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Molecular identification of local plant growth promoting rhizobacteria that act as resistance inducing agent against rice blast disease

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K E Y W O R D S : Plant growth promoting rhizobacteria, induced-resistance, Rice blast disease

ABSTRACT : Rice blast disease caused by Pyricularia oryzae Cav. frequently occurs as limiting factor for rice production in many parts of the world where rice is grown. The disease has been known to cause significant yield looses in rice including local varieties. Padi Merah Medang Putih, is one of Bali rice local varieties that popular among consumers, however this variety is highly susceptible to the blast disease. This study was done to find and identify plant growth promoting rhizobacteria (PGPR) that capable of inducing resistance against blast disease on Padi Merah Medang Putih. Ninety five isolates of rhizobacteria were isolated from diverse plant rhizospheres grown in Bali and tested for their ability to promote the rice growth and induce resistance against blast disease. Nine isolates have been proven serve as PGPR and among them four isolates namely KDDA, O38, OR3 and Al7Kla are capable of inducing resistance against blast disease on Padi Merah Medang Putih. Based on 16S rDNA sequence analysis, these PGPR are respectively identified as Bacillus methylotrophicus KDDA, Bacillus amyloliquefaciens O38, Pseudomonas aeruginosa OR3 and Achromobacter xylosoxidans Al7Kla. All of these PGPR can be further utilized to induced resistance against blast disease on Bali local rice variety, Padi Merah Medang Putih under green house as well as field condition.

Introduction

Indonesia has a diverse of rice local varieties which are distributed in many islands including Bali Island. However, the number of rice local varieties gradually decreased, from 12,000 varieties in 1970's to 3,800 varieties in 2008 (Anwar, 2008). This is partly due to the introduction of the new high yielding varieties with shorter growing period and higher productivity when compared to local varieties. In Bali Island, there are several local rice varieties are being cultivated such as Ijo Gading, Cicih Medang Selem, Cicih Medang Putih, Padi Merah Medang Selem, Padi Merah Medang Putih, Padi Del, Ketan Medang Selem, Ketan Medang Putih, and Ketan Hitam. The maximum productivity of Bali rice local varieties is about 4 ton/ha, while the high yielding varieties may reach 7 ton/ha or even more with growing period between 3 to 4 months (Swantantra, 2013; Suprapta et al., 2014a).

The low productivity of Bali rice local varieties may due to their low response to the fertilizer application. This characteristic probably because of the poor root system of the local varieties which have limited root hairs. In addition, several Bali rice local varieties are susceptible to the rice blast disease caused by Pyricularia oryzae Cav. The yield looses resulted from this disease varied in accordance with localities and rice varieties. In Japan the yield looses varied from 1 to 100% (Kato, 2001); in China about 70% (Chin, 1975), and in Indonesia varied from 15 to 35% (Suprapta et al., 2014a), and in the Philippines 50-85% (Delgado et al., 2015).

Several rhizobacteria have been known to serve as plant growth promoting rhizobacteria (PGPR), and some of them have been proven to increase the number of plant root hairs, increase the yield, and induce resistance against certain plant diseases (Wei, 1991; Press et al., 1997; Palukaitis et al., 1992; Sherata et al., 2008; Khalimi, 2009; Suprapta et al., 2014b). Treatment with formula containing three species of rhizobacteria namely Bacillus subtilis 5, Bacillus cereus 3S5 and Pseudomonas fluorescens 10S2 on rice cultivar UPLRi-5 could reduce the rice blast intensity by 33% when compared with control (Delgado et al., 2015). Other study done by Shyamala and Sivakumaar (2012) showed that treatment with a combination of rhizobacteria and salicylic acid significantly increased the resistance of rice to blast disease. This study was

done to find and identify the potential PGPR that can be used to induce resistance against blast disease on Bali local variety Padi Merah Medang Putih.

