral nutrient solubilization, or plant's tolerance improvement. On the other way, PGPB indirectly promote the growth of plants by preventing the deleterious effects of phytopathogenic microorganisms by producing the substances that inhibit pathogens or alter the metabolism of the host plant to increase its resistance to pathogen infection. They are referred to as biocontrol-PGPB [1, 3].

Among acetic acid bacteria classified in the family *Acetobacteraceae*, some species of the five genera, *Acetobacter*, *Gluconacetobacter*, *Asaia*, *Swaminathania*, and *Komagataeibacter* are recognized to possess some characteristics of PGPB [2-4]. *Acetobacter peroxydans* was the first *Acetobacter* species assigned as PGPB by the presence of *nifH* genes of both the type strain LMG 1635^T and isolates from wetland rice tissues [5]. *Ac. nitrogenifigens* RG1^T was isolated from nitrogen-free LGI (0.06 % KH₂PO₄, 0.02 % K₂HPO₄, 0.002 % CaCl₂, 0.001 % FeCl₃, 0.0002 % Na₂MoO₄, 10 % sucrose, pH4.5) agar containing 150 mg cycloheximide and 150 mg nystatin and was found to have nitrogenase activity and *nifH* gene [6].

Acetobacter diazotrophicus that was later renamed *Gluconacetobacter diazotrophicus* was considered as the first PGPB representative of the family *Acetobacteraceae* to be reported with the capacity of nitrogen fixation [7-9]. There are lots of reports on the plant growth promoting mechanisms of *G. diazotrophicus* as well as its different kinds of isolation sources [5, 10-13]. After the introduction of novel nitrogen-fixing acetic acid bacteria associated with coffee plants of the two species, *G. johannae* and *G. azotocaptans*, *G. azotocaptans* strain DS1 was reported to show additional plant growth promoting mechanisms such as producing IAA (indole-3-acetic acid, heteroauxin), solubilizing phosphate, and providing biological control against several fungal pathogens and to promote the growth of some corn varieties [14-16]. Though still not be surveyed for nitrogen fixing ability, the five recently introduced *Gluconacetobacter* species including *G. tumulicola*, *G. asukensis*, *G. tumulisoli*, *G. takamatsuzukensis*, and *G. aggeris* showed the growth on nitrogen-free LGI medium [17, 18].

Swaminathania salitolerans, the only species of the genus *Swaminathania*, strains of which were isolated from wild rice in India, was capable of fixing free nitrogen and of solubilizing phosphate [19].

The type strains of *Asaia bogorensis* and *As. siamensis* and the strains of four *Asaia* species, isolated from *Michalia champaca* flowers, *Anopheles* mosquito, and ant *Tetraoponera rufonigra* were found to have nitrogenase activity and *nifH* genes [20].

G. kombuchae (= *Komagataeibacter kombuchae* = *G. hansenii* = *K. hansenii*) strain RG3^T was reported to possess *nifH* gene [21-23].

The genus *Nguyenibacter*, the acetous group, the family *Acetobacteraceae* is comprised at presence of a single species, *N. vanlangensis*, in which strains TN01LGI^T and VTH-AC01 are reported [24]. The two strains were isolated by an initially enrichment procedure using nitrogen-free LGI medium to respective rhizosphere and root of Asian rice isolation sources. The genus *Nguyenibacter* is being expected as a candidate of plant growth promoters [4, 25, 26]. In order to elucidating the role, the present note reports some characteristics considered as plant growth promoting traits of the two strains in the genus *Nguyenibacter*.

2. MATERIALS AND METHODS

2.1. Materials

The two strains, *N. vanlangensis* TN01LGI^T and VTH-AC01, whose GenBank Accession numbers of 16S rRNA gene sequences are AB739062 and AB740269, respectively and that show the growth on LGI medium, were surveyed for some plant growth promoting characteristics [24]. Transgenic *Arabidopsis thaliana* PR1::GUS plants were used to test the ability to activate the plant defense system of the two strains.

