

**CHARACTERIZATION OF NITROGEN-FIXING AND IAA-PRODUCING  
PURPLE NON-SULFUR BACTERIA ISOLATED FROM RICE FIELDS  
IN NAM DINH, VIETNAM**

**Do Thi Lien<sup>1,\*</sup>, Cung Thi Ngoc Mai<sup>1</sup>, Dinh Thi Thu Hang<sup>2</sup>, Tran Thi Mai<sup>1</sup>,  
Nguyen Thi Kim Tien<sup>4</sup>, Le Thi Nhi Cong<sup>1</sup>, Bui Thi Kim Anh<sup>3</sup>, Nghiem Ngoc Minh<sup>1</sup>**

<sup>1</sup>Institute of Biology, Vietnam Academy of Science and Technology,  
18 Hoang Quoc Viet, Nghia Do, Ha Noi, Vietnam

<sup>2</sup>Graduate University of Science and Technology, Vietnam Academy  
of Science and Technology, 18 Hoang Quoc Viet, Ha Noi, Vietnam

<sup>3</sup>Institute of Science and Technology for Energy and Environment, Vietnam Academy  
of Science and Technology, 18 Hoang Quoc Viet, Ha Noi, Vietnam

<sup>4</sup>Phacogen Pharmaceutical Joint Stock Company, Ha Noi, Vietnam

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**ABSTRACT**

This study aimed to select purple photosynthetic bacteria (PPB) isolated from various paddy fields, including saline fields in Giao Thuy and paddy fields in Y Yen, based on their biofertilizer properties. Among 24 PPB isolates, strains GT10 and Y11 exhibited the highest efficiency in indole-3-acetic acid (IAA) production and nitrogen fixation. GT10 colonies were circular and convex, with smooth surfaces, reddish brown, diameter: 0.9-1.2 mm. The cell suspension appeared reddish-brown. Colonies of strain Y11 were spread out, reddish brown, slimy, diameter: 1.8-2.2 mm, with a purple-colored cell suspension. GT10 cells were oval-shaped (0.9-1.25  $\mu\text{m}$  long, 0.6-0.7  $\mu\text{m}$  wide) and reproduced by binary fission. In contrast, Y11 cells were rod-shaped (1.4-1.58  $\mu\text{m}$  long, 0.36-0.441  $\mu\text{m}$  wide) and reproduced by budding. Elemental sulfur globules were not observed in either strain. Both were Gram-negative and contained bacteriochlorophyll a as their primary photosynthetic pigment. The GT10 strain was able to use carbon sources such as acetate, propionate, lactate, succinate, formate, citrate, mannitol, sorbitol, glycerol, glutamate, isopropanol, sulfide, but not tartrate, methanol, or ethanol. These characteristics are consistent with the genus *Rhodobacter*. Y11 strain was able to use carbon sources such as acetate, propionate, lactate, succinate, formate, citrate, mannitol, sorbitol, glycerol, glutamate, or sulfide, but did not utilize tartrate, methanol, ethanol, or isopropanol. These capacities are similar to those of the *Rhodospseudomonas* species. Based on 16S rDNA sequencing, strain Y11 was identified as *Rhodospseudomonas palustris*, while strain GT10 was *Rhodobacter capsulatus*. The combined application of GT10 and Y11 strains may offer a sustainable biofertilizer alternative to chemical fertilizers in rice cultivation.

**Keywords:** IAA production, fixing molecular nitrogen, *Rhodospseudomonas palustris*, *Rhodobacter capsulatus*, Purple photosynthetic bacteria.

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\*Corresponding author email: [lien.ibt@gmail.com](mailto:lien.ibt@gmail.com)

## INTRODUCTION

Crop productivity and production efficiency are central goals in modern agriculture. Among the contributing factors, fertilizers are vital in enhancing crops' yield and quality. In rice cultivation (*Oryza sativa*), particularly under intensive farming systems, productivity cannot be sustained without fertilizer inputs (Kantha et al., 2015). Consequently, nitrogen-based chemical fertilizers have been widely applied to increase rice yield. However, prolonged use of these fertilizers is associated with several environmental concerns, including declining soil health and groundwater contamination by nitrate (Wang & Yang et al., 2003).

Increasing demands for organic farming products have recently encouraged farmers to utilize biofertilizers instead of chemicals. For instance, applying cultures of effective microorganisms (EM) to certain agricultural lands in Asia, including Vietnam, has been reported to improve soil quality, promote crop growth, and enhance crop yield. Recently, sustainable agriculture has been promoted, and plant growth-promoting bacteria and biofertilizers are among the substitutes for chemical fertilizers. Nitrogen is one of the major essential elements for plant growth. Biological nitrogen fixation by  $N_2$ -fixing microorganisms is an effective mechanism for converting  $N_2$  into ammonium ions ( $NH_4^+$ ), which can be used by plants (Olivares et al., 2013). Purple photosynthetic bacteria (PPB) are among nitrogen-fixing bacteria and are candidates for applications in paddy fields due to their extraordinary metabolic versatility, i.e. photoautotrophic, photoorganotrophic, chemoautotrophic and chemoorganotrophic (Sakarika et al., 2020). PPB not only fix  $N_2$ , but also produces plant growth-promoting substances (PGPSs) such as indole-3-acetic acid (IAA). IAA, commonly known as 'IAA', belongs to the group of auxins. Auxins are essential for plant development and have a cardinal role in regulating many growth and behavioural processes in the plant's life cycle. They are responsible for plant cell division, extension and specialization (Tsavkelova et al., 2006). Specifically, IAA is a phytohormone

playing a role in the growth activation of plants by inducing plant mineral uptake and root cell elongation (Sakarika et al., 2020). It also increases plant tolerance to salinity and low-temperature stress (Watanabe et al., 2000). Purple photosynthetic bacteria have been extensively used to enhance plant growth due to their metabolic versatility. PPB can utilize various organic compounds as carbon and energy sources during photoorganotrophic and chemoorganotrophic growth (Kim et al., 2004; Kantachote et al., 2005). There are claims that PPB can remove environmental contaminants such as  $H_2S$  and develop mechanisms of resistance to high concentrations of toxic metals such as Hg, As, Cd, Cu, Pb, and Zn (Ercal et al., 2001; Panwichian et al., 2010b), including Na (Panwichian et al., 2010a). Therefore, employing PPB in paddy fields could provide an attractive method to reduce chemical fertilizer use, aid rice plants in withstanding harsh conditions, and improve product quality. The experiment was set up in two fields, one as a control and the other as a PPB-treated field by Yen et al. (2022). PPB-treatment significantly increased root length (25%), root dry weight (57%), productive tillers per plant (26%), average grains per plant (38%), grain yield (33%), 1000 grain weight (1.6%), and harvest index (41%). Hence, from this research, it can be concluded that foliar application of PNSB on rice crops under field conditions improves crop growth and yield (Yen et al., 2022).