Materials And Methods

Isolation of Rhizobacteria

Isolation of rhizobacteria was done from rhizospheres of various plants grown in Bali, Indonesia. Ten grams of rhizospehere (containing roots and soil) was macerated in a mortal with 100 ml of saline phosphate buffer. A serial of dilution (10^{-2} to 10^{-7}) was done by adding sterile saline phosphate buffer. Cultural medium used for this isolation was tryptic soy agar (TSA) with composition in one liter : 3 g beef extract, 3 g yeast extract, 15 g peptone from casein, 5 g peptone from beef, 10 g lactose, 1 g glucose, 10 g sucrose, 0.5 g $NH3^{+}Fe^{3+}$ citrate, 5 g NaCl, 0.5 g sodium thiosulphate, 0.024 g phenol red, 12 g Bacto agar and distilled water to make the final volume of one liter. pH of the medium was adjusted to 7.4. This medium was added with 20 mg/ml of benomyl to avoid the fungal contamination (Basham et al., 1993). The colonies of rhizobacteria appeared on the medium were then isolated and maintained on slant agar media before they are used for further test.

Test for Plant Growth Promotion

All isolates of rhizobacteria obtained in this study were tested for their ability to promote the formation of root hair of rice variety Padi Merah Medang Putih, a local variety of Bali. The seed of rice was soaked in sterile distilled water for 24 h and then was drain out and placed on Petri dishes with wet Whatman No.2 filter paper. The germinated seed was then soaked in rhizobacterial suspension containing 10^6 CFU/ml, then was dry up in a laminar flow for an hour. This seedling was then grown in a test tube containing 0.1% KNO_3. This culture was maintained in cultural rack with 12 h fluorescence light at 28 ± 2^oC. The formation of root hairs was observed for 10 days and compared with control (the seed without rhizobacteria treatment).

Test for Induction of Blast Disease Resistance

test was done for rhizobacteria that capable of promoting root hair formation on rice variety Padi Merah Medang Putih. This variety was proven to be the most susceptible cultivar against rice blast disease. The seed was soaked in sterile distilled water for 24 h and then was drain and kept under moist condition in a Petri dish with wet Whatman No. 2 filter paper until germination was occurred. This germinated seed was then soaked in rhizobacterial suspension (10^6 CFU/ml) for 30 min. The germinated seed that was soaked only with sterile distilled water was prepared as control. This seed was then planted on a plastic cup (surface diameter : 5.5 cm, and height: 4 cm) filled with wet tissue with 0.1% KNO_3. Five seedlings were grown per cup. This culture was maintained in a rack with fluorescence light for 12 h at temperature of 28 ± 2^oC. The 9-day old rice seedling was inoculated with spore's suspension of P. oryzae (1×10^5 spores/ml) using mini hand sprayer throughout surface of seedling. After inoculation, the plant was maintained in a dark growth chamber with relative humidity (RH) 90% and temperature of 28^oC for 48 h. The plant was taken out from the chamber and maintained in a cultural rack with 12 h fluorescence light. Percentage of seedlings showing blast disease symptom was observed every day to know which of the PGPR isolate capable of inducing disease resistance.

DNA Extraction

All four isolates of PGPR that act as disease resistance-inducing agent were grown in an Erlenmeyer containing potato dextrose broth and peptone medium. These cultures were then incubated for 16 h in a shaker at 150 rpm under room temperature. Bacterial cell was harvested and put into 2-ml micro tube and then was centrifuged at 5,000x g for 10 min. The supernatant was discarded. Bacterial cell was re-suspended with 180 □l digestion solution, and added with 20 □l proteinase K solution. This suspension was mixed evenly using vortex. This culture was incubated at temperature of 56^oC until all cells lysis (the suspension is clear). This suspension was added with 20 □l RNase A solution and homogenized using vortex and then was incubated under room temperature for 10 min. A 200 □l lysis solution was added and homogenized with vortex for 15 seconds and added with 400 □l 50% ethanol and homogenized on vortex. This suspension was transferred into Genjet genomic DNA purification column which was placed in collector tube. The column was centrifuged for a minute at 6,000x g. The column was placed in a new collector tube, and added with 500 □l wash buffer I (which already added with ethanol), and then centrifuged for a minute at 8,000xg. The column was then put in a new collector tube and added with 500 □l wash buffer II and centrifuged for 3 min at 12,000x g. The column was placed in 1.5ml sterile micro tube and added 200 □l elution buffer in the center of column to dissolve DNA and incubated for 2 min at room temperature and centrifuged for a minute at 8,000xg. The column was disposed and the pure DNA obtained can be used for amplification or stored in -20^oC until use.