2.2. Nitrogen fixing ability analysis

Acetylene reduction activity analysis. The nitrogen fixing capacity of the two strains were studied by the acetylene reduction activity method described by Madhaiyan *et al.* [27] and Muthukumarasamy *et al.* [28].

2.3. Mineral nutrients solubilization analysis

Phosphorus solubilization capability examinations: Preliminarily examination was done according to Madhaiyan *et al.* [27] by using LGI plates containing either 1 % D-glucose or sucrose as a carbon source and 0.1 % calcium phosphate. For quantitative estimation of phosphorus solubilization capability, supernatants from Pikovskaya broths cultured at 30 °C were examined by the method described by Watanabe and Olsen [29] and by Alam *et al.* [30] for five days.

Zinc solubilization capability examinations: Clear zones of zinc oxide were estimated on LGI plates containing either 1 % D-glucose or sucrose as a carbon source and 0.1 % zinc oxide according to the method described previously [27].

2.4. Auxin production analysis

The strains were grown in broths of LGI medium containing either 1 % D-glucose or sucrose supplemented with 0, 50, 100, 200, or 500 µg/ml L-tryptophan at 30 °C for 2, 3, or 5 days. Auxin (IAA, heteroauxin) production by bacteria was assayed colorimetrically using a ferric chloride reagent in the presence of L-tryptophan by the method described previously [31, 32].

2.5. Inducing the expression of PR-1 gene in plant analysis

The broth cultures of *N. vanlangensis* TN01LGI^T and VTH-AC01 were detected for ability to induce the expression of *PR-1* gene, an encoding pathogenic-related protein gene, on *A. thaliana* PR1::GUS according to the method described by Beilmann *et al.* [33] and Iniguez *et al.* [34].

3. RESULTS AND DISCUSSION

3.1. Nitrogen fixing

In the acetylene reduction activity tested with 5 % sucrose as carbon source in liquid LGI medium, *N. vanlangensis* TN01LGI^T and VTH-AC01 were able to reduce acetylene to ethylene

with the amount of 21.61 nmol and 9.11 nmol of ethylene h^{-1} per culture, respectively.

Since the finding of the acetic acid bacteria that have characteristics of plant growth promoters, *G. diazotrophicus* was the most intensively surveyed and considered as a model of plant growth promoter in the family *Acetobacteraceae* [2, 3, 25]. The discovery of the genus *Nguyenibacter* was originated from screening and ecological studies on *Gluconacetobacter* plant growth promoting bacteria concerning rice field, an isolation source of the bacteria [5]. Up to now, all of the plant-growth promoting acetic acid bacteria were recognized as nitrogen fixers [5-7, 14, 19-20]. The two strains of *N. vanlangensis*, obtained initially from enrichment in nitrogen free LGI medium [24], also possessed nitrogen fixing ability.

3.2. Mineral nutrients solubilization

In the plate assays with LGI medium containing either D-glucose or sucrose as a carbon source and supplemented with 0.1 % calcium phosphate, *N. vanlangensis* TN01LGI^T and VTH-AC01 formed halozones. The diameters of solubilized halozones produced by the two strains were 17.33 mm and 15.33 mm, respectively, for three days with D-glucose as a carbon source. When grown on sucrose, the two strains produced levan-like polysaccharides, which were made by the bacterial cells that ran from the incubation paper discs. The halozones formed of calcium phosphate were not able to be measured for the diameter along with the growth of bacterial cells. During the five testing days, the two strains showed the highest phosphorus solubilizing activities of 230.95 μgml^{-1} and 230.66 μgml^{-1} on the third day (Fig. 1). The amounts of solubilized phosphorus were then reduced to 217.24 μgml^{-1} and 192.31 μgml^{-1} on the fourth day and 217.66 μgml^{-1} and 187.72 μgml^{-1} on the fifth day. The data obtained indicated the occurrence of phosphorus immobilization in the two strains.