Limited studies have explored the potential of PPB as biofertilizers for paddy fields in Vietnam. Therefore, this study aimed to isolate and select PPB strains that can fix nitrogen ( $NH_4^+$ ) and produce indole-3-acetic acid (IAA) to promote plant growth. The selected PPB strains may offer a practical and eco-friendly alternative to chemical fertilizers in rice cultivation, especially under challenging environmental conditions.

## MATERIALS AND METHODS

### Sampling

Soil and water samples were collected from saline paddy fields in Giao Thuy and non-saline fields in Y Yen, Nam Dinh,

Vietnam. Soil samples were collected from 0-0.5 cm below the soil surface, while water samples were taken from 0.5 cm below the water surface. The pooled soil samples were immediately transported to the laboratory under ambient conditions. The samples were subsequently subjected to enrichment culture to isolate purple non-sulfur bacteria.

#### **Enumeration and isolation of purple phototrophic bacteria from sample collections**

Purple phototrophic bacteria were enriched from the samples using cumulative cultivation in cylindrical plastic bottles (diameter = 5 cm, height = 35 cm), designed based on a modified Winogradsky column. Water and soil samples were introduced into the bottles at a ratio of 1:1 (for water samples) or 9:1 (for field soil samples). The bottles were then filled with a modified DSMZ-27 liquid medium, sealed, and incubated under continuous incandescent light at approximately 3000 lux. The composition of the DSMZ-27 medium (per 1 L) was as follows:  $K_2HPO_4$ , 1 g;  $KH_2PO_4$ , 0.5 g;  $MgSO_4 \cdot 7H_2O$ , 0.4 g; NaCl, 15 g;  $NH_4Cl$ , 0.4 g;  $CaCl_2 \cdot 2H_2O$ , 0.05 g; yeast extract, 0.3 g; sodium acetate, 1 g; succinic acid, 0.5 g; trace element solution, 1 mL; vitamin solution, 1 mL; agar, 20 g (for solid media); and distilled water up to 1000 mL. The initial pH was adjusted to 6.8-7.0 before autoclaving. The trace element solution (per 1 L) contained: HCl (25%), 6.5 mL;  $FeCl_2 \cdot 4H_2O$ , 1.5 g;  $H_3BO_3$ , 0.3 g;  $MnCl_2 \cdot 2H_2O$ , 0.03 g;  $CoCl_2 \cdot 6H_2O$ , 0.2 g;  $ZnSO_4 \cdot 7H_2O$ , 0.1 g;  $CuCl_2 \cdot 2H_2O$ , 17 mg;  $NiCl_2 \cdot 6H_2O$ , 24 mg; and  $Na_2MoO_4 \cdot 2H_2O$ , 36 mg; in distilled water to a final volume of 1 L. The vitamin solution (1 L) consisted of: thiamine, 500  $\mu$ g; niacin, 500  $\mu$ g; and biotin, 15  $\mu$ g, dissolved in distilled water. The solution was filter-sterilized and added aseptically to the medium before use. All media and containers were sterilized by autoclaving at 121 °C for 15 minutes before use.

#### **Isolation of purple phototrophic bacteria**

After one week of enrichment, pigmented biofilms ranging from brown-yellow to purple-red developed along the inner walls of the

culture flasks, indicating the presence of purple non-sulfur bacteria (PPB). Samples from these zones were streaked onto DSMZ-27 agar plates and incubated under nitrogen-flushed microaerobic conditions with continuous illumination (~3,000 lux). After 3–5 days, pigmented colonies (brown, pink, or purple) appeared. Repeated sub-culturing yielded pure isolates. Each pure culture was preserved by stabbing into DSMZ-27 agar at 4 °C or suspending in 20% glycerol and storing at -80 °C. Isolates were identified based on morphological, cultural, and physiological characteristics. Gram staining was performed, and bacteriochlorophyll content was measured using a UV-visible spectrophotometer (V-530, JASCO, Japan).

To evaluate carbon source utilization, a modified DSMZ-27 medium was prepared by replacing acetate and succinate with various test carbon sources (e.g., formate, citrate, tartrate, glycerol, glucose, methanol, ethanol, mannitol, sorbitol, propionate, sulfide, glutamate, isopropanol). Yeast extract was used as the sole nitrogen source. Each test medium was inoculated (1.0 mL culture per vial, in triplicate), sealed, and incubated under light (~3,000 lux) for 7 days. Growth was assessed via  $OD_{800}$  measurement.

#### **Culturing of purple phototrophic bacteria**

A single colony of purple non-sulfur bacteria was inoculated using a sterile platinum needle and put into a screw-cap tube under aseptic conditions. The tube was filled with liquid DSMZ-27 medium, sealed tightly, and incubated at room temperature (~25–28 °C) under continuous illumination from an incandescent lamp. Cells were harvested at the end of the exponential growth phase, and 10% of the culture volume was used as an inoculum for subsequent flask cultures. Flask cultivation was performed in 100 mL flasks under microaerobic-light conditions to acclimate the cells to the experimental environment.

#### **Growth of purple photosynthetic bacteria assessment**

The growth of the purple non-sulfur bacterial cultures was monitored by measuring

the optical density at 800 nm ( $OD_{800}$ ) using a UV-visible spectrophotometer (UV-160A, Shimadzu, Japan).

