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Screening of Endophytic Bacteria from Organic Rice Tissue for Indole Acetic Acid Production

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Abstract

The objectives of this study were to isolate and characterize endophytic bacteria from various rice tissues focusing on their ability to produce indole acetic acid (IAA). Rice tissue samples were collected from different three types of rice farm; 1 year, 3 years organic rice, and conventional rice farms in Udon Thani, Thailand. Seventy-one isolates of endophytic bacteria were screened using PDA and TSA medium. The majority of strains isolated from root tissues were totally 26 isolates, exclusively collected from 3 years organic rice farm. Phenotypic characteristics of all isolates illustrated that 34 isolates were identified as *Pesudomonas* sp., while other isolates were also identified as *Bacillus*, *Azotobacter,* and *Enterobacter* species. The study of IAA production indicated that 4 isolates efficiently produced IAA over than 10 µg/ml. The O-1-R-4 (2) isolate produced the highest IAA (14.58 µg/ml), and it was identified as *Bacillus* sp. This effective result will be used for further investigation on the feasibility of commercial production of IAA up-scale fermentation.

Keywords: Organic rice; Endophytic bacteria; Indole acetic acid; Root; Stem

1. Introduction

Organic rice is typically grown and processed without the use of any synthetic chemicals as found, for example, in fertilizers, insecticides, pesticides, herbicides, fungicides, preservatives, seed treatments and hormones growth regulators, etc. It mainly relies on organic sources to maintain soil health, supply plant

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nutrients and minimize insects, weeds and other pests[1]. Efficiency of organic rice expansion could be done by living microorganisms in ecosystem. Their metabolic activities will help to circulate many nutrients in soil.

Endophytic bacteria generally live in plant tissues without doing substantive harm or gaining benefit other than securing residency. They have been found in numerous plant species with most being members of common soil bacteria genera such as *Pseudomonas, Bacillus* and *Azospirillum* [2]. Endophytic bacteria can be isolated from surface-disinfected plant tissue or extracted from internal plant tissue [3]. Several reports indicated that both gram-positive and gram-negative bacterial endophytes have been isolated from difference plants such as soybean, wheat, corn, sorghum, cucumber fruits, sugar beet roots and rice [2, 3, 4, 5]. However some endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and induction of resistance to plants pathogens [6]. Endophytes also promote the growth of plants, especially through secretion of plant growth regulators; e.g. indole-acetic acid, via phosphate-solubilizing activity, by supplying biologically fixed nitrogen. In addition, endophytic bacteria supply essential vitamins to plants [7]. The production of auxin-like compounds increased shoot growth and tillering. Other effects of endophytes infection on the host plant include osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism [4, 8]. The aims of this study were to isolate and characterize endophytic bacteria from various rice tissues focusing on their ability to produce indole acetic acid (IAA). This information will be used for further investigation on the feasibility of commercial production of IAA up-scale fermentation.

2. Materials and methods

2.1 Isolation of endophytic bacteria.

The rice plants were collected from different three types of rice farm; 1 year, 3 years organic rice farms, and conventional rice farm in Udon Thani, Thailand. The healthy rice plants were carefully removed, washed under tap water to remove soil and separated to three parts; root, lower stem (5-10 cm. from root) and higher stem (10-15 cm. form root). All rice tissues were put in beaker, soaked in distilled water and drained. After that rice tissues were rinsed in 70% ethyl alcohol for 30 seconds and then sterilized with 0.2% $HgCl_2$ for 3 minutes for root and lower stem, 5 minutes for higher stem. The tissues were then washed ten times with sterile water, cut into small pieces and homogenized in a blender containing 90 ml sterile distilled water. Macerated tissues were diluted into 10^{-1} dilution by adding 9 volumes of sterile distilled water. Serial dilution was made up to 10^{-5} dilution by taking 1 ml of well-shaken suspension and adding into 9 ml water blank tubes. 100 µl from 10^{-4} and 10^{-5} dilutions were spread plated on two different media; PDA and TSA and then incubated at 35 °C for 24 hrs [6].

