Enhancement of Rice Seedling Growth with Rhizobacteria Inoculation in Response to Polyethylene Glycol (PEG)-induced Drought

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Abstract

Drought is a major constraint to rice production in the rainfed upland or rainfed lowland environments. Rhizobacteria, prevalent in rice root system, can produce enzymes and hormones that can aid in enhancing plant water retention. Drought tolerance of rice variety PSB Rc23 was assessed following inoculation with each of the 140 rhizobacterial isolates from Camarines Sur, Apayao, and Isabela. Isolates were screened using the stress substance polyethylene glycol (PEG) 8000. In response to 25% PEG, a more severe stress (= water potential of -7.5 bars), 4 of the 140 rhizobacterial isolates were selected as drought-tolerant. The selection was based on the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and indole-3-acetic acid (IAA), and plant's response in terms of mild leaf rolling, less leaf tip drying, and enhanced plant growth. ACC deaminase reduces ethylene level, thereby enhancing plant tolerance to drought stress. IAA enhances root formation; thus, influencing water absorption.

Keywords*:* ACC deaminase, drought, IAA, PEG, rhizobacteria

1. Introduction

Rainfed upland rice production in the Philippines is challenged by many abiotic stresses. Among any other environmental stress, drought is considered the single most devastating environmental stress which decreases crop growth (Lambers *et al.*, 2008). This adverse effect may be attributed to decreased

enzyme activities, loss of turgor, and decreased energy supply due to water insufficiency. Drought stress affect the plant water relation at cellular and whole plant level causing specific as well as unspecific reactions and damages.

In order to ameliorate this problem, inoculation of plants with native beneficial microorganisms that have plant growth-promoting capabilities such as ACC deaminase, IAA, siderophore production and phosphate solubilization may increase drought tolerance of plants that grow in these drought-affected areas. The growth promotion ability is mainly caused by the interaction between ACC-deaminase and both plant and bacterial auxin, IAA (Glick, 2014).

Bacteria can survive under stress conditions due to the production of exopolysaccharide (EPS), which protects microorganisms from water stress by enhancing water retention and by regulating the diffusion of organic carbon sources. EPS also help the microorganisms to irreversibly attach and colonize the plant roots due to involvement of a network of fibrillar material that permanently connects the bacteria to the root surface (Bashan *et al*., 2004). At the same time, this plant growth-promoting rhizobacteria (PGPR) can also improve the soil health conditions by making essential elements such as phosphorus and iron available for plant uptake (Sayyed *et al*., 2012; Sharma *et al*., 2013).

The potential of these beneficial microorganisms to improve the growth and yield of upland rice experiencing drought is vast. This study aimed to isolate and screen drought-tolerant rhizobacterial isolates and assess their impact on rice plant growth under drought conditions.

2. Methodology

2.1 Isolation of Bacteria from Plant Rhizosphere

Three upland rice samples per site were randomly collected from Camarines Sur, Apayao, and Isabela. The entire plant system of rice was collected. To isolate bacteria from the rhizosphere, the entire root systems were collected and carefully tapped to remove soil that adheres to the roots. The roots were then placed in 100 mL diluent and shaken thoroughly on a wrist action shaker. Soil suspension was diluted to make a series of four ten-fold dilutions. Similarly, 0.1 mL of each of the four dilutions was spread on duplicate trypticase soy agar (TSA) and AGS agar plates. Samples were incubated at

room temperature (28-30ºC). Each viable microorganism present in the sample develops into a visible colony (Black, 1965). Pure cultures were then transferred to agar slant.

2.2 In Vitro Screening of Rhizobacterial Isolates for Drought Tolerance using PEG

Drought tolerance of 140 rhizobacteria was tested using PEG 8000 as a stress substance. Isolates were tested for its ability to increase drought tolerance of PSB Rc23 rice seedling using 15% and 25% PEG solution at the Applied Biology Center for the Rice Environment, Philippine Rice Research Institute laboratory.