Selection of isolated purple phototrophic bacteria with  $N_2$ -fixing ability

PPB strains previously isolated from saline paddy fields were screened for their nitrogen-fixing capability. A nitrogen-free medium, slightly modified from Vatsala et al. (2011), was used for this purpose. The medium composition per liter was as follows: sodium acetate, 1.0 g;  $K_2HPO_4$ , 0.9 g;  $KH_2PO_4$ , 0.6 g;  $MgSO_4$ , 0.2 g;  $FeSO_4$ , 0.012 g; EDTA, 0.018 g;  $CaCl_2$ , 0.075 g;  $Na_2MoO_4 \cdot 2H_2O$ , 0.01 g;  $MnSO_4 \cdot 4H_2O$ , 2.8 mg;  $ZnSO_4$ , 0.75 mg;  $CuSO_4$ , 0.24 mg;  $H_3BO_3$ , 0.016 mg; and biotin, 0.001 mg. The final pH was adjusted to 7.0 using distilled water. Each isolate was inoculated into screw-cap tubes containing nitrogen-free medium at a volume ratio of 10% (v/v). The tubes were filled to eliminate headspace, while the uninoculated medium was a negative control. All cultures were incubated under microaerobic conditions at room temperature ( $\sim 25$ – $30$  °C) and illuminated with incandescent light ( $\sim 3,000$  lux) for 48 hours. According to Kolev (2007), nitrogen gas dissolves in water at concentrations of approximately 17–20 mg/L at 20–30 °C and 1 bar, which is presumed sufficient to support  $N_2$  fixation in sealed tubes. After incubation, the cultures were centrifuged at 8000 rpm for 15 minutes to collect cell-free supernatants. To screen for  $NH_4^+$  release, 0.5 mL of each supernatant was tested by adding a few drops of Nessler's reagent. The intensity of color development was used as a qualitative indicator of ammonium production: no color indicated absence of  $NH_4^+$ , pale yellow indicated low levels, yellow indicated moderate levels, and brown indicated high  $NH_4^+$  levels. Supernatants showing strong positive reactions were further quantified for ammonium concentration using a UV-visible spectrophotometer.

#### IAA production test

IAA production by PPB isolates was assessed following the method described by Ahmad et al. (2005). The isolates were

cultured in a glutamate-acetate (GA) medium in which L-sodium glutamate was replaced by 1 mM L-tryptophan as the IAA precursor. Cultures were incubated under microaerobic-light conditions for 5 days. After incubation, cultures were centrifuged at 8,000 rpm for 15 minutes, and the supernatants were collected for IAA determination. To quantify IAA, 1 mL of each culture supernatant was mixed with 2 mL of Salkowski's reagent (2% of 0.5 M  $FeCl_3$  in 35%  $HClO_4$  solution) in a test tube. The mixture was incubated at room temperature for 25 minutes, and the absorbance was measured at 530 nm using a spectrophotometer. IAA concentration was determined based on a standard curve generated with pure IAA. The GA medium used for IAA production contained (per 1 L distilled water, pH adjusted to 7.0): 3.8 g sodium glutamate, 5.44 g sodium acetate monohydrate, 2.0 g yeast extract, 0.5 g  $KH_2PO_4$ , 0.5 g  $K_2HPO_4$ , 0.8 g  $(NH_4)_2HPO_4$ , 0.2 g  $MgSO_4 \cdot 7H_2O$ , 53 mg  $CaCl_2 \cdot 2H_2O$ , 1.2 mg  $CoCl_2 \cdot 6H_2O$ , 1.2 mg  $MnSO_4 \cdot 5H_2O$ , 0.01 mg biotin, 2.5 mg ferric citrate, and 1.0 mg nicotinic acid.

#### Purple phototrophic bacteria identification

To identify the selected purple non-sulfur bacterial (PPB) strains, 16S rDNA sequence analysis was performed. The strains were cultivated in liquid DSMZ-27 medium, and cells were harvested by centrifugation. Genomic DNA was extracted from the cell pellets using the ADT DNA extraction kit according to the manufacturer's protocol. PCR amplification of the 16S rDNA gene was carried out in a 25  $\mu$ L reaction mixture containing: 1  $\mu$ L each of primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'); 2.5  $\mu$ L of 10 $\times$  PCR buffer; 2.5  $\mu$ L of  $MgCl_2$  (25 mM); 2  $\mu$ L of dNTP mix (2.5 mM); 2  $\mu$ L of DNA template ( $\sim 100$  ng); 0.25  $\mu$ L of Taq DNA polymerase (5 U/ $\mu$ L); and 13.75  $\mu$ L of nuclease-free water. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min, with a final extension step at

72 °C for 10 min. PCR products were visualized by electrophoresis on 0.8% agarose gel and detected under UV light using a gel documentation system (GelDoc/UV transilluminator). Target DNA bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, USA). Purified PCR products were sequenced using the Sanger method (Sanger et al., 1977) on an ABI Avant Genetic Analyzer (Applied Biosystems, USA) at the Key Laboratory of Gene Technology, Institute of Biology. The obtained sequences were analyzed using BLAST (NCBI) and DNASTAR software to identify closely related sequences. Multiple sequence alignments and phylogenetic analyses were performed using MEGA, ClustalX, and PHYLIP software packages.

### Statistical analysis

All experiments in this study were conducted in three replicates. Mean values and the standard deviations are presented. Data were analyzed using one-way ANOVA, and statistically significant differences were considered at  $p < 0.05$ .

## RESULTS

### Isolation of purple photosynthetic bacteria

From water and soil samples collected 1.5 to 2 months after rice transplantation in Nam Dinh, 24 purple photosynthetic bacteria (PPB) strains were successfully isolated. These isolates exhibited considerable diversity in colony morphology, including size, shape, and pigmentation variations. Colony colors ranged from brown, yellowish-brown, and reddish-brown to purple-red, dark red, and pink. Cellular morphology among the isolates was also heterogeneous, comprising short rods, oval-shaped cells, and spherical forms. No intracellular sulfur granules were observed in any of the strains. Most isolates reproduced via binary fission or budding. Based on cell shape and the absence of sulfur inclusions, all 24 isolates were preliminarily classified within the family Rhodospirillaceae, a group of purple non-sulfur bacteria.

