The Characteristics of Buprofezin Resistant Bacteria From Cassava Rhizosphere

Sapto Nugroho Hadi[1(✉)], Prita Sari Dewi[2], Ida Widiyawati[1], and Yugi R. Ahadiyat[1]

[1] Agroecology Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Indonesia
sapto.hadi@unsoed.ac.id

[2] Plant Breeding Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Indonesia

Abstract. Bacterial isolates from rhizosphere of cassava plant were isolated. The bacterial isolates are resistant to buprofezin. They have the potential to be used as bioremediation agents. The buprofezin-resistant bacteria isolates need to be carried out to determine the morphological, biochemical, Plant Growth Promoting Rhizobacteria (PGPR), molecular, and bioassays characteristics. This study aimed to determine the morphological, biochemical, molecular, PGPR, and bioassay characteristics of KR1 and PA11 isolates. The result showed that KR1 and PA11 were gram-negative and endospore forming bacteria. Biochemical characteristic of KR1 showed positive result for motility and catalase test, glucose fermentation, starch hydrolysis, and MR/VP test, while PA11 showed positive result for glucose, sucrose, and lactose fermentation, starch hydrolysis, MR/VP and oxidase test. KR1 was a fermenter bacteria, while PA11 was an oxidizer/fermenter bacteria based on the O/F test. Molecular characteristics showed KR1 was *Bacillus wiedmannii* and PA11 was *Bacillus cereus. PGPR characteristics showed* KR1 was phosphate solubilizing bacteria and PA11 was nitrogen fixing bacteria. Both KR1 and PA11 have ability to produce indole acetic acid (IAA). The bioassay characteristic showed that the consortium application of bacterial isolates (KR1 and PA11 isolates) had a significant effect on the root length of rice plants. The bacterial isolates application showed a better result on plant height than control (without bacterial isolate application).

Keywords: Rhizobacteria · Bacillus wiedmanii · Bacillus cereus · ultisol

1 Introduction

The rhizosphere of cassava plant is a good place for the growth and development of microorganisms such as soil bacteria. Rhizosphere are rich in nutritional sources, namely exudates (metabolites) released by plant roots into the soil such as sugar, amino acids, organic acids, glycosides, lipids, vitamins, etc. [1, 2]. This exudate acts as a chemical signal for motile bacteria to move towards the root surface, as well as being the main source of nutrients available to support bacterial growth and persistence in the rhizosphere. These bacteria can develop to form colonies efficiently in the rhizosphere soil of cultivated plants [3].

S. B. Sulistyo et al. (Eds.): ICSARD 2022, ABSR 30, pp. 373–382, 2023.
https://doi.org/10.2991/978-94-6463-128-9_37

The rhizosphere bacteria group is the best partner to improve plant nutrition from organic sources of Nitrogen, Phosphorus, and Sulfur [4, 5]. Some rhizobacteria produce several phytohormones such as indole-3-acetic acid (IAA), gibberellic acid, and cytokinin [6]. Those hormones are important for plant growth and development. This group of soil bacteria in the environment of the rhizosphere is known also to be able to help plants under stressful conditions [7].

Rhizosphere bacteria are also known to be useful in improving soil healthy. Soils contaminated with hazardous chemicals such as synthetic chemical pesticides can be rehabilitated by rhizosphere bacteria [8]. These rhizosphere bacteria play a role in degrading residues of active synthetic chemical pesticides into harmless compounds. Certain groups of soil bacteria even use synthetic chemical pesticides as carbon sources, nitrogen sources, and energy for their growth [9]. In the end, the land that has been successfully rehabilitated will increase its fertility rate so that it can be re-used in aquaculture activities optimally.

Cultivation of food crops, such as rice in Indonesia, generally uses synthetic fertilizers and pesticides. Some farmers apply synthetic fertilizers and pesticides unwisely. The use of synthetic fertilizers and pesticides is carried out excessively, and often exceeds the recommended dose. This condition causes the emergence of long-term problems in the agricultural system such as the destruction of agricultural land which leads to a decrease in land productivity due to the lower level of land fertility. The application of pesticides has an impact on the population of soil microbes which play an important role in supporting soil fertility [10].

To overcome the problem of damage to agricultural land in Indonesia due to unwise agrochemical inputs and to support efforts to optimize environmentally friendly cultivation systems, rice cultivation systems require the adoption of non-conventional technologies. Rice cultivation does not only focus on the use of synthetic fertilizers and pesticides, but also pays attention to land agro-ecosystem factors by using agricultural inputs that are more environmentally friendly.

