Environment and Natural Resources Journal

Volume 22
Issue 2
2024

Interaction between Rhizobacteria and *Andrographis paniculata* Under Water Limitation

Butsakorn Yodphet[1], Nisachon Jangpromma[2], Wanwipa Kaewpradit Polpinit[3], and Nuntavun Riddech[4*]

[1]*Graduate school of Microbiology Program, Faculty of Science, Khon Kaen University, Thailand 40002*
[2]*Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Department of Biochemistry, Faculty of Science, Khon Kaen University, Thailand 40002*
[3]*Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Thailand 40002*
[4]*Department of Microbiology, Faculty of Science, Khon Kaen University, Thailand 40002*

ARTICLE INFO

Received: 2 Nov 2023
Received in revised: 23 Jan 2024
Accepted: 26 Jan 2024
Published online: 14 Feb 2024
DOI: 10.32526/ennrj/22/20230310

Keywords:
ACC-deaminase/ IAA production/ PGPR/ *Andrographis paniculata*/ Water limitation

*** Corresponding author:**
E-mail: nunrid@kku.ac.th

ABSTRACT

Drought stress is a major agricultural problem that leads to increased accumulation of ethylene in plants. It also has negative effects on plant productivity and growth. *Andrographis paniculate* is an important herb widely used in medical applications to inhibit diseases caused by viruses. In order to improve the production quality and growth of the *A. paniculata*, ACC-deaminase plant growth-promoting rhizobacteria were isolated from rice rhizosphere soil. All bacterial isolates were screened for their plant growth-promoting properties, including ACC deaminase, IAA production, biofilm formation, and exopolysaccharide production. Among the bacterial isolates, Rh-01 and Rh-22 exhibited positive results (cutting-edge) in all tests and were identified as *Paenibacillus polymyxa* Rh-01 and *Stenotrophomonas maltophilia* Rh-22, respectively. These strains were selected for further pot experiment study. Our results revealed that treatment with chemical fertilizer showed the highest potential to promote *A. paniculata* seedlings under normal moisture conditions. However, under water limitation conditions, the application of ACC-deaminase plant growth-promoting rhizobacteria led to a higher chlorophyll content compared to the control treatment. In addition, under normal irrigation conditions, plant growth promoting rhizobacterial increased relative water content and total biomass. In terms of plant stress markers, the proline content in *Andrographis paniculate's* seedling stage was low under water limitation conditions. In conclusion, to enhance the growth of *A. paniculate* seedlings during water limitation stress, a combination of microbial biofertilizers and chemical fertilizers is beneficial.

HIGHLIGHT

- ACC-deaminase plant growth-promoting rhizobacteria offer sustainable solutions for mitigating biotic and abiotic stress.
- ACC-deaminase plant growth-promoting rhizobacteria were able to promote the growth of *A. paniculata* when cultivated under water limitation
- The first report to indicate that *Paenibacillus polymyxa* Rh-01 and *Stenotrophomonas maltophilia* Rh-22 can stimulate the growth of *A. paniculata* under normal irrigation and water limitation
- Combined application of chemical fertilizers and microbial bio-fertilizers is recommended for sustainable agricultural practices.

1. INTRODUCTION

Herbs or medicinal plants are natural products widely used in medical applications for their numerous health benefits. Thailand has a variety of native herbs, including *Andrographis paniculata*, which is known for its medicinal properties in treating fever, common cold, diarrhea, and acting as an

Citation: Yodphet B, Jangpromma N, Polpinit WK, Riddech N. Interaction between rhizobacteria and Andrographis paniculata under water limitation. Environ. Nat. Resour. J. 2024;22(2):171-183. (https://doi.org/10.32526/ennrj/22/20230310)

antioxidant. Recently, researchers have discovered its potential in treating fever caused by COVID-19 infections (Jiang et al., 2021). *A. paniculata* grows well in tropical areas. However, the changing climate and rising ambient temperatures are affecting plant growth, with drought stress becoming a significant agricultural problem.

Drought stress is abiotic stress caused by water deficit, and has severe implications for ecosystems, agriculture and connected livelihoods. Water is essential for plant growth as it is a key factor that influences photosynthesis, dissolution, and transportation of nutrients in the soil (Selvakumar et al., 2012). Drought affects plant cells, resulting in reduced nutrient absorption and pigment synthesis, and increased accumulation of ethylene and reactive oxygen species (ROS), causing damage to plant organs and plant development (Rahdari et al., 2012). These substances are damaging to growth (Smirnoff, 1993) and significantly impact crop production in arid and semi-arid regions (Ahmadizadeh et al., 2012). In general, plants respond to biotic and abiotic stress by scavenging ROS through antioxidant enzyme activity and accumulating compatible solutes like flavonoid, proline, and phenolic compounds, including secreted phytohormones such as ethylene. However, many previous studies found that high levels of ethylene in plants can lead to early flowering and aging. Hence, the bacterial community surrounding plant roots has a crucial role in influencing plant resistance to drought stress. Several studies have shown that ACC-deaminase-producing bacteria can alleviate the negative effect of drought stress by converting ethylene into α-ketobutyrate and ammonia. Additionally, indole-3-acetic acid (IAA), synthesized by plant growth-promoting rhizobacteria (PGPR), can improve root and shoot growth. Zhou et al. (2016) demonstrated that the *Bacillus megaterium* BOFC15 released polyamine and abscisic acid (ABA), which play important roles in plant resistance to water limitation conditions by controlling the plant stomata and preventing water loss from plant cells.

Ethylene is a gaseous phytohormone released in large amounts by plants, especially during ripening prior to leaf shedding and flowering. Ethylene plays an important role in regulating plant growth and development. High accumulation of ethylene in plants can accelerate plant maturity and senescent of plant cells. While ethylene is beneficial in agriculture, its gaseous form limits its utilization. Furthermore, ethylene is also responsible for other abiotic stress, such as salt stress, floods, and heavy metal contamination. Water deficit conditions induce ethylene biosynthesis in plants, and high ethylene content can limit root and shoot growth (Liu et al, 2013).