### Selection of PPB with the ability to release $\text{NH}_4^+$

Table 1. The concentrations of  $\text{NH}_4^+$  released by the isolated PPB strains under microaerobic-light conditions for 48 h

No.	PPB strains	$\text{NH}_4^+$ (mg/L)
1	GT <sub>1</sub>	$2.63 \pm 0.92^{\text{cd}}$
2	GT <sub>2</sub>	$3.05 \pm 0.60^{\text{cd}}$
3	GT <sub>21</sub>	$0.40 \pm 0.01^{\text{a}}$
4	GT <sub>11</sub>	$3.02 \pm 1.04^{\text{cde}}$
5	GT <sub>3</sub>	$3.97 \pm 1.03^{\text{def}}$
6	GT <sub>31</sub>	$3.67 \pm 0.99^{\text{def}}$
7	GT <sub>4</sub>	$4.19 \pm 0.73^{\text{def}}$
8	GT <sub>5</sub>	$00 \pm 0.00^{\text{a}}$
9	GT <sub>6</sub>	$0.33 \pm 0.10^{\text{a}}$
10	GT <sub>7</sub>	$2.70 \pm 1.05^{\text{cd}}$
11	GT <sub>8</sub>	$3.07 \pm 1.01^{\text{cde}}$
12	GT <sub>9</sub>	$3.80 \pm 0.90^{\text{def}}$
13	GT <sub>10</sub>	$4.34 \pm 0.85^{\text{ef}}$
14	Y <sub>1</sub>	$0.85 \pm 0.18^{\text{ab}}$
15	Y <sub>11</sub>	$4.63 \pm 0.71^{\text{f}}$
16	Y <sub>12</sub>	$2.12 \pm 0.78^{\text{bc}}$
17	Y <sub>2</sub>	$1.29 \pm 0.77^{\text{ab}}$
18	Y <sub>3</sub>	$4.15 \pm 0.64^{\text{def}}$
19	Y <sub>4</sub>	$3.77 \pm 0.85^{\text{cdef}}$
20	Y <sub>5</sub>	$3.54 \pm 0.71^{\text{def}}$
21	Y <sub>6</sub>	$3.55 \pm 0.95^{\text{def}}$
22	Y <sub>7</sub>	$3.72 \pm 0.67^{\text{cdef}}$
23	Y <sub>8</sub>	$2.40 \pm 0.89^{\text{cd}}$
24	Y <sub>9</sub>	$3.94 \pm 0.94^{\text{def}}$
25	Negative control	0,00

Note: Different lowercase letters (a, b, c, d, e, f) in the same column indicate a statistically significant difference (one-way ANOVA with Duncan's post hoc,  $p < 0.05$ ).

Nitrogen fixation by purple non-sulfur bacteria (PPB) is generally sufficient under strictly anaerobic conditions, as nitrogenase activity is vulnerable to oxygen (Masepohl et al., 2009). However, paddy fields are not completely anaerobic, even under flooded conditions, which is a common practice in rice cultivation. During the day, photosynthetic oxygen production and its subsequent transport from leaves to roots can introduce oxygen into the rhizosphere and surrounding

sediments (Larsen et al., 2015). While some of this oxygen is consumed in methane oxidation, a portion remains and hinders the establishment of strictly anaerobic environments (Gutierrez et al., 2014). To better reflect in situ field conditions, this study investigated the nitrogen-fixing capacity of PPB under microaerobic-light conditions. A total of 24 PPB strains previously isolated from sediment and water samples in rice paddies were evaluated for their ability to fix atmospheric N<sub>2</sub>, as inferred from ammonium (NH<sub>4</sub><sup>+</sup>) release in a nitrogen-free medium (Table 1).

The results demonstrated that 23 of 24 isolated strains exhibited nitrogen-fixing activity, as indicated by the ammonium accumulation (NH<sub>4</sub><sup>+</sup>) in the nitrogen-free culture medium. However, the extent of nitrogen fixation varied among the isolates. Of the 24 strains, 20 were classified as medium-level NH<sub>4</sub><sup>+</sup> producers, and three exhibited low-level production, while no NH<sub>4</sub><sup>+</sup> was detected in strain GT<sub>5</sub>. The 20 medium-level strains released between 1.29 ± 0.77 and 4.63 ± 0.71 mg/L of NH<sub>4</sub><sup>+</sup>. Notably, four strains exhibited high nitrogen-fixing capacity, producing more than 4.15 ± 0.64 mg/L NH<sub>4</sub><sup>+</sup>, and their performance significantly differed from the remaining strains (p ≤ 0.05).

Despite this, after 48 hours of incubation, biomass accumulation in nitrogen-free medium remained low across all tested strains, suggesting that NH<sub>4</sub><sup>+</sup> utilization was minimal under these conditions.

#### **Selection of PPB with the ability to produce Indole-3-acetic acid (IAA)**

Following isolation, the 24 PPB strains were cultured in liquid form to evaluate their indole-3-acetic acid (IAA) production capacity. Each strain was inoculated into an improved glutamate-acetate (GA) medium supplemented with 1 mM L-tryptophan at a 10% (v/v) inoculum ratio. The initial cell density in the experimental flasks reached approximately 10<sup>9</sup> CFU/mL. Flasks were sealed with rubber stoppers to maintain microaerobic conditions, and control flasks

containing only sterile medium were included. All cultures were incubated under continuous illumination at room temperature (25–30 °C) for five days. IAA concentrations in the culture supernatants were quantified using a modified Salkowski colorimetric assay. The IAA production levels of each PPB strain after five days of incubation under microaerobic-light conditions are summarized in Table 2.

The results indicated that all 24 isolated strains were able to grow well in the modified GA medium supplemented with 1 mM L-tryptophan. Among them, 21 strains produced detectable levels of indole-3-acetic acid (IAA), although IAA production varied significantly among isolates. IAA concentrations ranged from 54.33 ± 3.10 to 205.40 ± 5.85 mg/L, as determined using the colorimetric Salkowski assay (Table 2). These findings suggest that PPB strains isolated from rice fields in Nam Dinh possess considerable IAA-producing potential under microaerobic-light conditions. Three isolates did not produce detectable levels of IAA under the tested conditions. Among all strains, GT<sub>10</sub> and Y<sub>11</sub> exhibited the highest levels of IAA production and demonstrated strong nitrogen-fixing capacity (Tables 1 & 2). Consequently, these two strains were selected for further taxonomic identification and characterization.