DNA Amplification

DNA amplification was done using 2x Go Taq Green PCR Master (Pomega) with a pair of primers 16S (63F 5'-CAG GCC TAA CAC ATG CAA GTC-3' and 1387R 5'-GGG-CGG WGT GTA CAA GGC-3') in a PCR machine (SENSOQUEST Lancycler). The temperature condition of PCR are as follows : 94^oC for 5 min followed by 30 cycles in

sequence at 94^{o}C for 30 sec, 55^{o}C for 45 sec and 72^{o}C for 2 min, and at the last 72^{o}C for 10 min. PCR product was visualized on 1% agarose gel electrophoresis in TAE buffer stained with PeqGREEN. Sequencing of PCR product was performed with Automated DNA sequencer ABI PRISM 377 (Perkin Elmer Biosystem, USA). Results of sequencing were then compared with data of GenBank using BLAST-N (basic local alignment search tool-nucleotide) program accessed from NCBI (national center for biotechnology information). Construction of phylogeny three was done using MEGA 4.0 program with maximum parsimony with 1000x bootstrap.

Results And Discussion

Ninety five isolates of rhizobacteria were isolated from rhizospeheres of various plants grown in Bali such as rice (Oryza sativa), bamboo (Bambusa blumeana), soybean (Glycine max), ground nut (Arachis hypogaea), blady grass (Imperata cylindrica), crotalaria (Crotalaria spectabilis), and lemon grass (Cimbopogon citratus). Among them, nine isolates have been proven to be plant growth promoting rhizobacteria (PGPR) indicated by their capability to promote the formation of root hairs of rice as shown in Table 1.

Table 1. List of isolates of rhizobacteria that serve as plant growth promoting rhizobacteria (PGPR) to Bali rice local variety (Padi merah medang putih)

No.	Code of isolate	Name of plant from where samples (rhizospeheres) were collected	Capability to promote the formation of root hairs
1	O38	Rice	+
2	O39	Rice	+
3	OR3	Crotalaria	+
4	Al7Kla	Blady grass	++
5	Al2TT	Blady grass	+
6	KtE7	Ground nut	+
7	KDDA	Soybean	+
8	Bm5Sa	Bamboo	+
9	Sr2Ta	Lemon grass	+

\+ capable of increasing the number of root hairs from 20 to 30% when compared to control.
++ capable of increasing the number of root hairs more than 30% when compared to control.

Some rhizobacteria that colonize the rhizospeheres of plants are benefited to the plants that serve as plant growth promoting rhizobacteria (Kloepper et al., 1989). About 1-2% of rhizobacteria can promote the plant growth (Antoun and Kloepper, 2001). Several researchers reported that plant growth promoting rhizobacteria (PGPR) can promote the plant growth, induce disease resistance, and increase the yield (Wei, 1991; Press et al., 1997; Palukaitis et al., 1992; Sherata et al., 2008; Khalimi, 2009; Suprapta et al., 2014b). Mechanisms by which PGPR promote the plant growth are through 1) interfere with the hormonal balance in the plant, 2) capacity to fix nitrogen, and 3) solublilize phosphate to be available to the plant (Fernando et al., 2005; Cattelan et al., 1999).