The diverse microbial organisms in soil can be classified into two groups: beneficial microorganisms and non-beneficial microorganisms. Plant growth promoting rhizobacteria (PGPR) are beneficial microorganisms applicable in agriculture. Rhizobacteria live in the rhizosphere soil of plants and promote plant growth through various mechanisms. Direct mechanisms include phosphate solubilization, production of phytohormones, and nitrogen-fixing activities, while siderophore and antibiotic production are indirect mechanisms for improving the growth of plants. Several groups of PGPR exist, such as IAA-producing rhizobacteria, nitrogen-fixing rhizobacteria, phosphate-solubilizing rhizobacteria, and ACC deaminase-producing rhizobacteria. ACC-deaminase-producing rhizobacteria are capable of producing 1-aminocyclopropane-1-carboxylate (ACC)-deaminase enzyme, which degrades ethylene in plants by breaking down ACC into ammonia and α-ketobutyrate. Under water stress, ACC accumulates in plant cells. PGPR has shown significant ability to increase the fresh and dry weight of pepper and tomato seedlings whilst reducing ethylene content (Mayak et al., 2004). ACC deaminase-producing rhizobacteria promote plant growth and development and have proven to be effective strategies in alleviating drought-related problems. Therefore, the aims of this study were to explore the relationship between ACC deaminase-producing rhizobacteria and herb roots, as well as to evaluate the alleviation of water limitation stress using the ACC deaminase-producing rhizobacteria.

2. METHODOLOGY

2.1 Screening of rhizobacteria from rhizosphere soil samples under water limitation

Rice rhizosphere soil samples were collected from Ban Had, Khon Kaen, Thailand (GPS site: 16.166540°N 101.915853°W). Ten grams of the soil was mixed with 90 mL of 0.85% NaCl solution, and serially diluted using same solution as diluent. The samples were then spread on tryptic soya agar (TSA) (Himedia, India) supplemented with polyethylene glycol 6000 (PEG6000) up to 30% (w/w), and incubated at 30°C for 24-48 h. Rhizobacteria colonies that appeared on agar plates were selected based on different colony characteristics to pure culture and further tested for other plant growth-promoting traits.

2.2 Screening of ACC deaminase-producing rhizobacteria under water limitation

Thirty-seven bacterial isolates of rhizobacteria were obtained from rhizosphere soil. ACC deaminase-producing rhizobacteria were screened by using the modified DF salt minimal media (Dworkin and Foster, 1958). The media consisted of glucose, gluconic, citric acid, K_2HPO_4, Na_2HPO_4, $Mg.SO_4 \cdot 7H_2O$ amended with various concentrations of polyethylene glycol 6000 up to 35% by (w/v). The turbid in the medium was presented as a positive result and used for further study.

2.3 Measurement of the amount of ACC deaminase content in PGPR under water limitation

The two most effective ACC deaminase-producing rhizobacteria were grown in tryptic soya broth (TSB), and incubated at 28°C on a shaker incubator at 120 rpm for 24-48 h. The culture broth was diluted with 0.85% NaCl solution, and the optical density of the bacterial suspension was measured using a spectrophotometer at 500 nm, adjusted to OD 0.5 for use as inoculum size of starter culture. The suspension was then transferred into DF minimal salt broth supplemented with 3 mM ACC, incubated at 30°C, and shaken at 120 rpm for 24-72 h. After that, in the first step, the culture broth was centrifuged at 4,000 rpm for 10 min and the pellet was rinsed with 0.1 M Tris-HCl pH 7.5. In the second step, the bacterial cells were suspended in 0.1 M Tris-HCl pH 8.5, and 30 µL of toluene was added, and mixed on the vortex mixer for 30 s. In the third step, 200 µL of toluene and 20 µL of 0.5 M HCl were added and mixed on a vortex mixer, then incubated at 30°C for 30 min. In the fourth step, 1 mL of 0.56 M HCl was added and mixed on the vortex mixer and centrifuged at 13,000 rpm for 5 min. In the fifth step, 1 mL of supernatant was added to 800 µL of 0.56 M HCl and 300 µL dinitrophenylhydrazine, mixed, and incubated at 30°C for 30 min. For the last step, 2 mL of 2 N NaOH was added and absorbance was measured at 540 nm (Penrose and Glick, 2003). The experiment was conducted under two conditions: water limitation condition with the addition of 20% PEG6000 into the DF minimal salt medium, and normal condition without PEG6000.

2.4 Screening the biofilm formation and exopolysaccharide production in plant growth promoting rhizobacteria

The selected isolates were tested for biofilm formation using the crystal violet staining method (Latorre, 2016). The bacterial isolates were grown in TSB in a 15 mL centrifuge tube, and incubated at 30°C for 24 h. After incubation, a positive result was observed from the ring of biofilm which was adhered inside the surface of the tube, and the TSB medium was removed and the tube gently washed with distilled water. Then the 0.1% crystal violet solution was added to the tube to stain the adhered biofilm, then placed at room temperature for 25 min. After that, the crystal violet solution was discarded and the tube was washed with sterile distilled water. The detection of exopolysaccharide production in the bacterial isolates was performed by inoculating bacterial cells in TSB, incubating at 30°C for 24 h, then dropping the bacterial suspension on a paper disc and placing it on a minimal salt medium before incubation at 30°C for 4-5 days. After that, the paper disc was soaked in a test tube containing absolute ethanol. A positive result was indicated by the solution presenting a transparent color. This indicated that the bacteria could produce exopolysaccharides. (Paulo et al., 2012).

2.5 Study on the root colonization of PGPR in herb

2.5.1 Plant preparation

Two-month-old *A. paniculata* seedlings were washed with tap water to eliminate soil and debris.

2.5.2 Evaluation of root colonization in vitro by conventional method (spread plate technique)

In this experiment, *A. paniculata* seedlings were used. The seedlings were washed with sterile distilled water and soaked in bacterial suspension, both single isolates and co-inoculation, for 2 h. Then, the roots of the plant were transferred to 0.85% NaCl solution, diluted by 10-fold dilution up to 10^{-7} and spread on tryptone soya agar (TSA) and incubated for 24 h, after which the number of bacterial colonies on the agar plate was counted (Gamalero et al., 2004).

2.5.3 Microbial inoculant and plant preparation by scanning electron microscope (SEM) study

The two rhizobacterial isolates, in the form of single inoculum and co-inoculum (in the ratio of 1:1 (v/v)), were prepared in tryptone soya broth (TSB). The culture broth was centrifuged and the cell density was adjusted to 10^8 CFU/ mL. The rhizobacterial suspension was then transferred to Hoagland solution and the *A. paniculata* seedling was soaked in each rhizobacterial isolate's inoculum for 2 h. After that, the root of the plant was cut to 2-3 centimeters, fixed with 2.5% glutaraldehyde, and covered with

aluminum foil, before storage at 4°C for 2 h. The root sample was washed with sterile 0.1 M phosphate saline buffer for 10 min, three times. The sample was soaked in ethanol at varying concentrations up to 100% for 15 min, except for the 100% ethanol concentration which was soaked for 30 min, and this step was repeated. After that, the samples were stored in a desiccator before dehydration using the critical point dryer machine for 2 h. Finally, the sample was placed on carbon tape and stub respectively before being observed under the scanning electron microscope (modified from Kim and Krcmcr, 2005; Lombardi et al., 2018).