One of the agricultural inputs that are known to be environmentally friendly is the application of biological organic fertilizers (biofertilizer) [11]. Microorganisms such as rhizobacteria have the ability to support the growth and development of rice plants through the provision of nutrients needed by plants [12]. This type of rhizobacteria is known as Plant Growth Promoting Rhizobacteria (PGPR). The use of Biological Organic Fertilizers is not only able to support the availability of nutrients for rice plants but also supports efforts to develop environmentally friendly agricultural systems. This model of farming system is believed to be able to encourage sustainable cultivation of food crops. Several types of PGPR are also known to have the ability to heal agricultural land contaminated with harmful chemicals [13]. The use of PGPR which has two beneficial characters, providing nutrients and reducing levels of harmful chemicals in the soil strongly supports Indonesia's efforts to restore polluted agricultural lands as well as supports efforts to cultivate rice plants that are more environmentally friendly to meet sustainable food needs.

To find candidates for biological organic fertilizers that have PGPR characters as well as have the ability to reduce levels of synthetic pesticide contamination, the researchers explored local bacteria originating from the rhizosphere of cassava plants cultivated in

Banyumas Regency, Central Java Indonesia. This cassava plant is cultivated on marginal land. The results obtained 26 local bacterial isolates [14]. Two isolates of rhizobacteria (KR1 and PA11) were known to have dominant and resistant characters to synthetic pesticides with buprofezin as the active ingredient. Some isolates of rhizosphere bacteria were tolerant of synthetic pesticides [15].

In an effort to identify and further characterize this dominant rhizobacteria isolate, it is necessary to carry out morphological, biochemical, PGPR, molecular, and bioassay characteristics. Morphological characters were generally based on the gram and endospore staining test. Biochemical characters were based on enzymatic reactions carried out by bacteria when given certain tests. PGPR characters were based on the ability of bacteria in solubilizing inorganic phosphorus, nitrogen atmosphere fixation, and IAA production. Molecular characterization were based on 16S rRNA analysis. To see the potensial role of the target bacterial isolates for plant growth, related to PGPR characteristic, the application of the isolates to several upland rice cultivar is important to be known (bioassay characteristic). Bacterial isolates that have PGPR characteristics can be developed into biofertilizers [16]. Biofertilizer would play a key role in enhancing soil fertility and crop productivity [17]. Biofertilizers from soil bacteria that have strong PGPR characteristics are expected to help increase the productivity of upland rice cultivar, which has been known to have lower productivity compared to lowland rice cultivar [18].

This study aimed (1) to determine the morphological, biochemical, and molecular characteristics of KR1 and PA11 isolates, (2) to determine the PGPR characteristics of KR1 and PA11 isolates, (3) to know the respond of several upland rice cultivar after the isolates application.

2 Materials and Methods

2.1 Morphological and Biochemical Characteristics

Morphological characters include gram and endospores staining that refer to [19]. The biochemical character is determined by performing a series of tests, some of which refer to [19] such as simple carbohydrate fermentation (glucose, sucrose, lactose), starch hydrolysis, urease test, the IMViC series of tests (indole, methyl red, Voges-Proskauer, and citrate utilization), and H_2S production. Other biochemical test refer to [19] with modification such as motility and catalase test. For the motility test, modification of the method was carried out on the use of NA medium (Merck) to replace SIM media. For the catalase test, modifications were made to the use of NB (Merck) media to replace trypticase soy agar (TSA). Oxidase test refers to [20].

2.2 PGPR Characteristics

PGPR characters include testing the ability of bacteria to dissolve phosphate using pikovskaya medium, fix atmospheric nitrogen, and produce IAA.

In the test of the ability to dissolve phosphate, pure bacterial isolates were inoculated on steril pikovskaya agar (Himedia). The media was incubated for 48 h at 37 °C. The clear

zone that appears around the bacterial isolate was the positive indicator for phosphate solubilizing bacteria. On the test of the bacteria ability to trap atmospheric nitrogen, pure bacterial isolates were inoculated into sterile Norris Glucose Nitrogen Free Medium (Himedia) in a sterile test tube. Medium were incubated for 72 h at 37 °C After the medium becomes turbid, the sample was grown in sterile NA media (Merck) [21]. Bacteria that grow after incubation in NA medium at 37 °C for 72 h indicate the ability of bacteria for transforming nitrogen gas from the atmosphere into the compounds, that usable by plants such as ammonia. On the IAA production test, bacterial isolates were grown in NA medium containing 1000 ppm L-tryptophan (Merck). Samples were incubated for 5 days by shaking at 120 rpm, room temperature. After incubation, the culture was centrifuged at 5000 rpm for 15 min and the supernatant was separated. A total of 1 ml of the supernatant and 2 ml of Salkowski's reagent were put into a test tube, then stored in a dark room for 30 min. A positive result is indicated by a color change in the solution in the test tube to red [22].