2.2 Morphological and physiological characterization

Gram staining and capsule staining were carried out according to standard staining protocols.

Cellulase activity: the test isolates were spot-inoculation on cellulose agar plates and incubated for 1 week at 30 °C. Bacterial cellulose activity was observed. The medium was flooded with Congo red dye for 15 minutes, drained, rinsed with 1M NaOH. The Congo red reacts with cellulose to form a red-colored complex. Any clear area around the growth of the culture after the addition of the Congo red dye indicates the breakdown of cellulose by the organism due to its production of cellulase, an extracellular enzyme [7].

Pectinase activity test: the test isolates were spot-inoculated on the pectin agar plates and incubated for one week at 30 °C. The plates then were flooded with 1% aqueous Red ruthenium solution for one hour, drained, rinsed with water and observed. Red ruthenium is bound to unhydrolysed pectin and give the red color. Halo zone around isolate's colony was observed [9].

Motility test: each isolate was spot-inoculated on the center of semi-solid nutrient agar plates (0.2% agar) and incubated at 30 °C. The diffusion of colony was observed and recorded at 24 hours.

Antibiotic resistance test: the test isolates were spot-inoculated on the nutrient agar plates incorporated with filter sterilized streptomycin at the rate of 100 μg/ml and incubated for 48 hrs at 30 °C. The antibiotics resistance was recorded as positive if the test colony appeared on the plates, as compared to the control plate in which no antibiotic was added.

Fluorescence pigment production test: the test isolates were spot-inoculated on the King's B medium agar plates and incubated at 30 °C. The plates were exposed to UV light to examine the fluorescence ability after 24-48 hrs of incubation.

IAA production test: 5 μl of LB (Luria Bertani) broth alone and LB broth medium added with L-tryptophan at the rate of 100 μg/ml. The test tubes were covered with brown paper and incubated at 28 °C for 24 hours on a rotary shaker. The broth was centrifuged at 10000 rpm for 15 minutes. 2 ml of supernatant was collected and 2-3 drops of *o*-phosphoric acid were added. The aliquots were shaken, added 4 ml of reagent (1 ml of 0.5M $FeCl_3$ in 49 ml of 35% perchloric acid ($HClO_4$)) and vortexed thoroughly. The samples were incubated at room temperature for 25 minutes and their absorbance was read at 530 nm. Auxin quantification value was recorded by extrapolating calibration curve made by using IAA standard (10-100 μg/ml) [10].

3. Results

3.1 Isolation of endophytic bacteria

Seventy-one isolates of endophytic bacteria were isolated from three parts of rice tissue (root, lower stem and higher stem) using PDA and TSA medium. Rice tissue samples were collected from different three types of rice farm; 1 year, 3 years organic rice farms, and conventional rice farm in Udon Thani, Thailand. The serial dilutions of macerated rice samples were spreaded on TSA and PDA medium. The colony forming units (CFU) were determined after appropriate inoculation at 30 °C. A majority of the microorganisms were screened and isolated (26 strains) from root tissue of 3 years organic rice farm. As shown in Table 1. Bacterial isolates were identified based on Gram's staining and physiological tests.

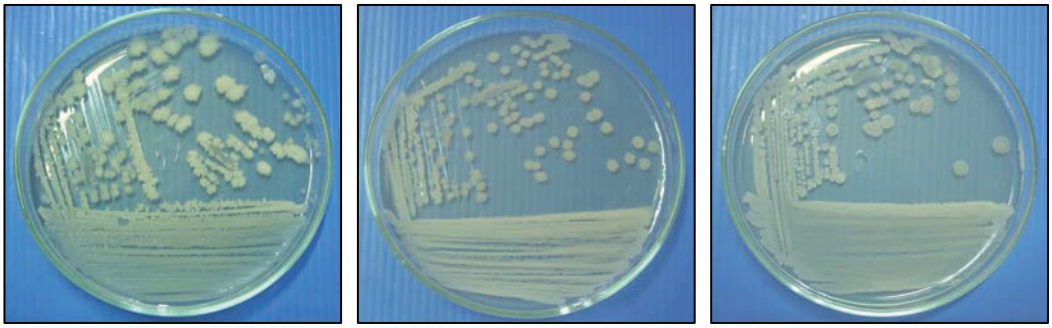
3.2 Characterization of bacterial colony