2.2.1 Preparation of Simplified Nutrient Addition Program (SNAP)-PEG Solution

SNAP solution obtained from the University of the Philippines Los Baños, Institute of Plant Breeding was prepared following the manufacturer's directions. PEG was dissolved in SNAP solution making a final concentration of 15% (water potential of -2.5 bars) or 25% (water potential of -7.5 bars) PEG in SNAP solution (S-P solution) (Michel, 1983).

2.2.2 Preparation of Isolate Broth Culture

Actinomycete isolates were inoculated in AGS broth while non-actinomycete isolates were inoculated in trypticase soya broth medium. Inoculated broths were incubated for seven days at room temperature.

2.2.3 Seed Surface Sterilization and Inoculation of Seeds

Rice seeds (cv PSB Rc23) were soaked in concentrated H_2SO_4 for 30 seconds and washed with sterile distilled water seven times to remove H_2SO_4. Rhizospheric isolates were the source of the inoculum. Surface sterilized seeds were pre-soaked in a seven-day old culture broth for 30 minutes.

2.2.4 Planting

Inoculated seeds were grown in duplicate sterilized test tube (2 seeds each per test tube which were tested per bacterial isolate) containing 0.25 ml of S-P solution and 0.25 ml bacterial broth culture. At seven days after sowing (DAS) plant height was measured and seedling growth was observed for the presence

of leaf rolling and leaf tip drying. Finally, at 14 DAS, plant height, root length and biomass were measured.

2.2.5 Selection of Potential Drought-Tolerant Rhizobacteria

Four isolates were selected among the 46 isolates based on their effects on total plant length at 25% PEG. Furthermore, the selected isolates were subjected to ACC deaminase and IAA assays to establish isolate ability to withstand drought stress.

2.3 In Vitro Screening of Selected Rhizobacteria Isolates for Growth Promoting Activities (GAP)

2.3.1 ACC Deaminase Activity

To test the ACC deaminase activity, the isolates were grown using the nitrogen-free Dworkin and Foster (DF) salts minimal agar medium. The medium was supplemented with either 2 g $(NH_4)_2SO_4$ or 3 mM ACC (Sigma) per liter as a sole nitrogen source. The heat-labile ACC was sterilized through sterile Millipore membranes and the filtrate was added to the salts medium after autoclaving.

Five-day-old isolates grown on rich oat meal yeast extract agar (OMYEA) were streaked in triplicate on DF agar medium plates amended with either $(NH_4)_2SO_4$ or ACC. The plates were incubated at 28+/-2°C in the dark for seven days. Growth and sporulation of the isolates on DF agar medium amended with ACC (DF-ACC agar) were taken as an indicator of the efficiency of selected isolates to utilize ACC and to produce ACC deaminase.

2.3.2 IAA Production

For the quantitative determination of IAA production, isolates were grown in AGS broth which consists of the following: arginine monohydrochloride, 1.0 g/l; glycerol, 10 ml/l; K_2HPO_4, 1.0 g/l; NaC_l, 1.0 g/l; AGS stock solution, 1.0 ml/l and $CaCO_3$, 1.0 g/l (El-Nakeeb and Lechevalier, 1962) supplemented with tryptophan. After seven days of incubation, the cultures were centrifuged at 5000 rpm for 15 minutes. One milliliter of the supernatant was mixed with 2 ml of Salkowski reagent and the appearance of a pink color indicated IAA production. The absorbance was measured at 530 nm and the quantity of IAA produced was estimated against the IAA standard.

3. Results and Discussion

3.1 Isolation of Bacteria from Plant Rhizobacteria

Figure 1 shows the 140 bacterial isolates obtained from the rice rhizosphere samples. Forty-two were isolated from Apayao upland rice rhizosphere samples. On the other hand, 49 isolates were obtained from the province of Camarines Sur and Isabela. The medium used were AGS and TSA media. Dhananjeyan *et al*. (2010) used AGS medium for the isolation of actinomycetes from corn and soya fields. The isolates were observed as filamentous, branching bacteria with a fungal type of morphology. El-Nakeeb and Lechevalier (1962) observed that when soil samples were treated with calcium carbonate and plated on the AGS medium, total and relative plate counts of actinomycetes obtained were higher than when other media and methods were used. In the present study, it proves that AGS medium can be used for actinomycete isolation.