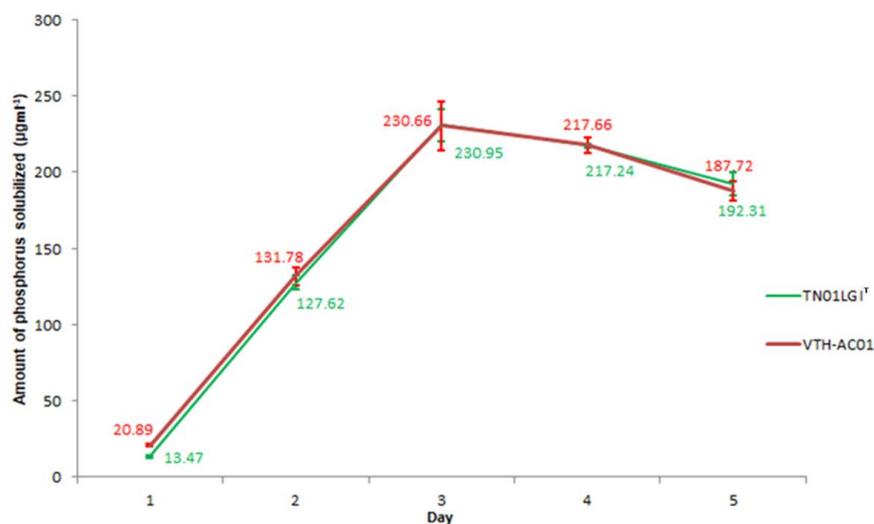


Figure 1. Phosphorus solubilizing capability of *Nguyenibacter vanlangensis* TN01LGI^T and VTH-AC01.

In the preliminary assessment for the zinc solubilization potential, *N. vanlangensis* TN01LGI^T and VTH-AC01 showed the presence of zinc oxide halozones on LGI plates containing either 1 % D-glucose or sucrose as a carbon source. The zinc oxide halozones were not measured on LGI medium containing sucrose due to the formation of levan-like polysaccharides on the agar plates. The diameters of zinc oxide halozones formed were 36.67 mm and 36.00 mm, respectively, on LGI medium containing 1 % D-glucose.

Phosphorus and zinc solubilizing abilities were found in *G. diazotrophicus* PA15^T and others in LGI medium amended with 1 % or 10 % D-glucose or sucrose as a carbon source [27, 35]. During the estimation of effects of *Pseudomonas putida*, *G. azotocaptans*, and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions, a clear zone was recognized in insoluble phosphates formed only by *G. azotocaptans* strain DS1 on NBRIP medium for 14 days at 28 °C [15]. The production of gluconic acid was considered to be due to solubilization of phosphorus and zinc, since acetic acid bacteria generally have an acid production capability [2, 25, 35]. In fact, *N. vanlangensis* TN01LGI^T and VTH-AC01 produced 2-keto-D-gluconic acid and 2,5-diketo-D-gluconic acid in addition to D-gluconic acid [24]. Noteworthy is that the two strains produced levan-like polysaccharides from sucrose as the only carbon source on LGI medium and solubilized phosphorus and zinc in spite of no production of the gluconic acids mentioned above. As a different consideration, the bacterial lipopolysaccharides appear to contribute to root colonization, and PGPB reach root surfaces by active motility facilitated by flagella in response to chemotactic [36]. The levan-like polysaccharides produced in LGI medium with 1 % sucrose obtained from the present study and the peritrichous flagellation described in the previous study [24] may improve root colonizing competition in the two strains that were isolated from rhizosphere or root of rice.

3.3. Auxin production

Nguyenibacter vanlangensis TN01LGI^T and VTH-AC01 did not produce phytohormone of IAA in the LGI medium without L-tryptophan but produced different amounts of IAA in the presence of 0.005 %, 0.01 %, 0.02 %, or 0.05 % L-tryptophan, indicating that the mechanism of IAA production in the two strains was dependent on the presence of L-tryptophan. The two strains showed an increasing IAA accumulation by the increasing addition of L-tryptophan in the LGI medium containing either 1 % D-glucose or 1 % sucrose as a carbon source.

Table 1. Auxin producing activity of the two *Nguyenibacter vanlangensis* strains TN01LGI^T and VTH-AC01.