#### **Morphological, physiological and biochemical characterization of strains**

For the identification of purple non-sulfur bacteria (PPB), several biological, physiological, and biochemical characteristics were examined, including colony and cellular morphology, bacteriochlorophyll absorption spectra, carbon source utilization, and 16S rDNA sequence analysis. Colonies of strain GT<sub>10</sub> were circular, convex, smooth-surfaced, and reddish-brown, with a diameter ranging from 0.9 to 1.2 mm. The corresponding cell suspension also appeared reddish-brown. In contrast, colonies of strain Y<sub>11</sub> were spreading, slimy, and reddish-brown, with diameters between 1.8 and 2.2 mm; the cell suspension appeared purple. Cell morphology, size, cell division mode, and

flagella presence were observed using scanning electron microscopy (SEM; Model S-4800, Hitachi, Japan). GT10 cells were oval-shaped, unicellular, and measured 0.6–0.7  $\mu\text{m}$  in width and 0.9–1.25  $\mu\text{m}$  in length. These cells reproduced via binary fission and no intracellular sulfur granules were detected (Fig. 1). Cells of strain Y<sub>11</sub> were rod-shaped,

measured 1.4–1.58  $\mu\text{m}$  in length and 0.36–0.44  $\mu\text{m}$  in width, and were observed either as single cells or in short chains. These cells reproduce by budding. Like GT<sub>10</sub>, no intracellular sulfur granules were observed in Y<sub>11</sub> cells. Both strains were determined to be Gram-negative. Representative colony and cell morphologies are shown in Figure 1.

Table 2. The concentrations of IAA production in GA medium supplemented with tryptophan by the isolated PPB strains under microaerobic-light conditions for 5 days

No.	PPB strains	$\Delta\text{OD}_{800}$	IAA productions ( $\mu\text{g/ml}$ )
1	GT <sub>1</sub>	1.88 ± 0.19	78.0 ± 4.92 <sup>def</sup>
2	GT <sub>2</sub>	2.63 ± 0.60	58.24 ± 5.60 <sup>b</sup>
3	GT <sub>21</sub>	1.98 ± 0.19	0.00 ± 0.00 <sup>a</sup>
4	GT <sub>11</sub>	2.04 ± 0.27	67.98 ± 5.04 <sup>c</sup>
5	GT <sub>3</sub>	2.45 ± 0.23	87.15 ± 7.03 <sup>fg</sup>
6	GT <sub>31</sub>	2.23 ± 0.29	118.87 ± 5.90 <sup>h</sup>
7	GT <sub>4</sub>	2.48 ± 0.35	188.96 ± 6.73 <sup>k</sup>
8	GT <sub>5</sub>	1.65 ± 0.24	73.05 ± 4.05 <sup>cd</sup>
9	GT <sub>6</sub>	1.99 ± 0.22	54.33 ± 3.10 <sup>b</sup>
10	GT <sub>7</sub>	1.97 ± 0.30	82.70 ± 5.05 <sup>egh</sup>
11	GT <sub>8</sub>	2.07 ± 0.30	90.07 ± 6.01 <sup>g</sup>
12	GT <sub>9</sub>	2.31 ± 0.25	83.80 ± 4.90 <sup>egh</sup>
13	<b>GT<sub>10</sub></b>	<b>2.53 ± 0.50</b>	<b>205.4 ± 5.85<sup>l</sup></b>
14	Y <sub>1</sub>	2.81 ± 0.41	59.22 ± 5.88 <sup>b</sup>
15	<b>Y<sub>11</sub></b>	<b>2.73 ± 0.24</b>	<b>191.63 ± 7.41<sup>k</sup></b>
16	Y <sub>12</sub>	2.81 ± 0.34	81.81 ± 3.78 <sup>def</sup>
17	Y <sub>2</sub>	1.95 ± 0.27	58.63 ± 3.77 <sup>b</sup>
18	Y <sub>3</sub>	2.93 ± 0.35	146.15 ± 5.64 <sup>i</sup>
19	Y <sub>4</sub>	1.85 ± 0.38	79.67 ± 4.85 <sup>def</sup>
20	Y <sub>5</sub>	2.71 ± 0.36	76.54 ± 5.71 <sup>cde</sup>
21	Y <sub>6</sub>	2.55 ± 0.29	0.00 ± 0.00 <sup>a</sup>
22	Y <sub>7</sub>	2.05 ± 0.31	0.00 ± 0.00 <sup>a</sup>
23	Y <sub>8</sub>	2.90 ± 0.42	79.48 ± 4.85 <sup>def</sup>
24	Y <sub>9</sub>	2.23 ± 0.35	68.18 ± 2.97 <sup>c</sup>
25	Negative control	0,00	0.00

Note: Different lowercase letters (a, b, c, d, e, f, I, k, g, h, I, k) in the same column indicate a statistically significant difference (one –way ANOVA with Duncan’s post hoc,  $p < 0.05$ ).

Based on colony morphology, cell suspension color, and cellular structure, strain GT<sub>10</sub> was preliminarily assigned to the genus *Rhodobacter*, while strain Y<sub>11</sub> was classified within the genus *Rhodospseudomonas*. A key feature distinguishing purple photosynthetic bacteria (PPB) from other phototrophic microorganisms is their ability to synthesize

bacteriochlorophylls (BChls) under illuminated conditions. Both GT<sub>10</sub> and Y<sub>11</sub> strains were cultivated in DSMZ-27 liquid medium under anaerobic conditions with continuous illumination from incandescent lamps to evaluate this characteristic. After four days of incubation, corresponding to the mid-exponential growth phase, the

bacteriochlorophyll content in the cell suspensions was analyzed spectrophotometrically. Absorption spectra were measured in the 400–900 nm range using Navaspec II (England) and UV-1650PC (Shimadzu, Japan) spectrophotometers. The absorption spectrum of strain GT<sub>10</sub> showed

characteristic peaks at 807 and 872 nm, while strain Y11 exhibited maximum absorption at 804 and 862 nm. These absorption maxima within the 800–900 nm range are typical of bacteriochlorophyll a in the cytoplasmic membranes of PPB (Plennig & Trüper, 1992), further supporting their taxonomic classification.

