Genetic Diversity of Antifungi-Producing Rhizobacteria of Pseudomonas sp. Isolated from Rhizosphere of Soybean Plant

ARI SUSILOWATI, ARIS TRI WAHYUDI*, YULIN LESTARI, SURYO WIYONO, AND ANTONIUS SUWANTO

Department of Biology, Faculty of Mathematics and Natural Sciences;
Institute of Environment and Agriculture, Faculty of Agriculture; Darmaga Campus, Bogor 16680, Indonesia

Abstract:
Antifungi-producing rhizobacteria have been recognized playing an important role in plant disease suppression. In our laboratory, 13 indigenous soybeans' rhizobacteria Pseudomonas sp. that showed strong growth inhibition of root pathogenic fungi, Rhizoctonia solani, Fusarium oxysporum and Sclerotium rolfsii, have been isolated from rhizosphere of soybean plant. For further understanding, the genetic diversity of the antifungi-producing Pseudomonas sp. was investigated using Amplified 16S rDNA Restriction Analysis (ARDRA) and 16S rRNA gene sequences analysis. 16S rRNA were amplified by PCR technique and digested with restriction endonuclease HaeIII, RsaI and AluI. Sequences of 16S rRNA gene were analyzed using the BLAST program for similarity searches on sequence databases. ARDRA based-dendrogram analysis was carried out by neighbor-joining of TREECON 1.3b software package. ARDRA indicated the variability of Pseudomonas sp. based on the digestion sites. Dendrogram analysis was based on the restriction enzymes profile of the amplified 16S rRNA distinguished Pseudomonas sp. into 7 ribotype groups. The sequences of 16S rRNA gene confirmed that the isolates belonging to Pseudomonas sp. and the phylogenetic tree formed 4 clusters. There was a quite overlap among ARDRA groups and 16S rRNA sequence clusters. This finding suggested that antifungi producing Pseudomonas sp. were present in the rhizosphere of soybean plant and the level of genetic diversity exist within these species. Sequence analysis of the 16S rRNA gene of the Pseudomonas sp. with an identical ARDRA pattern confirmed that members of an ARDRA group were closely related to each other.

Key words: antifungi producing rhizobacteria, ARDRA, genetic diversity, Pseudomonas sp., 16S rRNA

Disease suppressive soil suppresses the growth of phytopathogenic fungi or the induction of severe disease on susceptible plants (Schroth and Hancock 1982). This phenomenon, despite rarely happens, has been extensively known and strong evidences show that the disease is suppressed by specific rhizobacteria that have the ability to produce antifungal compounds. Several experiments have demonstrated that a number of Pseudomonas sp. strains with the ability to produce antifungal metabolite 2,4-diacyctlyphloroglucinol (DAPG) can be isolated with high frequency from black root rot disease suppressive soil applied to tobacco (Keel et al. 1996). These strains are able to suppress diseases in wheat (Raaijmakers et al. 1997). They are also involved in many plant disease suppression that can be related to the presence of phytopathogen-antagonistic functions in the soil microbiota (Garbeva et al. 2004; Mazzola 2004; Garbeva et al. 2006).

There is a great interest in plant-associated bacteria, particularly in the genus of Pseudomonas. Researchers aiming to improve crop responses emphasized on antibiotic producing bacteria indigenous to certain rhizosphere. Moreover, the components of suppressiveness have been described for multiple pathosystems, especially for those involving a specific pathogen and microbial antagonist (Wellert et al. 2002; Boronak and Becker 2007). Recently, antibiotic-producing bacteria have been recognized playing an important role in disease suppression. In our laboratory, we have screened the indigenous soybeans' rhizobacteria Pseudomonas sp. CRB (Cirebon) that are potential as a biocontrol for root rot disease caused by pathogenic fungi. Several Pseudomonas sp. CRB isolates strongly inhibited growth of root pathogenic fungi, i.e. Rhizoctonia solani, Sclerotium rolfsii or Fusarium oxysporum, in vitro.

Genetic diversity means the total number of genetic characteristics. Molecular tools such as ribotyping, in-situ hybridization, DNA sequence analysis and restriction fragment length polymorphism (RFLP) are now in common use to provide accurate genetic diversity information of microbes by using 16S rRNA gene. RFLP analysis on 16S rRNA gene is called amplified rDNA restriction analysis (ARDRA). This method is useful for genotype identification and can be used to infer genetic variability and similarity of microorganisms (Yang et al. 2007; Kidd et al. 2009). ARDRA can also be applied for characterizing a number of species including Clostridium botulinum toxinotype A strains (Pourshafie et al. 2009) and mycobacteria (De Baere et al. 2002). In this study, we described diversity of antifungi-producing Pseudomonas sp. CRB from rhizosphere of soybean plant employing ARDRA and 16S rRNA gene sequences analysis.