TSA medium was used in the isolation of non-actinomycete isolates. Timmusk *et al*. (2014) used TSA to determine the content of endospore-forming bacteria after heat treatment of the soil or plant material suspension at 80ºC for 30 min. TSA plates were inoculated with 100 mL of heat-treated bacterial suspensions, corresponding to 10^{-3} –10^{-5} g soil or plant rhizosphere material per plate (Timmusk *et al.*, 2014).

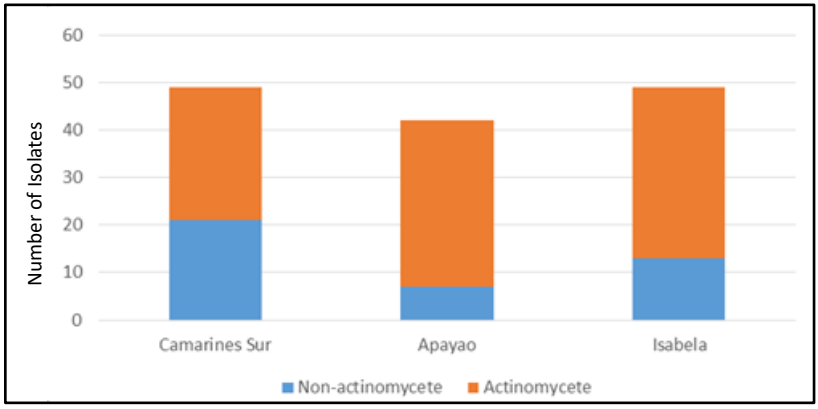

Figure 1. Rhizobacteria isolates obtained from the provinces Apayao, Isabela and Camarines Sur

3.2 In Vitro Screening of Rhizobacterial Isolates for Drought Tolerance using PEG

In this study, 140 rhizobacteria were screened for their drought tolerance using upland rice as the test crop. Isolates were tested for their ability to increase drought tolerance of PSB Rc23 rice seedling using 15% (water potential of -2.5 bars) and 25% (water potential of -7.5 bars) PEG solution.

At 15% PEG, a less severe stress, isolates A-CS_{4-1}, A-AP_{9-2}, A-CS_{11-1}, A-IS_{10-4}, A-IS_{4-0}, A-AP_{4-4}, A-AP_{1-3}, A-AP_{1-4}, A-IS_{2-5-3}, A-IS_{9-4}, T-CS_{7-I}, T-CS_{1-5}, A-CS_{10-12}, T-IS_{3-8}, A-AP_{7-8} and A-CS_{3-0} were able to inhibit leaf tip drying. Similarly, majority of rice seedlings did not exhibit leaf rolling.

Out of 140 isolates screened, 46 were selected as potential drought-tolerant bacteria based on their drought tolerance at 15% PEG, a less severe stress. Mild to moderate leaf rolling was observed in rice inoculated with rhizobacterial isolates. Inoculation with A-CS_{4-1}, A-AP_{9-2}, A-CS_{11-1}, A-AP_{8-5}, A-CS_{1-5} and A-IS_{2-5} increased plant length by 39%, 36%, 36%, 38%, 33%, and 31%, respectively, relative to uninoculated control. The 46 selected isolates were subjected to 25% PEG, a more severe stress, for further drought screening (Table 1 and Figure 2).

At 25% PEG (water potential of -7.5 bars), 15% of the 46 selected isolates showed no indication of leaf rolling (Table 1). Leaf rolling and leaf tip drying are indications of improved water assimilation in plants. Leaf rolling occurs as a mechanism of rice to reduce further water transpiration, while leaf tip drying is due to nutrient deficiency that may be caused by either insufficient supply or poor absorption.

In terms of plant length, inoculation with IS $_{4-7}$, AP $_{3-7}$, AP $_{2-1}$, CS $_{10-12}$, and IS $_{8-9}$ isolates increased total plant length by 8% to 23% relative to uninoculated control at 14 days after sowing (Figure 2). Less leaf rolling was observed in rice inoculated with IS $_{4-7}$, AP $_{3-7}$, AP $_{2-1}$, CS $_{10-12}$, and IS $_{8-9}$ isolates.