LGI medium containing	Amount of IAA produced (µgml ⁻¹)					
	<i>N. vanlangensis</i> TN01LGI ^T			<i>N. vanlangensis</i> VTH-AC01		
	Day 2	Day 3	Day 5	Day 2	Day 3	Day 5
1 % D-glucose + 0 % L-tryptophan	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
1 % D-glucose + 0.005 % L-tryptophan	1.52±0.03	1.10±0.03	0.80±0.02	2.00±0.02	1.06±0.02	0.82±0.02
1 % D-glucose + 0.01 % L-tryptophan	2.66±0.02	2.55±0.03	1.58±0.02	2.97±0.02	2.23±0.02	1.27±0.03
1 % D-glucose + 0.02 % L-tryptophan	4.33±0.03	4.09±0.02	2.69±0.03	4.26±0.05	4.10±0.05	1.82±0.02
1 % D-glucose + 0.05 % L-tryptophan	6.85±0.02	6.56±0.03	3.63±0.03	7.30±0.02	6.58±0.04	3.49±0.02
1 % sucrose + 0 % L-tryptophan	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
1 % sucrose + 0.005 % L-tryptophan	2.26±0.02	1.75±0.02	1.08±0.03	2.01±0.02	1.45±0.02	0.94±0.02
1 % sucrose + 0.01 % L-tryptophan	3.04±0.02	2.92±0.02	1.63±0.01	2.95±0.01	2.68±0.01	1.33±0.01
1 % sucrose + 0.02 % L-tryptophan	4.09±0.03	4.49±0.03	2.33±0.03	3.80±0.02	4.44±0.01	1.77±0.02
1 % sucrose + 0.05 % L-tryptophan	5.04±0.04	5.18±0.02	3.06±0.02	6.03±0.01	7.21±0.02	3.09±0.02

The maximum productions of IAA were 7.30 µgml⁻¹ in the medium containing D-glucose in the two-day growth and 7.21 µgml⁻¹ in the medium containing sucrose in the three-day growth

supplemented respectively with 0.05 % L-tryptophan in VTH-AC01 (Table 1). However, all the experimental data showed the decrease of IAA once accumulated in the growth medium on the fifth day.

Spaepen *et al.* [37] summarized seven bacterial IAA biosynthetic pathways, among which L-tryptophan was identified as a precursor using five different pathways. The IAA production was observed in *G. diazotrophicus* PA15^T and others from carrot, radish, beetroot, and coffee in LGI medium supplemented with 1 % D-glucose or 1 % sucrose in the presence of L-tryptophan. Among the strains tested, only PA15^T produced IAA in the absence of L-tryptophan [27]. However, the IAA production was about one-third, when compared with the presence of L-tryptophan. The data obtained above showed the possibility that the strain of *G. diazotrophicus* has a tryptophan-independent IAA production pathway, namely, an additional IAA synthetic pathway, or a weak L-tryptophan synthetic pathway for IAA production. The present study indicated that the two strains of *N. vanlangensis* apparently showed the tryptophan-dependent IAA production.

3.4. Inducing the expression of (*pr-1*) gene in plant

As mentioned above, *N. vanlangensis* TN01LGI^T and VTH-AC01 showed the capabilities of fixing atmospheric nitrogen, solubilizing phosphorus and zinc, and producing phytohormone auxin. This indicates that the two strains exhibit mechanisms to directly provide certain substances and to affect the metabolism of the plants. As the second, biocontrol-PGPB have the ability to indirectly promote plant growth by preventing the deleterious effects of phytopathogenic microorganisms including the production of substances that harms or inhibits pathogens, by limiting the availability of iron to pathogens or by altering the metabolism of the host plant to increase its resistance to pathogen infection [1].