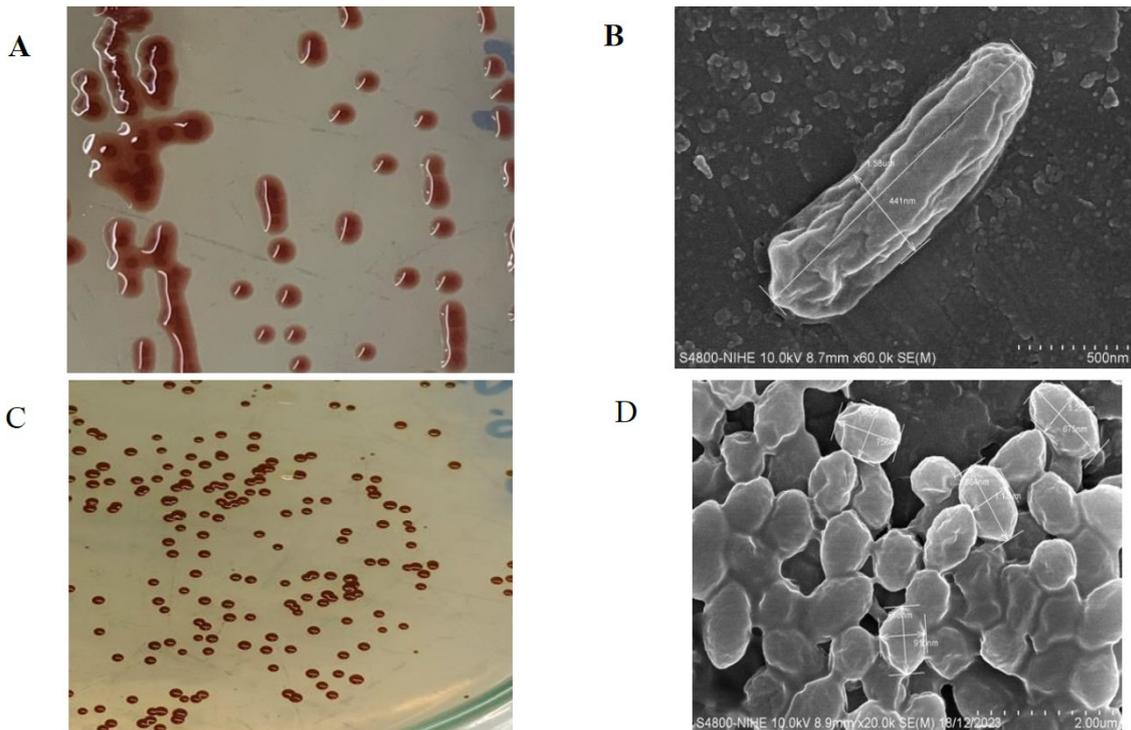


Figure 1. Colonies of strain (A), The cell morphology (B) of Y1 strains; Colonies of strain (C), The cell morphology (D) of GT10 strains

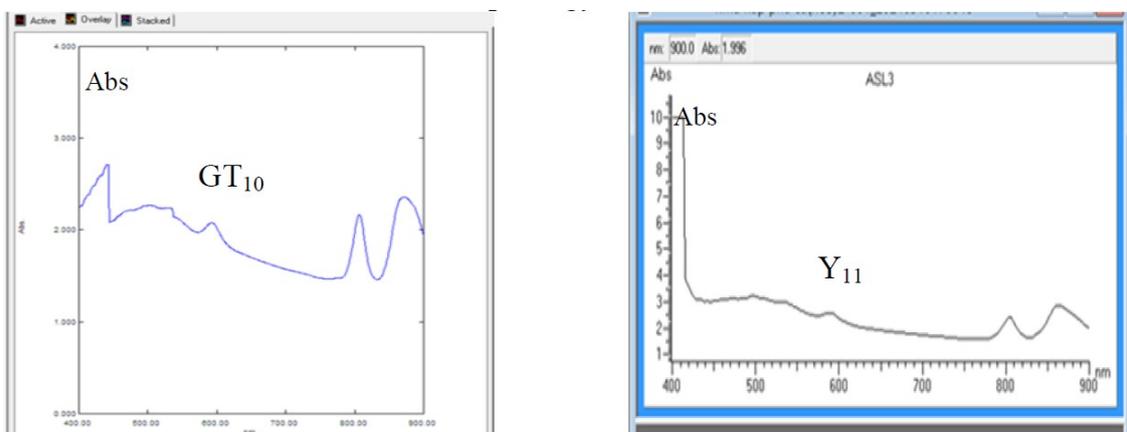


Figure 2. The absorption spectrum of GT<sub>10</sub> and Y<sub>11</sub> strains

### Ability to use carbon sources

In addition to their capacity for CO<sub>2</sub> fixation, purple photosynthetic bacteria (PPB) can utilize a wide range of organic carbon sources for growth. Some of these species-specific carbon sources can serve as taxonomic indicators for bacterial classification. To evaluate the carbon source utilization profiles of strains GT<sub>10</sub> and Y<sub>11</sub>, both isolates were cultured in a modified

DSMZ-27 medium, replacing sodium acetate with various alternative carbon sources. Cultivation was carried out under both anaerobic-light and aerobic-dark conditions, using liquid and solid media. The comparative ability of strains GT<sub>10</sub> and Y<sub>11</sub> to utilize different carbon sources was assessed about known profiles of *Rhodobacter capsulatus* and *Rhodopseudomonas palustris*, based on the studies by Girija et al. (2010) and Imhoff & Trüper (1989) in Table 3.

Table 3. Comparison of carbon source utilization by strains GT<sub>10</sub> and Y<sub>11</sub> concerning *Rhodobacter capsulatus* and *Rhodopseudomonas palustris* (Girija et al., 2010; Imhoff & Trüper, 1989)

Carbon source	GT <sub>10</sub>	<i>Rhodobacter capsulatus</i>	Y <sub>11</sub>	<i>Rhodopseudomonas palustris</i>
Acetate	++++	+	++++	+
Succinate	++++	+	++++	+
Formate	+	+	+	+
Tartate	-	-	-	-
Citrate	++	±	++	±
Glycerol	-	-	+++	+
Glucose	+++	+	++++	+
Methanol	-	-	-	±
Ethanol	-	-	-	±
Manitol	+++	±	++	±
Socbitol	++	±	+++	+
Propionate	+	+	+	+
Sulfide	+++	+	+++	+
Glutamate	++++	+	++++	+
Isopropanol	+	+	-	+

Note: Increased cell density at wavelength of 800 nm after 7 days of culture; (++++), ΔOD > 1.5; (+++), ΔOD from 1.0 to 1.5; (++), ΔOD from 0,5 to 1.0; (+), ΔOD from 0.1 to 0.5; and (-), ΔOD < 0.1 (no growth).