2.3 Molecular Characteristics

Bacterial pure isolates were analyzed for 16S rRNA sequences using oligonucleotides 27F (AGAGTTTGATCMTGGCTCAG), 785F (GGATTAGATACCCTGGTA), and 1429R (TACGGYTACCTTGTTACGACTT). The analysis was conducted by PT Genetika Science Indonesia. Sequencing results of 16S rRNA, were further analyzed using bioedit software version 7.0.5.3 [23]. The 16S rRNA sequences after bioedit analysis were used for n-blast for species identification compared with NCBI (Nation Center of Biotechnology Information) sequence database. 16S rRNA sequences of the bacterial isolate sample, 15 bacterial species from NCBI database, and 1 outgroup bacterial species were used to create phylogenetic trees using Mega version 10.1.1 [24].

2.4 Bioassay Characteristics

The bioassay was carried out by soaking rice seeds (Inpago Unsoed1, Unsoed Parimas, Inpago 8) in a suspension containing bacterial isolates (KR1, PA11, and the consortium) with a density of 10^9 cfu/ml [25]. The rice seeds were then planted in sterile ultisol soil in jars. Observation of plant growth response to the application of bacterial isolates was carried out when the plant was 14 days after planting (DAP). Observation variables such as plant height (cm), number of leaves, root length (cm), wet weight (g) and plant dry weight (g).

3 Result and Discussion

3.1 Morphological and Biochemical Characteristics

Morphological and biochemical characteristics of KR1 and PA11 were presented in Table 1.

Based on Table 1, the morphological characters showed that the isolates KR1 and PA11 were gram-negative bacteria that could form endospores. The character of the

Table 1. Morphological and biochemical characteristic

Characteristic	KR1	PA11
Gram staining	Negative	Negative
Endospore staining	+	+
Motility	+	-
Catalase	+	-
Glucose Fermentation	Negative	+, No gas
Sucrose Fermentation	+	+, No gas
Lactose Fermentation	+	+, No gas
Starch Hydrolysis	+	+
Urea Hydrolysis	-	-
MR/VP	+/+	+/+
Simmons Citrate	-	-
H_2S Production	-	-
Oxidase	-	+
Oxidative-fermentative (O/F)	O−/F+ Mannitol fermenter bacteria	O+/F+ Mannitol oxidizer/fermenter bacteria
Indole	-	+

endospore staining shows that this bacterial isolate has characteristics that can be gradual in unfavorable environmental conditions such as dry land. This result is consistent with the data that both bacterial isolates were obtained from sub-optimal land (ultisols) which had various limitations such as minimal nutrients and rainfall.

The biochemical characters showed that KR1 isolate was a motile bacterium, had catalase enzyme activity, was able to ferment glucose, hydrolyze starch, and was positive for the MR/VP test. Isolate KR1 including fermenter bacteria based on O/F test. Meanwhile, PA11 isolate showed positive results for simple sugar fermentation tests (glucose, sucrose, and lactose), starch hydrolysis, MR/VP, and oxidase. PA11 isolate was an oxidizer/fermenter bacteria based on the O/F test.

3.2 PGPR Characteristics

Based on Table 2, KR1 isolates was classified as phosphate solubilizing bacteria, while PA11 isolates was classified as nitrogen-fixing bacteria. Both bacterial isolates were IAA-producing bacteria. This results correspond to [26] that the upland soil (ultisol) contain phosphate solubilizing and nitrogen-fixing bacteria. The PGPR character also indicated that the two isolates were expected to be able to support the growth and yield of rice cultivation.

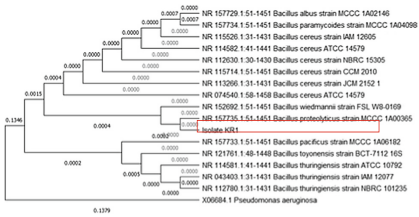

Fig. 1. Phylogenetic tree of KR1. KR1 was located in the same cluster with *B. wiedmnnii and B. proteolyticus* (red box).