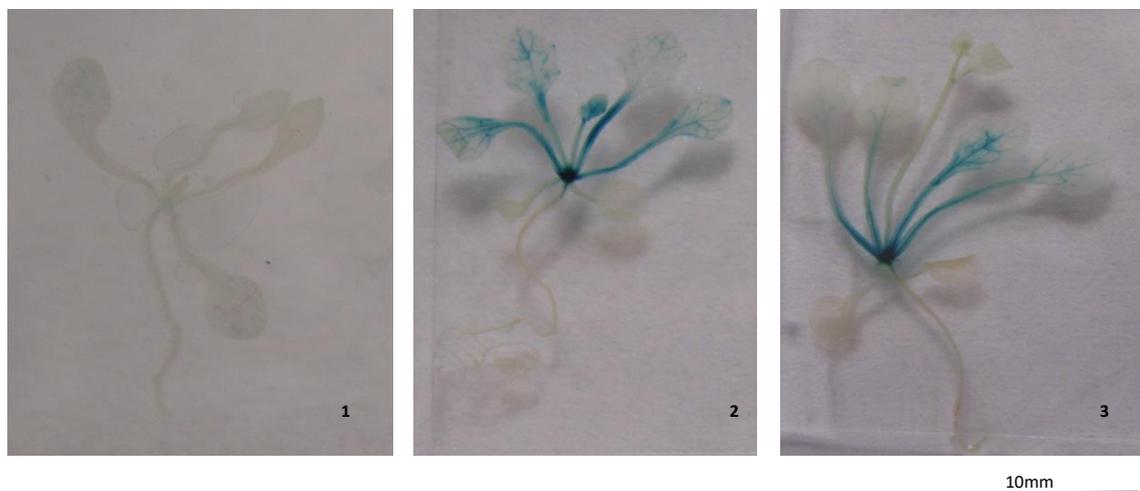


Figure 2. *Nguyenibacter vanlangensis* TN01LGI^T and VTH-AC01 activated the expression of *Gus* gene in one week growth *Arabidopsis thaliana* strain PR1::Gus. 1; *A. thaliana* PR1::Gus treated with 0.9 % NaCl; 2; *A. thaliana* PR1::Gus treated with *N. vanlangensis* TN01LGI^T; 3; *A. thaliana* PR1::Gus treated with *N. vanlangensis* VTH-AC01.

In order to initially elucidate the indirect plant growth promotion capability of the two strains of *N. vanlangensis*, the following experiments were done with *A. thaliana* PR1::GUS plant to induce the expression of *pr-1* gene, an encoding pathogenesis-related protein gene that

belongs to salicylic acid (SA) - independent plant defense system, in the presence or absence of the two strains.

The one week old *A. thaliana* PR1::GUS grown on MS medium containing 1 % sucrose was treated by spraying the broth cultures of the two strains with 0.01 at OD_{540nm} in logarithmic phase and then leaved for 96 h to induce PR1::GUS expression. The occurrence of reaction of X-Gluc reagent by β -glucuronidase (product from the expression of *gus* gene) led to retain blue colour after the **destained** (Fig. 2; 2 and 3). However, the one week old *A. thaliana* PR1::GUS treated only with 0.9 % NaCl did not show any blue colour (Fig. 2; 1). The data obtained above indicated that *N. vanlangensis* TN01LGI^T and VTH-AC01 induced PR1::GUS expression in *A. thaliana* PR1::GUS and therefore induced SA-mediated defense signaling pathway.

The *in vivo* results inducing SA-mediated defense signaling pathway indicated that the two strains of *N. vanlangensis* are designated as biocontrol-PGPB. Assays for antibacterial or antifungal activities may obtain interesting data in the two strains, as found in *G. diazotrophicus* and *G. azotocaptans* [15, 38].

4. CONCLUSIONS

The present study showed that *N. vanlangensis* TN01LGI^T and VTH-AC01 support the plant growth through different mechanisms including nitrogen fixing activity, phosphorus and zinc solubilization activities, and the production of auxin phytohormone. Besides, the two strains were preliminarily proved to have a capability of inducing plant protective system through SA-mediated defense signaling pathway. The genus *Nguyenibacter*, therefore, becomes additional genus in the family *Acetobacteraceae* belonging to the group of PGPB.

Further research on the roles as PGPB of the species *N. vanlangensis* might give more interesting results and application in future is prospected.

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TG- hỏi anh Bình kiểm tra chỉnh sửa lại chữ destained (trước CÓNCLUSION)?