The results showed that under both anaerobic-light and aerobic-dark conditions, strain GT<sub>10</sub> could utilize a wide range of carbon sources, including acetate, propionate, lactate, succinate, formate, citrate, mannitol, sorbitol, glycerol, glutamate, isopropanol, and sulfide. However, it was unable to utilize tartrate, methanol, or ethanol. This carbon utilization profile closely resembled that of the genus *Rhodobacter*, particularly *R. capsulatus*, which, unlike other members of the genus such as *Rhodobacter sphaeroides*, *Rhodobacter veldkampii*, and *Rhodobacter adriaticus*, is capable of metabolizing both formate and

isopropanol (Imhoff & Trüper, 1989). Similarly, strain Y<sub>11</sub> demonstrated the ability to utilize acetate, propionate, lactate, succinate, formate, citrate, mannitol, sorbitol, glycerol, glutamate, and sulfide, but not tartrate, methanol, ethanol, or isopropanol. This metabolic profile is consistent with the genus *Rhodopseudomonas*, and most closely aligned with *R. palustris*. Based on these physiological characteristics, it is likely that strain GT<sub>10</sub> belongs to the species *R. capsulatus*, while strain Y<sub>11</sub> belongs to *R. palustris*. 16S rDNA gene sequencing was subsequently conducted to confirm their precise taxonomic positions.

### Bacterial identification

All selected strains were analyzed based on their 16S rRNA gene sequences to validate these taxonomic predictions, and phylogenetic relationships were constructed as shown in Figure 3. Comparative analysis using the GenBank database revealed that strain GT<sub>10</sub> shared the highest sequence similarity with *R. capsulatus*, displaying over 99% homology with sequences from strains of this species. Specifically, GT10 exhibited 99.12% sequence identity with *R. capsulatus* ATCC 11166

(GenBank accession no. D16428.1). Similarly, strain Y<sub>11</sub> showed high sequence homology with *R. palustris*, sharing over 99% identity with reference sequences, including *R. palustris* strain D12700 (GenBank accession no. D12700). The results of molecular identification were consistent with previous phenotypic characterization, further supporting the classification of GT<sub>10</sub> as *R. capsulatus* and Y<sub>11</sub> as *R. palustris*. Phylogenetic analysis was performed using the neighbor-joining method with 1,000 bootstrap replications to assess the robustness of the tree topology.

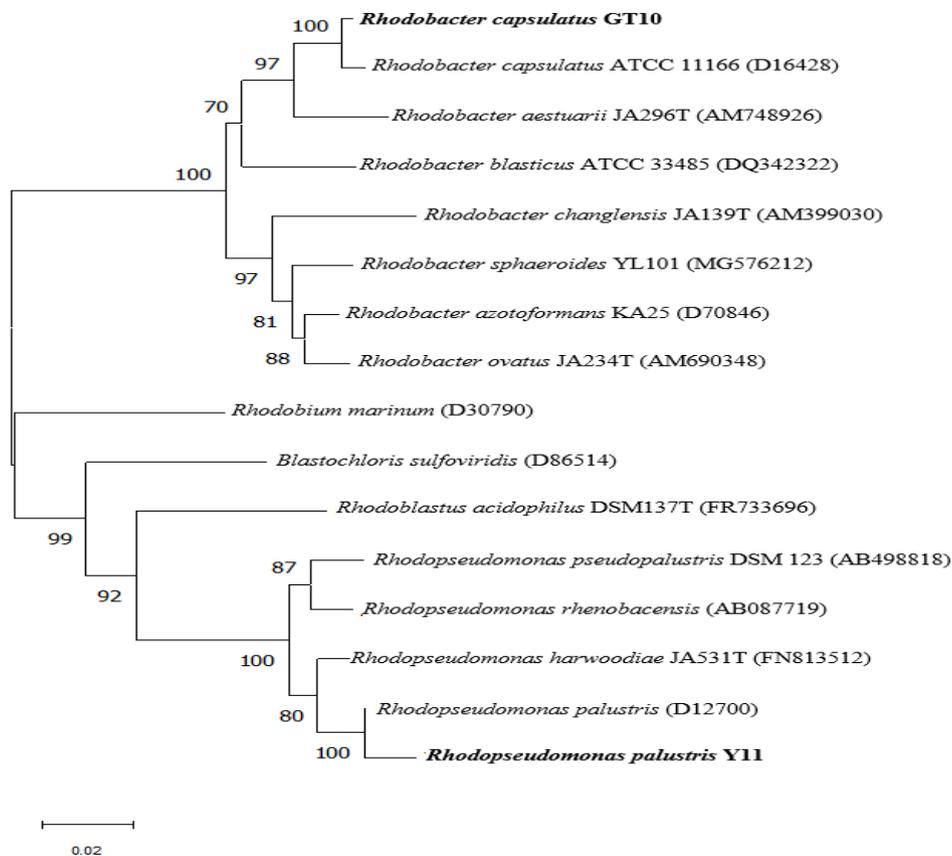


Figure 3. Phylogenetic tree constructed using the neighbor-joining method based on 16S rRNA gene sequences, illustrating the relationships of strains GT<sub>10</sub> and Y<sub>11</sub> with reference taxa within the domain Bacteria. Bootstrap values (based on 1000 replicates) are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site

### DISCUSSION

Over the past two decades, the trend of overusing chemical fertilizers in agriculture

has increased, with excess nitrogen fertilizer becoming standard in rice production; however, the fertilization principles have not been adhered to, resulting in low fertilizer use