Among nine isolates obtained in this study, only four isolates namely KDDA, O38, OR3 and Al7Kla capable of inducing resistance against rice blast disease on Padi Merah Medang Putih. All seedlings (100%) treated with PGPR followed by inoculation with spores suspension of P. oryzae grew well without blast disease symptom, while all rice seedlings without treatment of PGPR (control) showed severe blast disease symptom as shown in Fig. 1.

Figure 1. Healthy Rice seedlings of cultivar Padi Merah Medang Putih treated with PGPR isolate Al7Kla (left) and rice seedlings of control showing severe blast disease symptom (right).

Several works have been done related to the induce resistance against plant diseases. Van Peer et al. (1991) reported that carnation was systemically protected from infection of Fusarium oxysforum f.sp. dianthi after it was treated with Pseudomonas fluorescens strain WCS417r. Other work done by Wei et al. (1991) found that rhizobacteria protected cucumber plant from anthracnose disease caused by Colletotrichum orbiculare. Capability of rhizobacteria to induce resistance is depend on specific interaction between rhizobacteria and plant (Van Loon, 2007). For example, Pseudomonas putida WCS358r and P. fluorescens WCS374r work in different ways. In Arabidopsis, P. putida WCS358r could induce resistance, but could not in carnation and radish (Van Peer et al., 1991; Van Peer and Schippers, 1992; Van Wees et al., 1997). In contrast, radish plant was responsive to P. fluorescens WCS374r, but was not for Arabidopsis (Leeman et al., 1995; Van Wees et al., 1997).

In the present study isolates KDDA, O38, OR3, and Al7Kla could induce resistance against blast disease. Separation pattern of 16S rDNA-specific fragments amplified with primers 63F and 1387R of PGPR isolates Al7Kla, O38, KDDA, and OR3 is shown in Fig. 2. Labeled band of approximately 1,300 bp is corresponding to 16S rDNA.

A comparative 16S rRNA gene sequence-based phylogenetic analysis placed isolate KDDA in a clade with the species Bacillus methylotrophicus strain RA, B. methylotrophicus MSL_3065, and B. methylotrophicus MSL_3012 and revealed pairwise similarities ranging from 99 to 100% (Table 2, Fig.3). Based on this result, the name Bacillus methylotrophicus strain KDDA is proposed to indicate PGPR isolate KDDA. Isolate O38 located at a clade with Bacillus amyloliquefaciens strain SL-10, B. amyloliquefaciens ASAGI, and B. amyloliquefaciens BChi1 with pair wise similarity 99%. The proposed name of this isolate is Bacillus amyloliquefaciens strain O38. Isolate OR3 located at a clade with Pseudomonas aeruginosa strain DM14, P. aeruginosa strain DM11, and P. aeruginosa strain DM2 with pair wise similarities 100%. Pseudomonas aeruginosa strain OR3 is proposed to indicate isolate OR3. Other result placed the isolate Al7Kla in a clade with Achromobacter xylosoxidans Fb15, A. xylosoxidans NSBx10 (JWB2), and A. xylosoxidans TPL14 with pair wise similarities 92%. Achromobacter xylosoxidans strain Al7Kla is proposed as the species name of isolate Al7Kla.

Most of rhizobacteria that can induce disease resistance belong to the Genus Pseudomonas and Bacillus (Kloepper et al., 2004; Van Wees et al., 2008; Delgado et al., 2015) which is in accordance with our present finding, in which two isolates viz. KDDA and O38 belong to the Genus Bacillus, and one isolate, OR3 belong to the Genus Pseudomonas. Study done by Delgado et al. (2015) showed that treatment with formula containing three species of rhizobacteria namely Bacillus subtilis 5, Bacillus cereus 3S5 and Pseudomonas fluorescens 10S2 on rice cultivar UPLRi-5 could reduce the rice blast intensity by 33% when compared with control (Delgado et al., 2015). In the present study we found other potential inducing agent viz. Achromobacter xylosoxidans strain Al7Kla isolated from Imperata cylindrica (blady grass). This is the first report on Achromobacter xylosoxidans isolated from the rhizosphere of blady grass with potential induced resistance against rice blast disease. Other study reported the antagonistic potential of A. xylosoxidans LK391696 against fungal pathogens such as Alternaria solani, Curvularia lunata, and Fusarium oxysforum (Devi and Mohan, 2015). This bacteria was isolated from tannery effluent sludge, and obviously inhibited the growth of C. lunata, A. solani and F. oxysforum with inhibitory activities respectively by 95, 85, and 80% (Devi and Mohan, 2015).