MATERIALS AND METHODS
Microorganism and Culture Condition. Pseudomonas spp. were isolated from soybean's rhizosphere from a soybean plantation in Cirebon area, West Java, Indonesia. The rhizosphere's soil was diluted in 10 mL 0.85% NaCl solution and then serial dilution was made to obtain the appropriate bacterial number so that various individual bacteria would grow separately on agar surface. Diluted bacteria (100 µL) were then spread onto King's agar (King et al. 1954) and incubated for 24 hours at room temperature (27-28°C). Each visible colony with different appearance was picked and streaked on fresh medium to obtain pure
culture. Identification of Pseudomonas sp. was conducted based on morphological and physiological characters. Microgen system (DNA and GNB) that employs 24 standardized biochemical substrates in microwell was also used to complete the test. Gram negative, rods, motile, aerobic, catalase positive, and oxidize positive were the characters that lead to Pseudomonas identification (Holm et al. 1994). Eighty one isolates named Pseudomonas sp. CRB were collected. Thirteen isolates that showed strong inhibition of fungal pathogen in vitro were used in this study (Table 1). Pseudomonas sp. were were routinely cultivated on agar plate of King's medium B (20 g L \text{-1} \text{ bacto peptone, 1.5 g L} \text{-1} \text{ KH}_{2} \text{PO}_{4}, 1.5 g L \text{-1} \text{ MgSO}_{4}, 15 g L \text{-1} \text{ bacto agar (Difco, France), 15 mL } \text{ glycerol (in room temperature (27-28°C)).}

DNA Extraction and PCR Amplification. Genomic DNA was isolated from overnight cultures of Pseudomonas sp. CRB by using a cetyl trimethyl ammonium bromide (CTAB)-based protocol (Sambrook and Russell 2001). DNA coding for 16S rDNA of each isolate was amplified with primer 23F (5'-CAT GCC TAA CAC ATGCAA GTC-3') and 1387R (5'-GGG CCG WGA CAA GGC-3') (Invitrogen, Japan). These primers amplify approximately 1 kb of the 16S rDNA gene, specific to consensus regions that are considered as universal bacterial domains (Marchesi et al. 1998). PCR reactions (Takara, Japan) were done in a total reaction volume of 50 µL containing 25 µL GC buffer II, 8 µL dNTP mix (2.5 mM each), 20 pmol of each primer, 1.5 g L \text{-1} \text{ KH}_{2} \text{PO}_{4}, 1.5 g L \text{-1} \text{ MgSO}_{4}, 15 g L \text{-1} \text{ bacto agar (Difco, France), 15 mL } \text{ glycerol (in room temperature (27-28°C)).}


dna sequence analysis. The robustness of the inferred trees was evaluated by using bootstrap analysis of 100 resamples. The isolates that occupy the same cluster are considered to share the same attributes. Therefore, isolates belonging to the same cluster are similar in some sense. Other isolates forming other groups differ from ribotype 7. Cluster dendrogram showed the ARDRA pattern of ribotype 1 was similar to ribotype 2 and differ from ribotype 7 (Fig 1).

Cluster Analysis Based-ARDRA. The results of ARDRA using HaeIII, RsaI, and AluI were subjected to cluster analysis. Neighbor-joining method was used to construct the dendrogram of bands resulting from endonuclease digestion. In this method, similar variables were grouped. The ARDRA clustering revealed a considerable level of genetic diversity among the isolates, since 7 clusters, designated as ribotype 1-7, were identified (Fig 1). Pseudomonas sp. CRB-16, CRB-17, CRB-80, CRB-82 and CRB-102 are clustered in one group, i.e. ribotype 7. The isolates that occupy the same cluster are considered to share the same attributes. Therefore, isolates belonging to the same cluster are similar in some sense. Other isolates forming other groups differ from ribotype 7. Cluster dendrogram showed the ARDRA pattern of ribotype 1 was similar to ribotype 2 and differ from ribotype 7 (Fig 1). In this result, Pseudomonas sp. CRB-3 is the most unique isolate among others.