2.5.4 Characterization of PGPR isolates by morphology study and molecular assay

Bacterial isolates were characterized by studying the colony morphology and observing the appearance trait. The identification of PGPR was performed by PCR amplification and sequencing of bacterial 16s rRNA gene. Bacterial genomic DNA was obtained from the overnight grown cells using a genomic DNA extraction kit (Tiangen biotech (Beijing, China)). The sequence of nucleotide primer used for amplification of 16s rRNA genes was universal 8F primer: 5'-AGA GTT TGA TCM TGG CTC AG-3'and reverse universal 1512R primer: 5'-ACG GYT ACC TTG TTA CGA CTT-3'. DNA samples were purified and amplified on thermal PCR machine (PCR MJ Research-PTC-200). The program was used for the amplification of 16s rRNA gene: preheating at 95°C for 10 min denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.30 min, and final extension at 72°C for 10 min, followed by cooling at 4°C when the PCR product was purified and sequenced at services of ATCG Co., Ltd., Thailand. The obtained nucleotide sequences were compared with the NCBI database using the BLASTN program. The nucleotide sequence was compared by using MEGA 7.0.9 software, and a phylogenetic tree was constructed. Additionally, 16s rRNA nucleotide sequences in both strains were deposited in the DDBJ database following the submission instructions at https://www.ddbj.nig.ac.jp/index-e.html.

2.5.5 Pot experiment

A. paniculata seedlings were transferred to pots containing a mixture of soil and coconut fiber in a 1:1 ratio, with a total weight of 5 km. One month after transplantation, the seedlings were exposed to drought stress, and their growth parameters were measured two months later. The treatment of the pot experiment was shown in Table 1.

Table 1. Experimental design for growing *A. paniculata*

No.	Treatments
1	Control without PGPR + irrigated 100% FC
2	Control without PGPR + irrigated 50% FC
3	PGPR + irrigated 100% FC
4	PGPR + irrigated 50% FC
5	Organic fertilizer + irrigated 100% FC
6	Organic fertilizer + irrigated 50% FC
7	Chemical fertilizer + irrigated 100% FC
8	Chemical fertilizer + irrigated 50% FC

2.5.6 Statistical analysis

Mean and standard deviation were calculated for the experiment data. Statistical analysis of the data was carried out by using the Statistix 10 program. Analysis of variance (ANOVA) and Least Significant Difference (LSD) were used for comparison of significant differences among all experimental treatments.

3. RESULTS AND DISCUSSION

3.1 Isolation and screening of plant growth promoting rhizobacteria

The total microorganisms in the soil sample are shown in Table 2. Thirty-two bacterial isolates were isolated from the rice rhizosphere soil. Among them, 16, 16, and 17 bacterial isolates were able to grow in TSB supplemented with polyethylene glycol 6000 (PEG6000) at concentrations of 20%, 30%, and 35%, respectively. Of all these bacterial isolates, only 17 isolates were able to produce the ACC-deaminase enzyme.

Table 2. Total microorganisms in rice rhizosphere soil sample

Sample	Log CFU/g soil
1	6.63±0.33
2	6.57±0.23
3	6.59±0.42

All of the bacterial isolates that showed ACC-deaminase activity were selected for detecting other plant growth-promoting properties, including IAA production (Table 3), biofilm formation, and exopolysaccharide production. The most effective ACC-deaminase plant growth-promoting rhizobacteria were chosen for quantitative analysis in a further experiment.

Table 3. IAA content in plant growth promoting rhizobacteria under normal and water limitation condition. ANOVA was performed with mean and standard deviation in each column with the same letter indicating no significant difference ($p<0.05$) by LSD test.

isolates	IAA content (µL/mL) without PEG (normal condition)	IAA content (µL/mL) with PEG (water limitation)
Rh-01	16.49±0.92^{b}	20.37±0.38^{c}
Rh-06	16.52±1.52^{b}	14.14±0.92^{d}
Rh-10	11.94±0.42^{c}	11.92±0.22^{ef}
Rh-13	7.29±0.88^{ef}	10.91±0.93^{fg}
Rh-22	29.78±0.88^{a}	56.75±1.52^{a}
Rh-23	5.48±0.59^{f}	13.13±0.22^{de}
Rh-28	12.31±0.70^{c}	24.82±0.09^{b}
Rh-32	8.87±0.12d^{e}	11.82±0.18^{ef}
Rh-37	10.38±1.39^{cd}	9.70±1.44^{g}

Two high-potential isolates of PGPR containing ACC-deaminase were examined for quantitative ACC-deaminase enzyme activity under normal and drought stress conditions. The results showed that the rhizobacteria could produce the ACC-deaminase enzyme under both conditions (Table 4).

Table 4. Quantitative estimation of ACC-deaminase activity in plant growth-promoting rhizobacteria. ANOVA analysis was performed with Mean and standard deviation in each column with the same letter indicating no significant difference ($p<0.05$) and by LSD test.

isolate	ACC-deaminase activity (µmol of α-ketobutyrate/mg protein/h)	
	With PEG6000	Without PEG6000
Rh-01	21.66±0.37^{b}	39.72±1.35^{b}
Rh-22	27.01±0.07^{a}	50.20±0.64^{a}

The most efficient ACC-deaminase plant growth-promoting rhizobacteria were further evaluated for quantitative ACC-deaminase enzyme activity under both normal and water limitation conditions. Under normal irrigation, all bacterial isolates showed a more effective production of the ACC-deaminase enzyme compared to the drought stress condition. However, PGPR was also able to produce the ACC-deaminase enzyme even under water limitation. Bacterial isolate Rh-22 seemed to produce more ACC-deaminase enzyme than isolate Rh-01 under both conditions.

Observation of root colonization by ACC-deaminase-producing rhizobacteria with plant growth-promoting properties under a scanning electron microscope revealed that both rhizobacteria isolates were capable of colonizing roots (Figure 1). This was similar to the results found in conventional experiments, where spreading plates were used to count the number of rhizobacteria invading plant roots; we found that the number of bacteria for both isolates was in a similar profile. Interestingly, co-inoculated treatment resulted in higher bacteria numbers compared to other treatments (Table 5).

Table 5. Root colonization of plant growth-promoting rhizobacteria by viable plate count

isolate	Log CFU/root	
	With PEG	Without PEG
control	6.08±0.022	6.19±0.043
Rh-01	9.28±0.034	9.19±0.043
Rh-22	10.18±0.071	9.29±0.034
Co-inoculated	10.10±0.089	9.78±0.300

3.2 Colony morphology and molecular identification of bacterial isolates

Characteristics of colony morphology that appeared on culture medium Nutrient agar (NA) plant growth-promoting rhizobacteria isolate Rh-01 colonies are round, flat, white, small-sized, jagged edged, gram-positive, and rod-shaped. For Rh-22 isolates, the colonies are white, jagged edged, wrinkled, gram-positive isolate (Table 6 and Figure 2). For molecular identification, the sequence of the 16S rRNA gene was analyzed. Rhizobacteria isolate Rh-01 was identified as *Paenibacillus polymyxa* Rh-01 with 99.6% similarity. Rh-22 showed 100% similarity with *Stenotrophomonas maltophilia* Rh-22 species (Table 7). The nucleotide accession number of strains Rh-01 and Rh-22 are LC775011 and LC775012, respectively. The phylogenetic tree was constructed from the 16S rRNA sequence of genus *Bacillus* and *Stenotrophomonas* (Figure 3).