Further study is necessary to evaluate the capability of four species of local PGPR in inducing resistance against blast disease on Bali rice local variety, Padi Merah Medang Putih under green house and field condition. In addition, the mechanism through which they can induce resistance against blast disease should be investigated

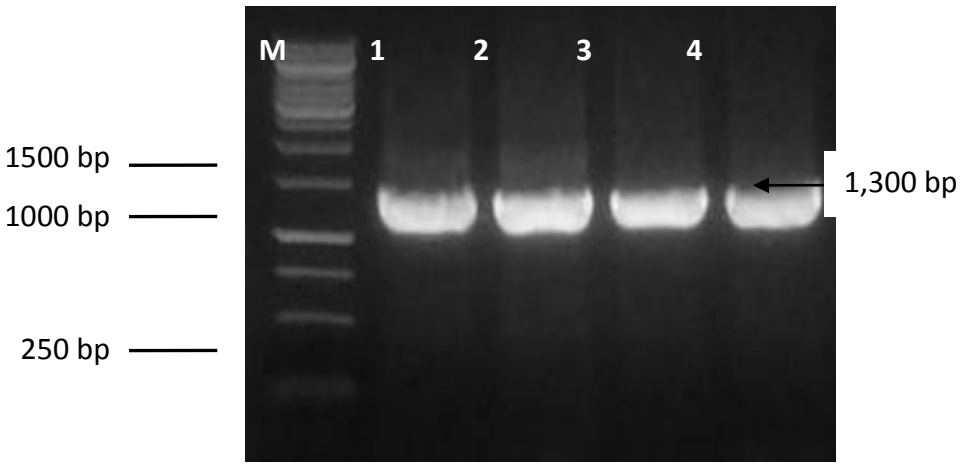

Figure 2. Agarose gel electrophoreses of 16S rDNA of PGPR M1= 1 kb gene ladder; Lane 1= fragment of 16S rDNA isolate Al7Kla; Lane 2= isolate O38, Lane 3= isolate KDDA, and Lane 4= isolate OR3

Table 2. Maximum scores, E values, and percentage of similarities of four isolates of local plant growth promoting rhizobacteria

Isolate	Species homolog	Identity	Max Score	Query Cover	E value	Accession Number
KDDA	Bacillus methylotrophicus strain RA	100%	2266	99%	0.0	KU146559.1
	Bacillus methylotrophicus MSL_3065	99%	2259	99%	0.0	KT719890.1
	Bacillus methylotrophicus MSL_ 3012	99%	2259	99%	0.0	KT719862.1
O38	Bacillus amyloliquefaciens strain SL-10	99%	2264	100%	0.0	KT026596.1
	Bacillus amyloliquefaciens ASAG1	99%	2268	100%	0.0	FJ597542.1
	Bacillus amyloliquefaciens BChi1	99%	2264	100%	0.0	KT306960.1
OR3	Pseudomonas aeruginosa strain DM14	100%	2269	100%	0.0	KT229747.1
	Pseudomonas aeruginosa strain DM11	100%	2269	100%	0.0	KT229744.1
	Pseudomonas aeruginosa strain DM2	100%	2269	100%	0.0	KT229735.1
AL7Kla	Achromobacter xylosoxidans Fb15	92%	1335	98%	0.0	JN162396.1
	Achromobacter xylosoxidans NSBx.10 (JWB2)	92%	1323	98%	0.0	JF330161.1
	Achromobacter xylosoxidans TPL14	92%	1323	98%	0.0	EU373389.1