Table 2. PGPR characteristic

PGPR Characteristic	KR1	PA11
Phosphate solubilizing bacteria	+	-
Nitrogen-fixing bacteria	-	+
IAA production	+	+

Table 3. N-BLAST characteristic

Isolate code	E Value	Percentage Identity (%)	Probably Identity
KR1	0.0	100.00	*Bacillus wiedmannii*
PA11	0.0	100.00	*Bacillus cereus*

3.3 Molecular Characteristics

The result showed that the 16S rRNA sequence lengths for KR1 was 1401 bp and 1399 bp for PA11. Based on N-BLAST analysis, we could compare sequences obtained with nucleotide sequences from NCBI's database.

Table 3 showed the N-BLAST analysis result. KR1 has a high identity with *Bacillus wiedmannii* and PA11 with *B. cereus.* For bacterial samples using 16S rRNA markers, it was said to be identical at the species level if the percentage identity is above 97.5%, and at the genus level if the percentage identity is above 95% [27].

Phylogenetic tree of the isolate were presented in Fig. 1 and 2. KR1 was located in the same cluster with *B. wiedmnnii* and *B. proteolyticus.* PA11 was located in the same cluster with *B. cereus.* This result corespond to previous N-BLAST analysis that *KR1* with *B. wiedmannii* and *PA11* with *B. cereus* and *B. paramycoides.*

3.4 Bioassay Characteristics

The analysis result of the application of bacterial isolates to the rice plant with different cultivar was presented in Table 4.

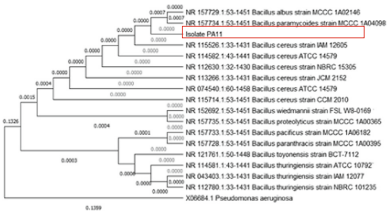

Fig. 2. Phylogenetic tree of PA11. PA11 was located in the same cluster with *B. cereus* dan *B. paramycoides* (red box).

Table 4. Bioassay characteristic

Treatment	Plant Height (cm)	Number of Leaves	Root Length (cm)	Wet weight (g)	Dry weight (g)
B0	19.778	2.389	9.611 a	0.038	0.027
B1	22.111	2.389	12.556 bc	0.041	0.028
B2	21.333	2.444	10.778 ab	0.035	0.029
B3	22.444	2.278	13.333 c	0.038	0.028

Remark: B0 = without bacterial application, B1 = application of KR1, B2 = application of PA11, and B3 = application of consortium (KR1 and PA11).

Based on Table 4, the results of the variance analysis showed that the application of different bacterial isolates had a significant effect on rice growth, namely on the variable root length. Isolate B3 (a consortium of isolates KR1 and PA11) showed the best value with a root length of 13,333 cm. This was presumably due to the ability of KR1 and PA11 isolates to produce growth hormone IAA (Table 4). The presence of the IAA hormone supplied by bacteria causes the process of elongation, division and differentiation of cells to occur better so that it can support the growth of lateral roots of plants. IAA-producing bacteria have a positive effect on rice seeds, so that rice sprouts have a higher IAA secretion ability and are more sensitive. IAA will cause pectin to dissolve and the cell wall becomes soft so that it can increase water absorption and the cell will expand [28].

In addition, the length of plant roots is thought to be influenced by the adequacy of nutrients such as phosphate supplied by bacterial isolates which are phosphate solubilizing bacteria. This type of bacteria is important for providing phosphorus nutrients for plants cultivated in sub-optimal land such as ultisols.

4 Conclusions

The result showed that KR1 and PA11 were gram-negative and endospore forming bacteria. Biochemical characteristic of KR1 showed positive result for motility and catalase test, glucose fermentation, starch hydrolysis, and MR/VP test, while PA11 showed positive result for glucose, sucrose, and lactose fermentation, starch hydrolysis, MR/VP

and oxidase test. KR1 was a fermenter bacteria, while PA11 was an oxidizer/fermenter bacteria based on the O/F test. Molecular characteristics showed KR1 was *Bacillus wiedmannii* and PA11 was *Bacillus cereus. PGPR characteristics showed* KR1 was phosphate solubilizing bacteria and PA11 was nitrogen fixing bacteria. Both KR1 and PA11 have ability to produce indole acetic acid (IAA). The bioassay characteristic showed that the consortium application of bacterial isolates (KR1 and PA11 isolates) had a significant effect on the root length of rice plants. The bacterial isolate application showed a better result on plant height than control (without bacterial isolate application).

Acknowledgments. The authors would like to thank the Center for Research and Community Service, Universitas Jenderal Soedirman for the research funding support.

Authors' Contributions. SNH designed the study, SNH and IW carried out laboratory work and analysed data, and PSD, AYR wrote the manuscript and conducted proof-reading. All authors read and approved the final version of the manuscript.

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