Figure 1 showed the colony characteristics of some isolates on TSA medium. They were classified into three group of colony morphology depending on color, form, elevation, and margin. The members of group one were white circular, convex and entire; those in group two were pale orange, circular and undulate; and those in group three were light yellow circular, undulate with creamy colonies.

Table 1. Isolation frequency and total population of endophytic bacteria in rice tissues

Rice farm type	Type of tissue	Number of bacterial isolates		
		1st time of isolation	2nd time of isolation	Total numbers of both isolation
C[1]	R[4]	3	8	11
	N[5]	1	4	5
	S[6]	3	6	9
O-1[2]	R	1	4	5
	N	0	4	4
	S	4	7	11
O-3[3]	R	3	8	11
	N	2	6	8
	S	3	5	7
Total number of endophytic bacteria				71

[1]C : Conventional rice farm
[2]O1 : 1 year organic rice farm
[3]O3 : 3 year organic rice farm
[4]R : Root
[5]N : Lower stem
[6]S : higher stem



(a) (b) (c)

Fig. 1. (a) The colony characteristics of O-3-R-2 isolate on TSA medium (b) The colony characteristics of O-3-R-3 isolate on TSA medium (c) The colony characteristics of O-1-S-3 isolate on TSA medium

3.3 Morphological and physiological characterization

The result from Gram's staining clearly exhibited 40 gram-positive and 31 gram-negative isolates. However, the result fairly differenced from that of recently researched by Hung et al. (2004) and found that gram-positive and gram-negative isolates were equally distributed between two species of soybean. The result also found that 6 isolates formed capsule (Table 2). When grown on 0.2% agar, 30 isolates were found to be motile. 71 isolates of endophytic bacteria were screened for growth on NA amened with Streptomycin at the rate of 100 µg/ml. 43 isolates were able to grow in the presence of the antibiotic. When grown on King's B medium (specific for fluorescent Pseudomonads), 34 isolates were found to be

putative fluorescent Pseudomonads. Twenty-five isolates gave a clear zone of hydrolysis on pectin agar plate. When grown on cellulose agar medium, there were 13 isolates able to grow.

Table 2. Isolation frequency and total population of endophytic bacteria in rice tissues

Rice farm type	Gram's staining		Capsule staining	Motility	Antibiotic resistance	Fluorescence	Pectinase activity	Cellulase activity	IAA Production
	Positive	Negative							
Conventional	15	10	0	10	0	13	5	0	21
1 year organic	10	10	1	8	19	10	10	7	18
3 year organic	15	11	5	12	24	11	10	6	27
Total	40	31	6	30	43	34	25	13	66

The IAA production ability of endophytic bacteria was detected. 66 isolates produced IAA in both of presence and absence of the precursor tryptophan. 4 isolates efficiently produced IAA over than 10 μg/ml (Table 3). The O-1-R-4 (2) isolate produced the highest IAA (14.58 μg/ml).

Table 3. The ability of IAA production of the first 4 isolates of endophytic bacteria

The Endophytic bacterial isolate	Ability of IAA production (μg/ml).
O-1-R-4 (2)	14.58
O-1-N-2 (2)	13.54
C-R-8 (2)	13.17
C-R-5 (2)	12.68

The characteristics of O-1-R-4 (2) isolate were determined. The colony was white and circular, rod-shaped, motile, gram positive, endospore forming, not showed the ability of pectin hydrolysis, cellulose hydrolysis and fluorescence ability. The O-1-R-4 (2) isolate was identified as *Bacillus* sp.