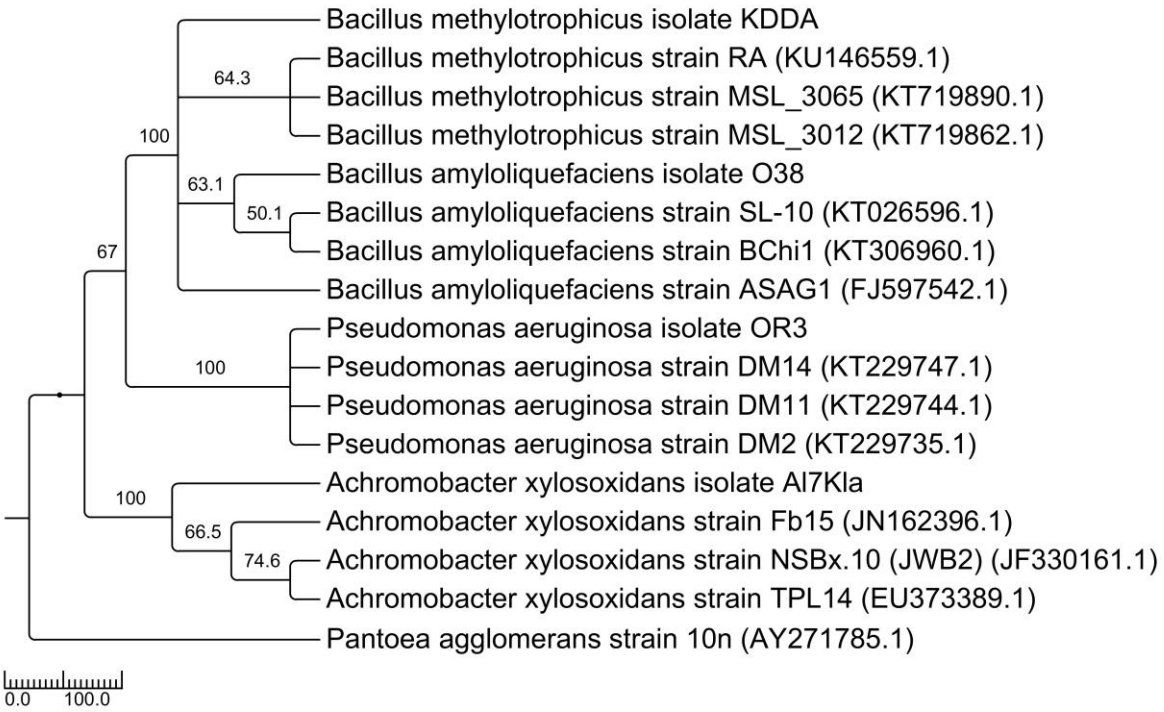

Figure 3. Phylogenetic relationship between rhizobacteria isolates KDDA, O38, OR3 and Al7Kla with other bacteria available from GenBank database.

Conslusion

Four isolates (KDDA, O38, OR3, and Al7Kla) of local plant growth promoting rhizobacteria isolated from rhizospheres of several crops grown in Bali Island, Indonesia were proven to act as resistance inducing agent against rice blast disease caused by Pyricularia oryzae Cav. on Bali local rice variety, Padi Merah Medang Putih. Based on the sequence of 16S rDNA isolate KDDA was identified as Bacillus methylotrophicus strain KDDA; isolate O38 was identified as Bacillus amyloliquefaciens strain O38; isolate OR3 was identified as Pseudomonas aeruginosa strain OR3, and isolate Al7Kla was identified as Achromobacter xylosoxidans strain Al7Kla. All of these PGPR can be further tested and evaluated for their capability to induce resistance against rice blast disease under green house and field condition.

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