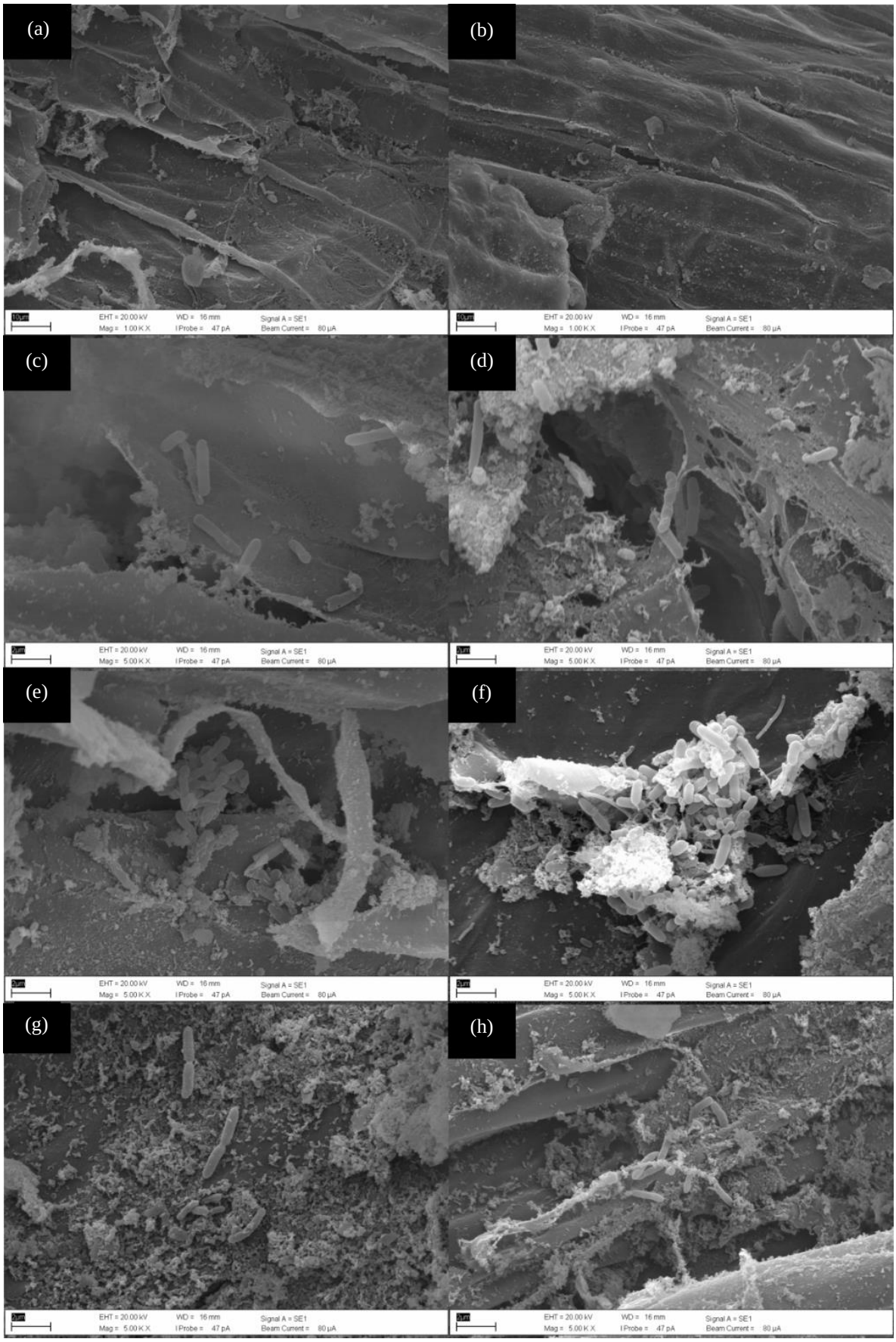

Figure 1. Root colonization of plant growth-promoting rhizobacteria observed under a scanning electron microscope, control without PEG and with PEG (a, b), strain Rh-01 without PEG (c), strain Rh-01 with PEG (d), strain Rh-22 without PEG (e), strain Rh-22 with PEG (f), mix of 2 strains without PEG (g), co-inoculation of 2 strains with PEG (h), with magnification 5000X.

3.3 Appearance of *A. paniculata* growth when simulated with plant growth-promoting rhizobacteria containing ACC-deaminase.

After 2 months of planting, A. paniculata plants were harvested and measured for growth parameters and stress markers (proline and relative water content).

Table 6. Morphological of plant growth-promoting rhizobacteria containing ACC-deaminase activity

Colony morphology	PGPR isolates	
	Rh-01	Rh-22
Form	Circular	Irregular
Color	White	Yellow
Margin	Entire	Curled
Structure	Opaque	Opaque
Surface	Smooth	Rough

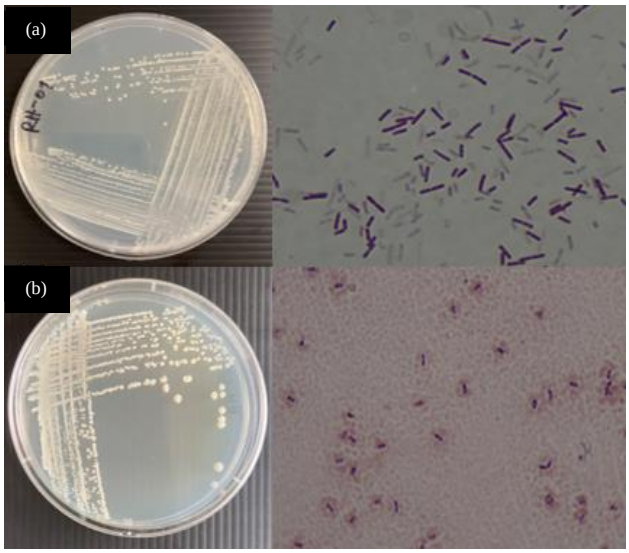

Figure 2. Colony morphology and gram stain of isolate Rh-01 (a), Rh-22 (b)

Table 7. Molecular identification of ACC-deaminase plant growth-promoting rhizobacteria