16S rDNA Gene Sequences and Phylogenetic Relationship. BLASTN results of the partial sequence of 16S rDNA gene (about 600 nucleotides) showed high similarity with Pseudomonas spp. (83-100%) (Table 3). The phylogenetic relationship among 13 isolates were presented as a dendrogram using neighbor joining method (Fig 2). Four clusters were generated when Pseudomonas sp. CRB isolates were grouped according to their 16S rDNA sequences. Isolates found in the same cluster seem to have close phylogenetic relationship. Many isolates with the same

![Figure 1](image-url)
that they were phylogenetically placed in the same cluster, i.e. cluster 6. It is interesting to note that the Pseudomonas sp. CRB-11, CRB-17, CRB-80, CRB-82, and CRB-102, except CRB-3, were found in rhibotype 7 group. Pseudomonas sp. CRB-109 and 111 those were grouped as ribotype 6 were also found in the same cluster, i.e. cluster 6. Therefore, they were likely to be closely related.

**DISCUSSION**

Bacteria responsible for soilborne plant disease suppression can be found among microbial community of the rhizosphere. There were 13 isolates of Pseudomonas sp. CRB possessing antifungal activity from rhizosphere of soybean plant. The antifungal activity was confirmed in vitro and in planta against target fungal pathogens. Considering that antifungal activity, these bacteria are possibly naturally occurring disease suppressing bacteria. The number of the isolates was not many, but these antifungal-producing Pseudomonas sp. CRB isolates are promising for biocontrol agent. For comparison, there were 39 fluorescent pseudomonads producing DAPG (Ramette et al. 2001). Among antifungal compounds synthesized by pseudomonads, DAPG is a key secondary metabolite associated with disease suppression in several pathosystems. Benitez and McSpadden-Gardner (2009) identified novel Mycobacterium and Pelobacter species involved in damping-off disease suppression caused by oomycete pathogens toward tomatoes and soybeans.

ARDRA pattern had sufficiently similar 16S rRNA sequence that they were phylogenetically placed in the same evolutionary line (Fig. 2). For example, cluster 4, represented by Pseudomonas sp. CRB-16, CRB-17, CRB-80, CRB-82, and CRB-102, except CRB-3, were found in the same cluster. Pseudomonas sp. CRB-109 and 111 those were grouped as ribotype 6 were also found in the same cluster, i.e. cluster 6. Therefore, they were likely to be closely related.

**ACKNOWLEDGEMENT**

This study was financially supported by the Incentive Program for Basic Research from the Ministry of Research and Technology (Program Insentif Riset Dasar, RISTEK-KNRT), the Republic of Indonesia 2008 to 2011. We are grateful for this funding and support of this research.

**REFERENCES**


Other researches that employed ARDRA showed more diversity at the genotype level. In Saldana et al. (2003), ARDRA analysis revealed 3 different genotypes among fast-growing rhizobia that nodulate soybean, even though all belonged to a subgroup to which included Sinorhizobium sactorii and S. fredii (Chénèry et al. 2000) showed that denitrifying isolates from soils were grouped according to the similarity of their restriction patterns into 26 ARDRA types. Interestingly, ARDRA results suggest that some denitrifying isolates are specific to a soil type, while others seem to be genetically widespread. Tian et al. (2009) demonstrated that a total of 28 ARDRA patterns were identified among the 299 siderophore-producing bacterial isolates from the rhizosphere of bamboo. These 28 ARDRA patterns represented bacteria of 14 different genera belonging to 6 bacterial families, namely: β-, γ- Proteobacteria, Sphingobacteria, Bacillii and Actinobacteria. Indeed, from all the examples analyzed, ARDRA could describe the genetic diversity of bacteria studied. If the bacteria have lower genetic diversity, it will be represented by the same band pattern of the enzyme digestion even though many enzymes were used and if the bacteria have greater genetic diversity, different band patterns will be generated. The 16S rRNA gene sequence is about 1550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms to provide distinguishing measurement (Clifford 2004; Woeae 2006). The single copy of the 16S rRNA gene sequences allows differentiation between strains at multiple levels, including what we now call the species and subspecies level. Previous reports showed that the phylogenetic assignment obtained from partial and full-length sequences were very similar (Massana et al. 1997; Xiang et al. 2005). Since we were interested only in determining the group of pseudomonads, a partial sequence analysis was justified. In line with this reason, the most clinical bacterial isolates the initial 500 bp sequence provide adequate differentiation for identification (Hall et al. 2003). Sequence analysis of approximately 600-bp 16S rDNA fragment allowed the rapid identification of this isolates that lead to genu Pseudomonas. However, for discrimination of closely related species sequencing of the entire 16S rRNA gene is essential.