Table 1. Plant response to drought (using 25% (water potential of -7.5 bars) PEG, a more severe drought stress) in terms of leaf rolling and leaf tip drying after rhizobacterial inoculation

Isolate	Leaf rolling	Leaf tip drying
Control	+ + +	-
AP 7-1	++	-
IS 10-7	+	+
CS 11-1	+++	-
CS 4-2	+	-
IS 5-10	+	-
IS 10-4	++	-
IS 10-9	n.d.	n.d.
AP 4-4	++	+
AP 4-4	-	-
CS 10-8	+	-
IS 6-2	-	-
AP 2-1	-	-
IS 9-4	+	-
AP 3-A	++	+
AP 3-2	+	+
AP 9-2	-	+
AP 1-3	n.d.	n.d.
IS 5-4	+	-
CS 10-12	-	-
AP 1-4	-	+
CS 4-1	+	-
AP 8-5	+	-
AP 7-8	++	-
IS 2-7	+	-
AP 3-6	+	-
CS 3-3A	+	-
AP 7-7	+	+
IS 2-8	+++	-
CS 1-5	+	-
CS 9-1	+	-
IS 4-7	+	-
AP 9-6	+++	-
CS 11-2	++	-
IS 4-3	+	-
CS 3-8	+	++
AP 3-7	+	+
IS 8-9	-	-
IS 8-0	+	++
CS 2-4	+++	-
CS 7-3	+++	-
CS 9-3	n.d.	n.d.
IS 2-9	+	+
CS 2-9	+	++
CS 2-11	++	-
AP 2-10	n.d.	n.d.
CS 1-1	+	+

(+) mild, (+ +) moderate and (+ + +) severe, n.d.: not determined

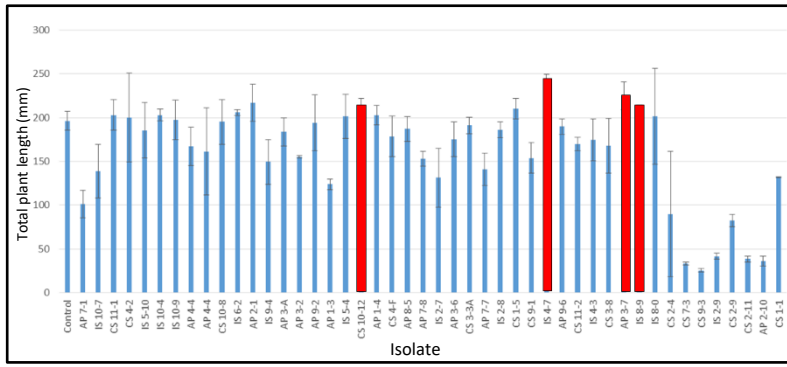

Figure 2. Total plant length of PSB Rc23 seedlings as affected by forty-six selected isolates at 25% PEG concentration, a more severe drought stress or lower water potential = -7.5 bars, 14 days after sowing (DAS).
Note: Initial screening started with 140 isolates.

3.3 Selection of Potential Drought-Tolerant Rhizobacteria

Isolates were selected from among 46 isolates based on their effects on total plant length at 25% PEG. Inoculation with IS $_{4\text{-}7}$, AP $_{3\text{-}7}$, CS $_{10\text{-}12}$, and IS $_{8\text{-}9}$ isolates increased total plant length by 8% to 23% at 14 days after sowing (Figure 2). Figure 3 shows the four selected isolates: (a) IS $_{4\text{-}7}$, (b) AP $_{3\text{-}7}$, (c) CS $_{10\text{-}12}$, and (d) IS $_{8\text{-}9}$ grown in AGS agar medium. Furthermore, the selected isolates were subjected to ACC deaminase and IAA assays to establish isolate ability to withstand stress.