Bacterial isolates	Molecular identification		
	Closet NCBI match	Accession NO.	Similarity
Rh-01	*Paenibacillus polymyxa* Rh-01	LC775011	99.64%
Rh-22	*Stenotrophomonas maltophilia* Rh-22	LC775012	100%

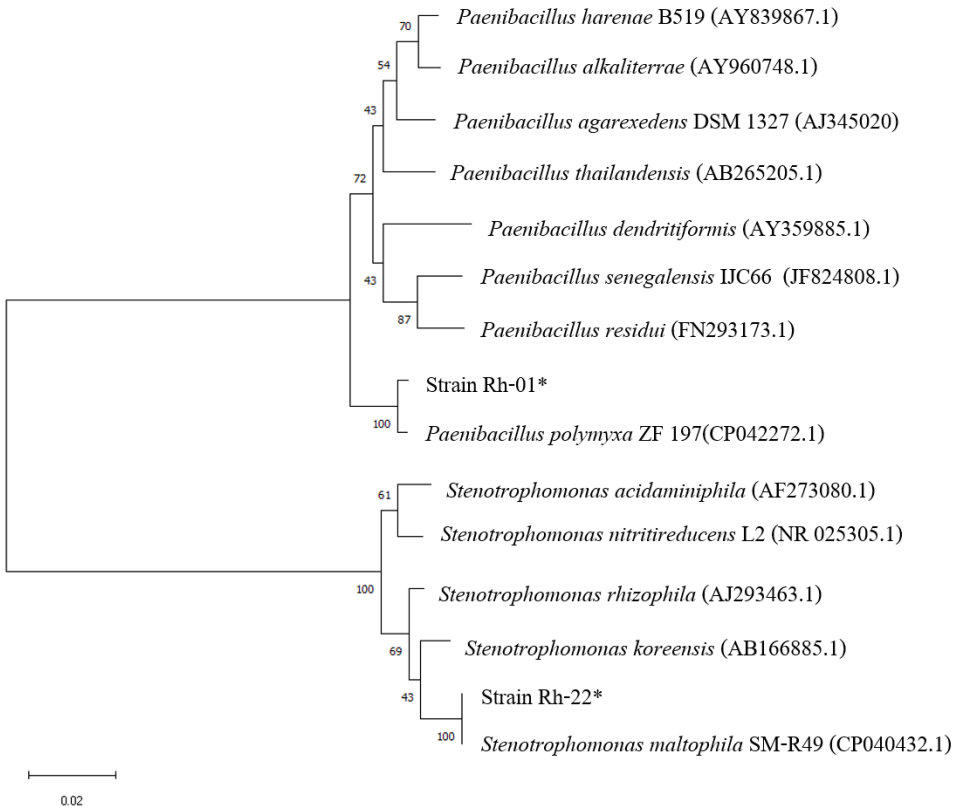

Figure 3. The phylogenetic tree was constructed from the 16S rRNA gene sequence from selected bacterial isolates by using Mega 11 software

3.4 Plant height and chlorophyll content

Water limitation at 50% FC of irrigation in all treatments had an effect on plant height, especially with plants treated with organic fertilizer (T6) and chemical fertilizer (T8). The reduction in plant height might be due to the limited water content available to solubilize nutrients in the soil. However, a non-significant difference was observed in treatment T3 (supplemented with PGPR, 100% FC, and 50% FC irrigation). Moreover, the plant height in T4 (PGPR and 50% irrigation) and T8 (Chemical and 50% irrigation) revealed a similar profile with a non-significant difference in statistical calculation. This indicated that PGPR and chemical fertilizer have equal potential to stimulate plant height under water limitation conditions (Figure 4).

For the chlorophyll content in plant leaves, the result showed that at 50% FC of irrigation in treatments, the chlorophyll content of the plants was lower than in treatment T7, which was treated with chemical fertilizer (100% FC irrigation). Moreover, our results indicated that PGPR in both treatments T3 (50% FC) and T4 (100% FC)) were able to enhance the growth of plants by expressing a higher content of chlorophyll than in the control (T2 with 50% FC irrigation) (Figure 4).

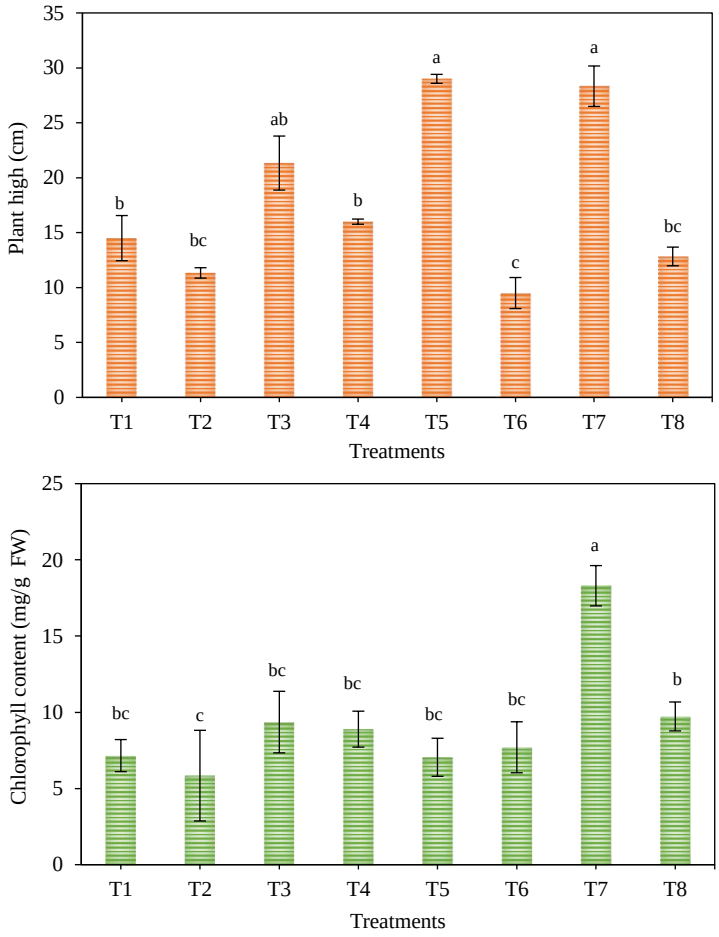

Figure 4. Effect of PGPR inoculant on Plant height and Chlorophyll content (T1=control + 100% FC, T2=control + 50% FC, T3=PGPR + 100% FC, T4=PGPR + 50% FC, T5=Organic Fertilizer + 50% FC, T6=Organic fertilizer + 100% FC, T7=Chemical fertilizer + 100% FC, T8=Chemical fertilizer + 50% FC).