efficiency (Bui Ba Bong, 2013). The overapplication of chemical fertilizers has been linked to increased disease susceptibility, greater dependence on pesticides, reduced product quality, environmental pollution, and higher greenhouse gas emissions (Truong Hop Tac, 2009). To mitigate these negative impacts, it is essential to investigate integrated fertilization strategies that combine inorganic, organic, and microbial fertilizers. Organic and microbial fertilizers are crucial in crop production, providing vital nutrients and maintaining long-term soil fertility. Among microbial inoculants, the most extensively studied nitrogen-fixing groups in rice cultivation include free-living cyanobacteria, heterotrophic bacteria, and cyanobacteria in symbiotic association with aquatic ferns (Choudhury et al., 2004). Additionally, purple photosynthetic bacteria (PPB) have emerged as promising candidates due to their nitrogen-fixing abilities and production of plant growth-promoting substances. Laboratory studies have demonstrated their potential to enhance cereal growth and increase rice yield (Montano et al., 2009; Harada et al., 2005). To produce fertilizer for rice, particularly in saline rice areas, we have isolated 24 strains of photosynthetic purple bacteria with varying morphological characteristics. These strains were evaluated for their ability to fix molecular nitrogen. The results indicated that nineteen medium-level  $\text{NH}_4^+$  releaser strains released between  $0.33 \pm 1.10$  and  $4.63 \pm 0.71$  mg/L. Notably, four strains were capable of fixing  $\text{N}_2$ , achieving over  $4.15 \pm 0.64$  mg/L, and exhibited a statistically significant difference ( $p \leq 0.05$ ) compared with all other tested bacterial strains. These findings align with those by Nookongbut et al. (2019), who isolated PPB from peat swamp forests in Thailand. In contrast to purple photosynthetic bacterial strains previously isolated from rice fields by Sakpirom et al. (2017) the strains obtained in the present study demonstrated a higher nitrogen-fixing capacity after 48 hours of incubation under light microaerobic conditions. Similarly, compared to the survey conducted by Nguyen Thi Thu Hang et al. (2015), in which

*Azotobacter* strains AZT1 and AZT7 were cultured in liquid Ashby medium supplemented with 2% glucose (pH 7.0) at 30 °C for 72 hours, nitrogen fixation capacities of 3.36 and 3.32 mg/L were reported, respectively. The nitrogen-fixing performance of the strains isolated in this study surpassed these values under comparable experimental conditions. Our six selected strains exceeded these figures, with  $\text{NH}_4^+$  production surpassing 3.94 mg/L. According to the study of Thai Thanh Duoc & Nguyen Huu Hiep (2022), screening 25 strains on Nfb liquid medium without a nitrogen source after 48 hours, the nitrogen-fixing bacteria strains isolated from corn roots all had the ability to synthesize  $\text{NH}_4^+$  ranging from 0.35–4.33 mg  $\text{NH}_4^+$ /L. This synthesis ability is equivalent to the photosynthetic purple bacteria strains isolated by us. According to the research results of Pham Thi Ngoc Lan & colleagues (2020), the study had two strains with strong growth and nitrogen fixation ability in Ashby liquid medium, strains N55 and N96. The  $\text{NH}_4^+$  content was 21.14 mg/mL and 59.60 mg/mL. The molecular nitrogen fixation capacity of these two strains was higher than that of the PPB strains we isolated.

The IAA content produced by our PPB isolates was also significant. It exceeded the IAA levels (63.11–73.87 ppm). Hoang Kim Chi et al. (2019) reported in root-zone bacterial strains isolated from turmeric. Compared with the IAA production ability of the PPB group isolated from a field contaminated with Cd and Zn in Thailand, published by Sakpirom & colleagues (2017), the IAA content produced by the isolated strains was 25–60 times higher. However, compared with the acid-tolerant PPB strains isolated in Thailand by Nookongbut & colleagues (2019), the IAA content produced by the acid-tolerant strains from  $(43.5 \pm 0.67)$ – $(302.6 \pm 1.77)$  mg/L was higher than that of the isolated PPB strains. Compared with the *Enterobacteria*, *Azotobacteria* & *Pseudomonas* species isolated from the rice rhizosphere soil in Colombia by the research group Torres & colleagues (2000), which produced the highest

IAA of 32.2 µg/mL, the IAA production ability of the 24 screened strains, of which 21 strains produced higher IAA.

This study initially screened 24 PPB strains, of which four were identified as potential biofertilizers based on NH<sub>4</sub><sup>+</sup> release (Table 3). Following further screening for indole-3-acetic acid (IAA) production (Table 2), three strains (GT4, GT10, and Y11) were identified as the most effective producers. Among them, strains GT10 and Y11 demonstrated high IAA production and strong nitrogen-fixing capabilities. These findings suggest that identifying strains suitable for field application requires broad (extensive) and targeted (intensive) screening efforts. Nevertheless, such efforts are justified, given the multifunctional potential of the selected isolates. Subsequent characterization of GT10 and Y11 included assessments of cell morphology, photosynthetic pigment profiles, and carbon source utilization. The carbon utilization analysis revealed that both strains were capable of metabolizing various carbon sources, including species-specific substrates. Notably, both strains could grow using sulfide as an electron donor. These combined physiological traits support the high potential of strains GT10 and Y11 as biofertilizer candidates for enhancing rice growth under diverse field conditions.

Under anaerobic conditions, the rhizosphere often supports the growth of sulfate-reducing bacteria (SRB), leading to the production of hydrogen sulfide (H<sub>2</sub>S) (Harada et al., 2001b). H<sub>2</sub>S at a concentration of 0.05 to 1 mg/mL adversely impacted rice cultivars by reducing the uptake rate of nutrients. This is due to H<sub>2</sub>S inhibiting respiration and the oxidative power of rice roots. GT<sub>10</sub> and Y<sub>11</sub> strains can use H<sub>2</sub>S as an electron donor for photosynthesis (Harada et al., 2001a, b). This ability may reduce sulfide toxicity in rice roots and enhance nutrient assimilation, improving grain production.

Strains GT<sub>10</sub> and Y<sub>11</sub> were further characterized based on biological, physiological, and biochemical features, including colony and cell morphology,

bacteriochlorophyll absorption spectra, carbon source utilization, and 16S rDNA sequence analysis. Based on these analyses, strain Y<sub>11</sub> was identified as *R. palustris*, while strain GT<sub>10</sub> was most closely related to *R. capsulatus*.

## CONCLUSION

In summary, two purple non-sulfur bacterial strains, GT<sub>10</sub> and Y<sub>11</sub>, were successfully isolated from rice fields in Nam Dinh, Vietnam. Both strains demonstrated significant abilities to fix atmospheric nitrogen and produce indole-3-acetic acid (IAA). Based on their biological characteristics and 16S rDNA sequence analysis, strain Y<sub>11</sub> was identified as *R. palustris*, while strain GT<sub>10</sub> was identified as *R. capsulatus*. These two strains exhibit promising potential for development as microbial biofertilizers to enhance rice cultivation. Further field-based studies are recommended to evaluate their effectiveness under real soil and environmental conditions, especially in saline-affected rice-growing areas.

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