4. Discussion

This investigation is to describe the indigenous endophytic bacteria isolated from three types of rice tissue, which collected from conventional, 1 year organic rice, and 3 year organic rice farms. Seventy-one isolates of endophytic bacteria were screened from those samples, using PDA and TSA medium. There was significant phenotypic variation in the types of indigenous bacteria. Several factors may explain these differences, including rice tissue types and the type of rice farm. Previous researches have reported that there were both phenotypic and genotypic variations in the types of endophytes isolated from two species; one cultivated (*Glycine max*) and another wild (*G. soja*) of soybean [6]. Moreover, Stoltzfus et al. (1997) selected 133 isolates of endophytic bacteria from root and stem of rice tissues of diverse varieties grown in different soil types [4].

The diversity of a collection of seventy-one putative endophytic bacteria was assessed using phenotypic characterization methods. Colony morphology gave an indication of the variation among the endophytes. The isolates studied were chosen for their dominance as well as uniqueness or difference with other in colony morphology. The studying of all isolates morphological and physiological characteristics indicated that 34 isolates to be *Pseudomonas* because they grown on King'B medium, the selective medium for *Pesudomonas.* The other isolates to be *Bacillus*, *Azotobacter* and *Enterobacter* species. Result of the previous study evidenced that most of the endophytic bacteria isolated from

medicinal plants belonged to *Bacillus* and *Pseudomonas* species [2]. Bai et al. (2002) also isolated *Bacillus* sp. from nodules of soybean [11]. In addition, the recent study of endophytic bacteria isolated from rice tissue found the species of *Pseudomonas*, *Bacillus*, *Azospirillum* and others [4].

In addition to this study, 25 isolates secreted pectinase and 13 isolates secreated cellulase. It can be summarized that hydrolytic enzymes, pectinase and cellulose may play an importance role in the mechanisms by which endophytic bacteria penetrate into and persist in the host plant. Also, these enzyme might be involved in the invasion of host plants by endophytes, as reported for *Azoarcus* sp. [12].

The motility of endophytic bacteria was also studied. 26 isolates were motile. Due to the motility and pectinolytic activity may confer an advantage for intercellular ingress and spreading of endophytic bacteria into the host plants, the cell wall of the host plants contain cellulose, whereas the middle lamella between cell walls contain mainly pectin [13].

The ability of IAA production was investigated. There were 66 isolates could produced IAA, but only 4 isolates efficiently produced IAA over than 10 μg/ml. The O-1-R-4 (2) isolate was produced the highest IAA (14.58 μg/ml). Bandara et al. (2006) found that endophytic bacteria and fungi isolated from rice also produced IAA with variable quantity [8]. In addition, the previous research reported that there were 15 isolates of endophytic bacteria produced IAA over than 25 μg/ml [6]. IAA has many different effects, as all auxins do, such as inducing cell elongation and cell division with all subsequent results for plant growth and development.

This study significantly demonstrated the occurrence and diversity of culturable endophytic bacteria in various rice tissues. This can be utilized in future application, such as effect of rice seed treatment with endophytic bacteria, delivery of degradative enzymes for controlling certain plant diseases, and production of IAA in large scale including other useful products.

5. Conclusion

Seventy-one isolates of endophytic bacteria were screened from three parts of rice tissue using PDA and TSA medium. Rice tissue samples were collected from different three types of rice farm; 1 year, 3 years organic rice, and conventional rice farms in Udon Thani, Thailand. The isolates were examined for colony forming, Gram's staining, physiological characteristics, and indole acetic acid productivity. The majority of strains isolated from root tissues were totally 26 isolates, exclusively collected from 3 years organic rice farm. Phenotypic characteristics of all isolates illustrated that 34 isolates were identified as *Pesudomonas* sp., while other isolates were also identified as *Bacillus*, *Azotobacter,* and *Enterobacter* species. The study of IAA production indicated that 4 isolates efficiently produced IAA over than 10 μg/ml. The O-1-R-4 (2) isolate produced the highest IAA (14.58 μg/ml), and it was identified as *Bacillus* sp.

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