3.5 Plant yield

Maximum total biomass was yielded in the treatment which applied chemical fertilizer with 100% FC irrigation (T7). It decreased when plants were treated with 50% FC irrigation in all treatments. However, when these values were compared to the control T1 and T2, there was a non-significant difference in the treatment that was treated with PGPR (T4), organic fertilizer (T6), and chemical fertilizer (T8). It seems that the PGPR's ability to solubilize nutrients in soil was similar to when the soil was applied with organic and chemical fertilizer. Moreover, we found that our PGPR was able to promote the biomass of roots when plants were treated

with PGPR under 100% FC irrigation. On the other hand, it decreased when treated with 50 % FC water irrigation. This indicates that water is important for supporting the microbial activities in soil that affect plant growth (Table 8).

Table 8. Effect of PGPR inoculant on root and shoot fresh weight, root & shoot dry weight, root and shoot biomass, and total biomass of *Andrographis paniculate (burm.f.) nees* under water limitation. ANOVA was performed with Mean and standard deviation in each column with the same letter indicating no significant difference *($p<0.05$) and **($p<0.01$) by LSD test.

Treatments	Shoot fresh weight (g)	Shoot dry weight (g)	shoot biomass (g)	Root fresh weight (g)	Root dry weight (g)	Root biomass (g)	Total biomass (g)
T1	3.583^{b}	1.003^{b}	2.580^{b}	2.013	0.338^{cd}	1.680	4.260^{bc}
T2	1.923^{cd}	0.790^{b}	1.133^{c}	1.620	0.346^{cd}	1.273	2.407^{d}
T3	2.910^{bcd}	0.823^{b}	2.087^{bc}	3.757	0.599^{ab}	3.157	5.243^{b}
T4	2.107^{bcd}	0.630^{b}	1.478^{bc}	3.757	0.323^{cd}	1.180	2.657^{cd}
T5	3.380^{bc}	0.887^{b}	2.493^{b}	3.517	0.573^{abc}	2.943	5.437^{b}
T6	1.777^{d}	0.533^{b}	1.243^{c}	2.350	0.265^{d}	2.083	3.327^{cd}
T7	13.467^{d}	3.797^{a}	9.670^{a}	2.637	0.690^{a}	1.947	11.617^{a}
T8	2.59^{3bcd}	0.940^{b}	1.653^{bd}	1.730	0.400^{bcd}	1.330	2.983^{cd}
%CV	22.52	23.44	25.67	47.92	32.03	56.27	21.40
F-test	**	**	**	ns	*	ns	**

3.6 Relative water content and accumulation of proline

Relative water content (RWC) refers to the current water content in the leaf of the plant. Normally, the reduction of relative water content was observed in drought stress condition. Our data showed that a lower content of RWC was found in all 50% FC irrigation. The RWC was in the range of 28-48% (Figure 5).

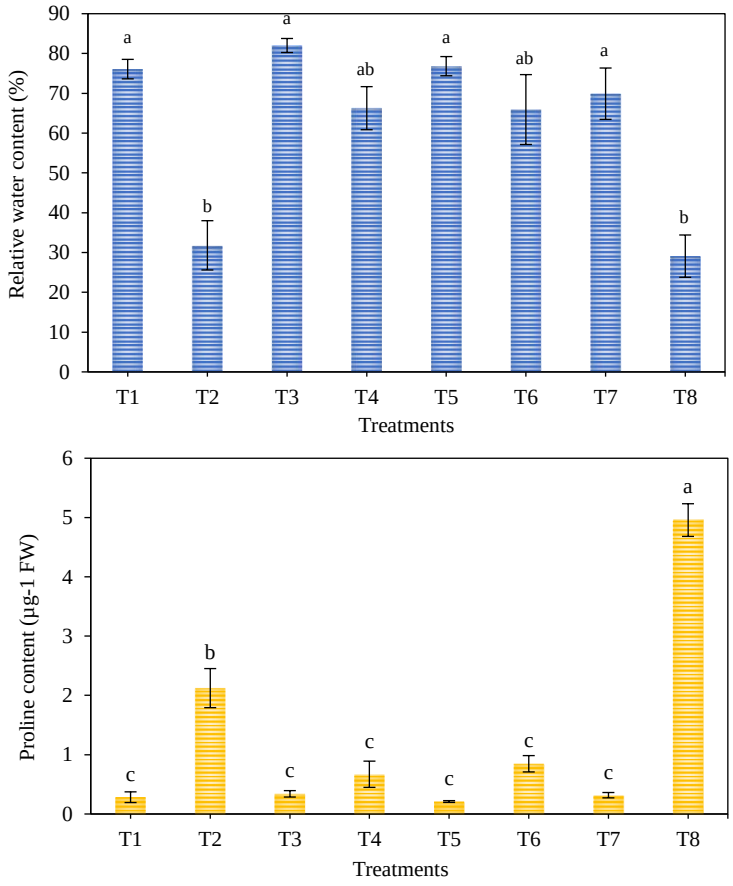

Figure 5. Effect of PGPR inoculant on proline content and relative water content in plant leaf (T1=control + 100% FC, T2=control + 50% FC, T3=PGPR + 100% FC, T4=PGPR + 50% FC, T5=Organic Fertilizer + 50% FC, T6=Organic fertilizer + 100% FC, T7=Chemical fertilizer + 100% FC, T8=Chemical fertilizer + 50% FC).

The growth of *A. paniculata* was affected by water limitation conditions as observed from the increasing accumulation of proline in plant leaves. The highest proline content was recorded in the treatment of chemical fertilizer with 50% FC irrigated (T8). However, the application of microbial inoculant (PGPR) for growing plants under water limitation (T3 and T4) presented a significantly decreased proline content when compared to the control treatment (T2) and T8 (supplemented with chemical fertilizer and 50% FC irrigation) (Figure 5). Meanwhile, there was no significant difference in other treatments when compared to the normal irrigation control treatment (T1). This indicated that irrigation had an effect on the accumulation of proline in plants, with the plant encountering stress due to low water content.

4. DISCUSSION

Water deficit affects plant growth, as water is an important factor for photosynthesis in plants and is necessary for the uptake and transport of nutrients in the soil (Lisar et al., 2012). The application of ACC-deaminase plant growth-promoting rhizobacteria is an environmentally sustainable option to alleviate the effect of biotic and abiotic stress on plant growth and productivity (Glick, 2005). In most higher plants the synthesis of ethylene is one of the response mechanisms utilized to cope with biotic and abiotic stress. A high level of ethylene has an impact on the growth and development of plants.