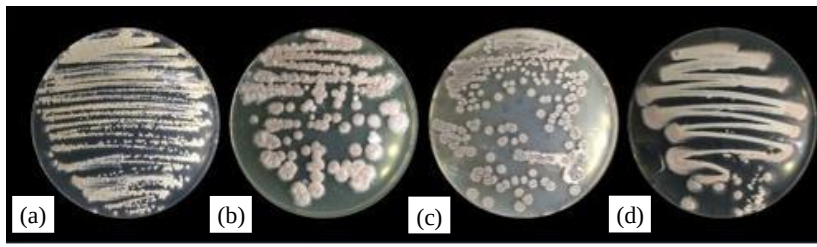

Figure 3. Isolates, a) IS $_{4\text{-}7}$; b) AP $_{3\text{-}7}$; c) CS $_{10\text{-}12}$; and d) IS $_{8\text{-}9}$ isolates, grown in AGS agar medium

3.4 In vitro Screening of Selected Rhizobacterial Isolates for GPA

3.4.1 ACC Deaminase Activity

All isolates produced ACC deaminase except for isolate IS $_{8\text{-}9}$ as shown in Table 2. According to El-Tarabily (2008), growth and sporulation of the isolates on DF agar medium amended with ACC (DF-ACC agar) were taken

as an indicator of the efficiency of selected isolates to utilize ACC and to produce ACC deaminase.

Bacteria that produce ACC-deaminase promote plant growth by utilizing plant-produced ACC, precursor of ethylene. This causes the level of ethylene to decrease in the plant. Glick (2005) emphasized that decreased ethylene levels allow the plant to be more resistant to a wide variety of environmental stresses.

Table 2. Production of ACC deaminase and IAA by IS $_{4-7}$, AP $_{3-7}$, and IS $_{8-9}$, isolates

	Isolate	ACC Deaminase	Indole-3-acetic acid (ppm)
1.	IS $_{4-7}$	+	5.4
2.	AP $_{3-7}$	+	11.5
3.	CS $_{10-12}$	+	7.8
4.	IS $_{8-9}$	-	12.9

(+) ACC deaminase producer; (-) non ACC deaminase producer

ACC deaminase-producing bacteria have been known to promote plant growth by decreasing ethylene inhibition of various plant processes (Husen, 2011). They can increase root growth by lowering endogenous ACC levels (Glick, 2005). However, bacteria, lacking ACC deaminase, have also been shown to increase plant growth, and such observations cannot be explained by known mechanisms. It is presumed that under such conditions bacterial cells possess certain surface components or secrete compounds that act as 'elicitors' of plant growth. Plant roots must be able to perceive and recognize such elicitors in ways similar to the recognition of elicitors from plant pathogens. In fact, plant pathogens might interfere with the action of PGPR by being perceived by similar receptors (Husen, 2011).

3.4.2 IAA Production

IAA production of the selected isolates ranged from 5.4 ppm (IS $_{4-7}$) to 12.9 ppm (IS $_{8-9}$) at 7 days after incubation (Table 2). Khamna (2010) observed that quantification of phytohormones by actinomycetes indicated that they produce auxin. Auxin productions of some actinomycetes are as follows: *Micromonospora* sp., 9,030 ppm; *Actinoplanes*, 270 ppm; *Streptomyces* sp., 750 ppm, and *Frankia* sp., 920 ppm (Solans, 2011). Some actinomycetes such as *Streptomyces* sp. have an auxin production ranging from 11.03 ppm to 144 ppm.

The production of growth promoting substances such as IAA is part of the metabolism of various bacteria associated with plants causing modifications in the morphology of roots, influencing nutrient and water absorption, and consequently promoting plant growth (Bashan and Holgiun, 1997).

Figure 4 shows the upland rice as affected by inoculation with each of the four selected isolates: (a) IS $_{4-7}$, (b) AP $_{3-7}$, (c) CS $_{10-12}$, and (d) IS $_{8-9}$ at 14 DAS.

Water stress is only one of various environmental stresses in which plant suffers from. Environmental stresses affect plants in many ways. One of which is by causing plant to produce ethylene beyond its threshold. Application of rhizobacteria can lower the level of growth-inhibiting stress ethylene within the plant through the action of the enzyme ACC deaminase. Rhizobacteria acts as a sink for ACC preventing heightened levels of ethylene in plant.