In the present study, two bacterial isolates, Rh-01 and Rh-22, exhibited large amounts of ACC deaminase activity and were selected from rice rhizosphere in arid regions for further assessment of growth promoting abilities such as IAA production, biofilm formation, and exopolysaccharide production. Phytohormones such as IAA, which are released by plant growth promoting bacteria, can promote plants by stimulating root elongation, the formation of lateral roots, and root hairs. This mechanism improves root system and enhances nutrient uptake in plants (Gupta and Panday, 2019).

According to our results, various types of fertilizer and water irrigation affect the growth of *A. paniculata* plant differently. Under normal irrigation conditions, the results showed that chemical fertilizer is the best fertilizer for promoting the growth of *Andrographis paniculate* in terms of improvement in plant height and chlorophyll content. In contrast, under water limitation conditions (50% FC irrigation), there was no significant difference in chlorophyll content among all three fertilizers, but a significant difference was found when compared to the water stress control treatment (T2; 50% FC irrigation without fertilizer). However, the highest increase in plant height was shown in the treatment with microbial bio-fertilizer (PGPR) (T4). This result suggests that at the 50% FC irrigation, ACC- deaminase plant growth-promoting bacteria could help to promote plant height under water stress treatment when compared to the control (without supplemented with fertilizer, T2), added organic fertilizer (T6) and chemical fertilizer. (T8). The improvement in plant height might be due to the reduction of stress ethylene levels by ACC-deaminase plant growth promoting bacteria. Danish et al. (2020) also demonstrated that ACC-deaminase producing bacteria enhanced the plant height of maize under drought stress. In addition, Yuan et al. (2023) reported that ACC-deaminase-producing bacteria *Pseudomonas* DY1-3 increased root growth and height of maize. They indicated the potential of plant growth-promoting bacteria to improve resistance to abiotic stress.

For the results of other plant growth parameters, chemical fertilizer treatment showed the highest increase in shoot fresh weight, shoot dry weight, root dry weight, and total biomass which was similar to the findings of Forouzandeh et al. (2012) who also reported that chemical fertilizers showed the highest effect on essential oil content in the medicinal plant Basil.

Biofilm formation of rhizobacteria promotes plants directly. Root exudate secreted by plants contains different types of sugar, vitamins, organic acid, and mucilage that attract PGPR for root colonization and form a biofilm around the root of the plant. The role of biofilm could promote root colonization of the microorganism located around the plant's root. Root colonization plays an important role in the survival of bacteria and protects the plant from the adverse effects of drought stress (Ansari and Ahmad, 2018). In addition, biofilm maintains the water and nutrient-holding capacity in the rhizosphere soil around the plant roots, improving the uptake of water and soluble nutrients. Exopolysaccharide (EPSs) production is one of the important traits of PGPR that has benefits on the formation of biofilm. Exopolysaccharides look like high molecular weight polymers of sugar, which contain both homopolysaccharides and heteropolysaccharides. Normally, EPSs are a key factor for promoting and protecting the microbial cell which maintains moisture, and thus increasing drought-tolerant

capability. Many research reports state that exopolysaccharides play a significant role in biofilm formation and also serve as an important component in biofilm formation (Salas-Jara et al., 2016; Lebeer et al., 2007; Czaczyk and Myszka, 2007).

The molecular identification and phylogenetic tree analysis revealed that the rhizobacterial isolate Rh-01 and isolate Rh-22 belong to *Paenibacillus polymyxa* Rh-01 and *Stenotrophomonas maltophilia* sm-Rh-22 respectively. A similar result was observed by Gupta and Pandey (2019), who reported the ACC-deaminase-producing ability of *Peanibacillus*. Moreover, Majeed et al. (2015) also indicated multiple plant growth promoting traits and the potential of *Stenotrophomonas* spp. for biofertilizer production.

According to pot experiment results, different types of fertilizer and different levels of water irrigation affected the growth of *A. paniculata* plant. Under the normal water conditions, chemical fertilizer best promoted the growth of *A. paniculata* in terms of improvement in plant height and chlorophyll content. In contrast, under water limitation conditions (50% FC), there was a non-significant difference in chlorophyll content among all three kinds of fertilizers, but a significant difference was found when compared to the water stress control treatment (T2). However, the highest increase in plant height was shown in microbial bio-fertilizer treatment (T4). This result suggested that ACC- deaminase plant growth-promoting bacteria could help promote plant height when compared to organic fertilizer with water stress treatment (T6), which showed the highest decrease in plant height. This result was consistent with the previous study of Ratnaningsih et al. (2023) who investigated ACC deaminase isolated from the rhizosphere of pine apple plants and found that these microbes promoted the growth of soybean. Similarly, Gupta et al. (2022) indicated that ACC deaminase producing PGPR could alleviate the adverse effect of osmotic and salinity stress in *Pisum sativum*.

Plant stress markers indicated the stress level in plants. In this study, we investigated relative water content and proline content in *A. paniculata* leaves. Our result revealed that relative water content exhibited a non-significant difference among all of the normal irrigation conditions (100% FC), supplemented with each type of fertilizer. On the other hand, under drought stress conditions the relative water content decreased when compared to normal irrigation as a control treatment (T1). A similar result was presented by Aslam et al. (2021), who found a reduction in leaf water content when using chemical fertilizer alone under severe evaporation for improved Canola growth, decreasing relative water content as a result of water limitation. In contrast, the application of microbial biofertilizer (PGPR) maintained relative water content in plant leaves.

Proline content is one of the most important osmoprotectants in higher plants. Our results showed proline content increased in water limitation treatment (T2). Furthermore, chemical fertilizers with water limitation condition also increased proline content in *A. paniculata* leaves, indicating that the accumulation of proline under stress in plants might be due to the high acidic and electro conductivity (pH and EC) of chemical fertilizer, while the application of microbial bio-fertilizer triggered a reduction in proline content in *A. paniculata* leaves. These results demonstrated that microbial bio-fertilizer alleviates stress in plants by increasing relative water content and decreasing proline accumulation in the plant. Furthermore, our results were consistent with a previous report showing that exopolysaccharide-producing bacteria significantly reduced proline content in inoculated plants (Khan and Bano, 2019). In our study indicates that ACC-deaminase-producing bacteria showed potential as a microbial biofertilizer for improving plant growth under biotic and abiotic stress.

5. CONCLUSION

Our results suggest that drought stress has a negative impact on the physiological and biochemical growth markers of the *A. paniculata* plant compared with the control treatment. However, plant growth promoting rhizobacteria can ameliorate plants via various mechanisms. These findings lead to a recommendation for the combined application of chemical fertilizers and microbial bio-fertilizers to accomplish sustainable agricultural goals.

DECLARATION OF COMPETING INTEREST

The authors confirm that they have not encountered any interpersonal or financial disputes that would have appeared to have an impact on the data presented in this study.