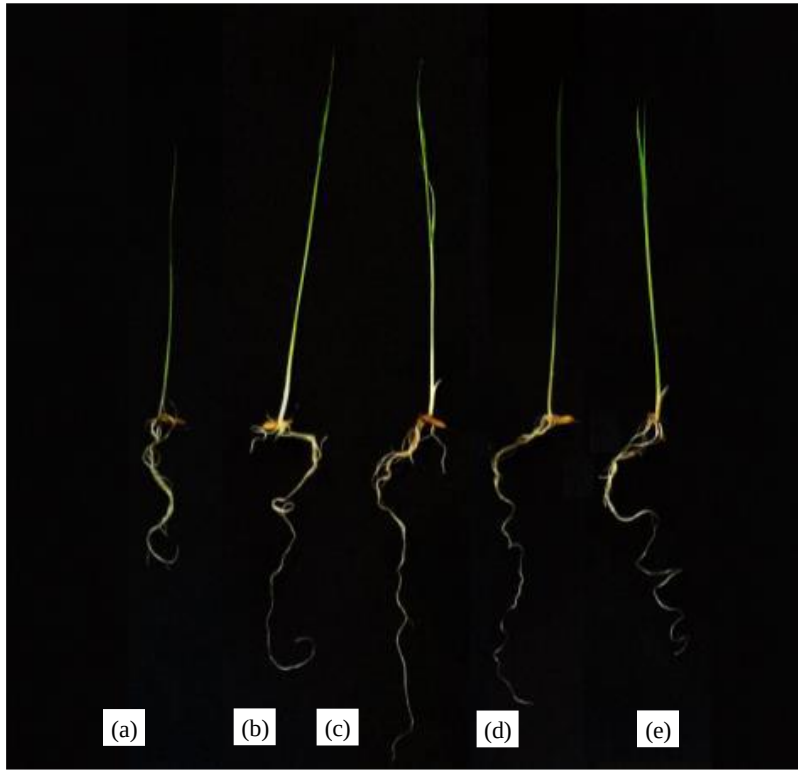

Figure 4. Upland rice as affected by inoculation with each of the four selected isolates: Uninoculated, (b) IS $_{4-7}$, (c) AP $_{3-7}$, (d) CS $_{10-12}$, and (e) IS $_{8-9}$ at 14 DAS

They produce plant growth-promoting compounds which enables the plant to resist wide variety of environmental stresses like water stress. Moreover, rhizobacteria are also able to promote plant growth directly, usually by providing the plant with the phytohormone IAA.

In this study, it is possible that the increase in plant growth under drought conditions is attributed to drought-tolerant rhizobacteria indicating an improved water assimilation. However, further screening of rhizobacterial isolates for growth-promoting activities and for drought tolerance under a higher water potential conditions should be conducted.

4. Conclusions

Drought tolerance of 140 rhizobacteria was assessed using PEG 8000 that induced mild to severe drought stresses. This method was found to be practical and reliable. In this study, isolates were tested for their ability to increase drought tolerance of PSB Rc23 rice seedling using 15% (water potential of -2.5 bars) and 25% (water potential of -7.5 bars) PEG solution. Of 140 isolates screened, 46 were selected as potential drought-tolerant bacteria based on their drought tolerance at 15% PEG, a less severe stress. Isolates were then selected from among 46 isolates based on their effects on total plant length at 25% PEG. Inoculation with IS $_{4-7}$, AP $_{3-7}$, CS $_{10-12}$, and IS $_{8-9}$ isolates increased total plant length by 8% to 23% at 14 days after sowing.

The selected rhizobacterial isolates, inoculated in pre-germinated rice seeds in the PEG system, produced growth-promoting compounds such as ACC deaminase (reduces ethylene level, thereby enhancing plant tolerance to drought stress) and IAA (enhances root formation, thereby influencing water absorption) and enhanced the drought tolerance of rice variety PSB Rc23 in terms of visual and growth responses. The four rhizobacterial isolates that were found to enhance the drought tolerance of the rice variety PSB Rc23 will have to be evaluated in soil conditions with varying stress levels.

5. Acknowledgement

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