ACKNOWLEDGEMENTS

This study has been supported by Microbial Fertilizer Lab group, the Department of Microbiology, Faculty of Science Khon Kaen University and Protein

and Proteomics Research Center for Commercial and industrial Purposes (ProCCI). We would to thank Dr. Pornrapee Sarin and Dr. Natthawat Sritongon for assisting with the recommendations and statistical analysis. The author would like to thanks Mr. Matthew Savage for proofing English writing of this manuscript.

REFERENCES

Ahmadizadeh M, Valizadeh M, Shahbazi H, Nori A. Behavior of durum wheat genotypes under normal irrigation and drought stress conditions in the greenhouse. African Journal of Biotechnology 2012;11(8):1912-23.

Ansari FA, Ahmad I. Biofilm development, plant growth promoting traits and rhizosphere colonization by *Pseudomonas entomophila* FAP1: A promising PGPR. Advances in Microbiology 2018;8(3):Article No. 235.

Aslam MM, Farhat F, Siddiqui MA, Yasmeen S, Khan MT, Sial MA, et al. Exploration of physiological and biochemical processes of canola with exogenously applied fertilizers and plant growth regulators under drought stress. PloS One 2021;16(12):e0260960.

Czaczyk K, Myszka K. Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. Polish Journal of Environmental Studies 2007;16(6):799-806.

Danish S, Zafar-ul-Hye M, Mohsin F, Hussain M. ACC-deaminase producing plant growth promoting rhizobacteria and biochar mitigate adverse effects of drought stress on maize growth. PLoS One 2020;15(4):e0230615.

Dworkin M, Foster JW. Experiments with some microorganisms which utilize ethane and hydrogen. Journal of Bacteriology 1958;75(5):592-603.

Forouzandeh M, Fanoudi M, Arazmjou E, Tabiei H. Effect of drought stress and types of fertilizers on the quantity and quality of medicinal plant Basil (*Ocimum basilicum* L.). Indian Journal of Innovations and Developments 2012;1(9):696-9.

Gamalero E, Lingua G, Giusy Caprì F, Fusconi A, Berta G, Lemanceau P. Colonization pattern of primary tomato roots by *Pseudomonas fluorescens* A6RI characterized by dilution plating, flow cytometry, fluorescence, confocal and scanning electron microscopy. FEMS Microbiology Ecology 2004;48(1):79-87.

Glick BR. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiology Letters 2005;251(1):1-7.

Gupta A, Rai S, Bano A, Sharma S, Kumar M, Binsuwaidan R. ACC deaminase produced by PGPR mitigates the adverse effect of osmotic and salinity stresses in Pisum sativum through modulating the antioxidants activities. Plants 2022;11(24):Article No. 3419.

Gupta S, Pandey S. ACC deaminase producing bacteria with multifarious plant growth promoting traits alleviates salinity stress in French bean (*Phaseolus vulgaris*) plants. Frontiers in Microbiology 2019;10:Article No. 1506.

Jiang M, Sheng F, Zhang Z, Ma X, Gao T, Fu C, et al. *A. paniculata* (Burm. f.) Nees and its major constituent andrographolide as potential antiviral agents. Journal of Ethnopharmacology 2021;272:Article No. 113954.

Khan N, Bano A. Exopolysaccharide producing rhizobacteria and their impact on growth and drought tolerance of wheat grown under rainfed conditions. PLoS One 2019;14(9):e0222302.

Kim SJ, Krcmcr RJ. Scanning and transmission electron microscopy of root colonization of morning glory (*Lpomoea* spp.) seedlings by rhizobacteria. Symbiosis 2005;39:117-24.

Latorre JD, Hernandez-Velasco X, Wolfenden RE, Vicente JL, Wolfenden AD, Menconi A, et al. Evaluation and selection of Bacillus species based on enzyme production, antimicrobial activity, and biofilm synthesis as direct-fed microbial candidates for poultry. Frontiers in Veterinary Science 2016;3:Article No. 95.

Lebeer S, Verhoeven TL, Perea Vélez M, Vanderleyden J, De Keersmaecker SC. Impact of environmental and genetic factors on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG. Applied and Environmental Microbiology 2007;73(21):6768-75.

Lisar SY, Motafakkerazad R, Hossain MM, Rahman IM. Causes, effects and responses. Water Stress 2012;25(1):1-14.

Liu F, Xing S, Ma H, Du Z, Ma B. Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in *Platycladus orientalis* container seedlings. Applied Microbiology and Biotechnology 2013; 97:9155-64.

Lombardi N, Vitale S, Turrà D, Reverberi M, Fanelli C, Vinale F. Root exudates of stressed plants stimulate and attract Trichoderma soil fungi. Molecular Plant-Microbe Interactions 2018;31(10):982-94.

Majeed A, Abbasi MK, Hameed S, Imran A, Rahim N. Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Frontiers in Microbiology 2015;6:Article No. 198.

Mayak S, Tirosh T, Glick BR. Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Science 2004;166(2):525-30.

Rahdari P, Hoseini SM. Drought stress: A review. International Journal of Agronomy and Plant Production 2012;3(10):443-6.

Ratnaningsih HR, Noviana Z, Dewi TK, Loekito S, Wiyono S, Gafur A, et al. IAA and ACC deaminase producing-bacteria isolated from the rhizosphere of pineapple plants grown under different abiotic and biotic stresses. Heliyon 2023; 9(6):e16306.

Paulo EM, Vasconcelos MP, Oliveira IS, Affe HMDJ, Nascimento R, Melo ISD, et al. An alternative method for screening lactic acid bacteria for the production of exopolysaccharides with rapid confirmation. Food Science and Technology 2012; 32(4):710-4.

Penrose DM, Glick BR. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant 2003;118(1):10-5.

Salas-Jara MJ, Ilabaca A, Vega M, García A. Biofilm forming Lactobacillus: New challenges for the development of probiotics. Microorganisms 2016;4(3):Article No. 35.

Selvakumar G, Panneerselvam P, Ganeshamurthy AN. Bacterial mediated alleviation of abiotic stress in crops. In: Bacteria in Agrobiology: Stress Management. Heidelberg, Germany: Springer; 2012. p. 205-24.

Smirnof N. The role of active oxygen in the response of plants to water deficit and desiccation. NewPhy-Tologist 1993; 125:27-58.

Yuan Y, Shi Y, Liu Z, Fan Y, Liu M, Ningjing M, et al. Promotional properties of ACC deaminase-producing bacterial strain DY1-3 and its enhancement of Maize resistance of salt and drought stresses. Microorganisms 2023;11(11):Article No. 2654.

Zhou C, Ma Z, Zhu L, Xiao X, Xie Y, Zhu J, et al. Rhizobacterial strain *Bacillus megaterium* BOFC1 5 induces cellular polyamine changes that improve plant growth and drought resistance. International Journal of Molecular Sciences 2016;17(6);Article No. 976.